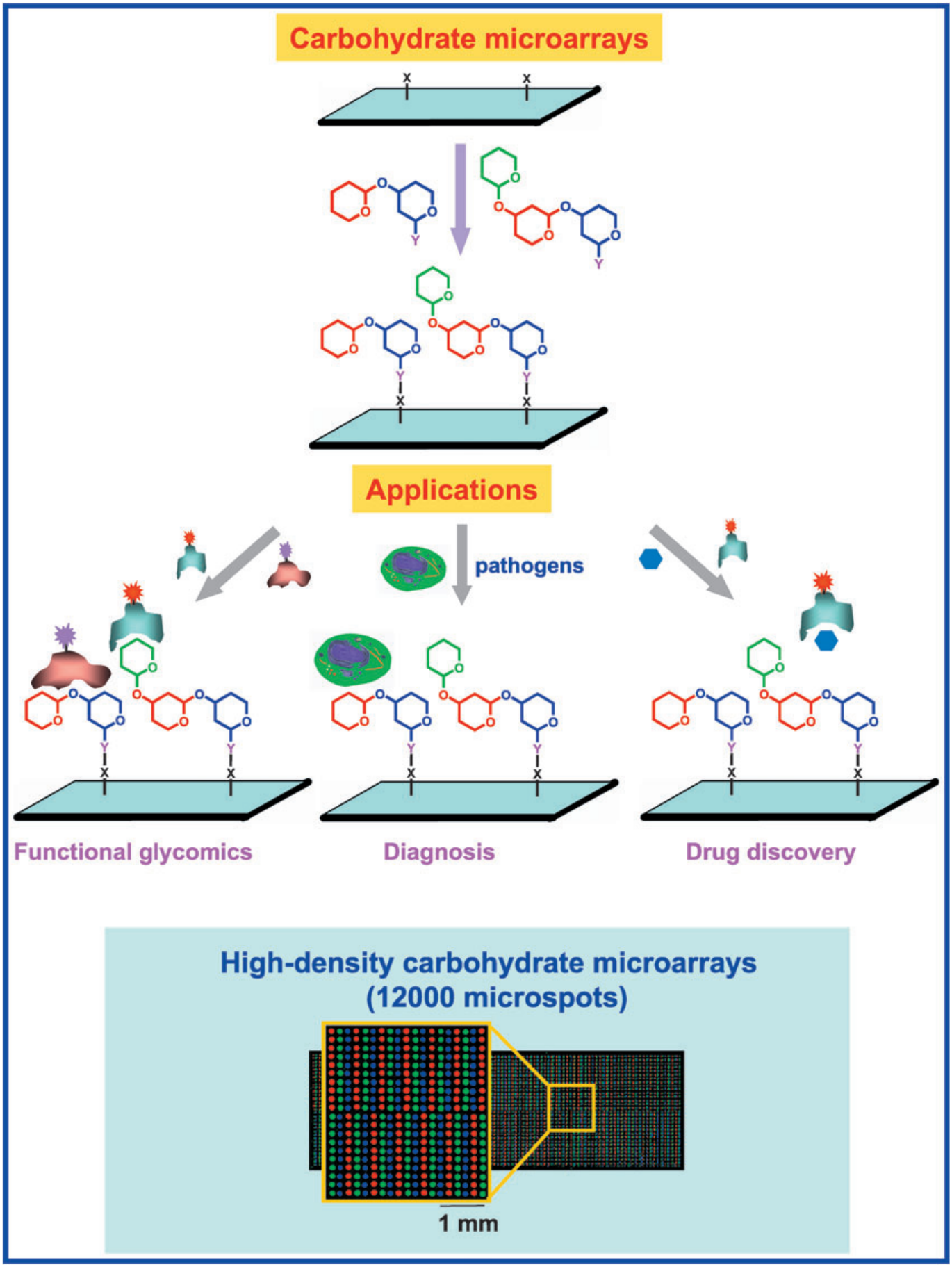

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Carbohydrate Microarrays: An Advanced Technology for Functional Studies of Glycans

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Abstract: The biological significance of glycans in the post-genomic era requires the development of new technologies to enable functional studies of carbohydrates in a high-throughput manner. Recently, carbohydrate microarrays have been exploited as an advanced technology for this purpose. Efficient immobilization methods for carbohydrate probes on the proper surface are essential for the successful fabrication of carbohydrate microarrays. Up to date, several techniques have been developed to attach simple or complex carbohydrates to a solid surface. The developed glycan microarrays have been applied for functional glycomics, drug discovery, and diagnosis. In this concept article, we discuss the progress of immobilization methods of carbohydrates on solid surfaces, their potential uses for biological research and biomedical applications, and possible solutions for some remaining challenges to improve this new technology.

Keywords: carbohydrates · glycoconjugates glycosylation · immobilization · oligosaccharides

Introduction

In the post-genomic era, functional studies of carbohydrates (functional glycomics) in living organisms have received great attention for biological research and biomedical applications. The cell surface is highly decorated with diverse structures of glycans, mainly present in the forms of glycoconjugates such as glycoproteins and glycolipids. The cell surface glycans vary in different cell types and states. They act as biomolecular recognition markers for a variety of important biological functions, including cell communication, cell adhesion, fertilization, development, differentiation, and immune response through to specific interactions with proteins.[1] These interactions are also involved in detrimental disease processes, such as inflammation, tumor metastasis, and viral or bacterial infections.[1] Interestingly, carbohydrate–carbohydrate interactions are also known to mediate biological processes such as cell adhesion, signal transduction, and melanoma cell metastasis.[2] Therefore, functional studies of glycans may provide invaluable information on understanding biological phenomena and exploiting more effective therapeutic agents and diagnostic tools.[3]

For over a decade, microarray-based technologies have been extensively developed as high-throughput analytic tools for studying biological processes (Figure 1). These technologies facilitate fast, quantitative, and simultaneous analyses of a large number of biomolecular interactions. For instance, DNA microarrays, which were the first to be developed, have been applied for analyzing mutation of genes,

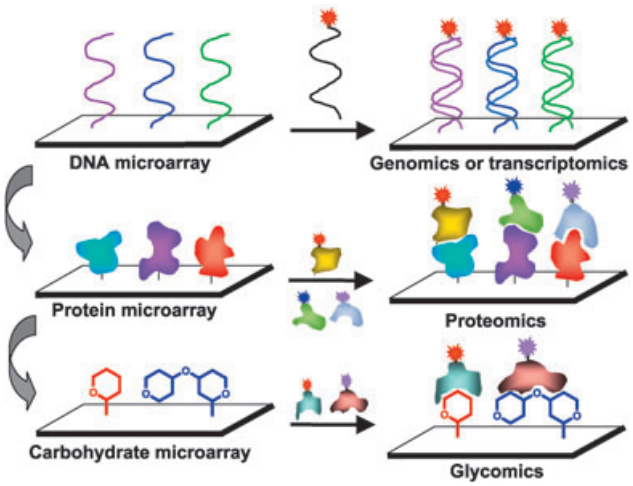

Figure 1. Microarray-based technologies for studies of biological processes.

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studying change of patterns of gene expression in disease, and tracking the activities of many genes at the same time.[4] Protein microarrays, which were developed after DNA microarrays, have been used for the high-throughput studies of protein–protein interactions and profiling of protein expression in normal and diseased states.[5] Although these technologies have been widely used for genomic, transcriptomic, and proteomic research during last decade, it is only recently that carbohydrate microarrays were exploited for glycomic research.[6]

In general, DNA–DNA (or DNA–RNA) and protein–protein interactions are strong enough for detection with DNA and protein microarrays. However, carbohydrate–protein interactions are known to be relatively weak and thus may not be easily detected with carbohydrate microarrays.[7] For the successful applications of carbohydrate microarrays, carbohydrates immobilized on the solid surface should be strongly recognized by proteins to allow detection. One possible solution for achieving strong binding of carbohydrates to proteins is to immobilize carbohydrate probes with proper spacing and orientation on the solid surface, resulting in their multivalent interactions (cluster effect).[7] As described below, the lectin-binding experiments with carbohydrate microarrays indicate that carbohydrate-binding proteins strongly interact with the corresponding carbohydrates on the surface. This shows that the immobilized carbohydrates on the surface appear to display multivalency unlike in solution. On the other hand, the immobilized carbohydrates on the solid surface may act as cell-surface carbohydrates and can be recognized by proteins that are similar to a cell surfaces. As a consequence, carbohydrate microarrays are ideal for the functional studies of glycans.

In this article, we first discuss the developed immobilization methods of carbohydrates on the solid surface, and then mention potential applications of carbohydrate microarrays for functional glycomics. Finally, we address possible solutions for some remaining challenges to improve this new technology. Low-density microtiter arrays may be of use for analyzing a relatively small number of samples. However, high-density microarrays have an advantage over microtiter arrays, since tens of thousands of very small quantities of samples can be simultaneously analyzed. For these reasons, we focus on the high-density carbohydrate microarrays in this article.

Immobilization Strategies for Carbohydrates on a Solid Surface

Efficient immobilization techniques for carbohydrates on a solid surface are a prerequisite for the successful preparation of carbohydrate microarrays. Four general methods can be used for immobilizing simple or complex carbohydrates: 1) nonspecific and noncovalent immobilization of chemically unconjugated carbohydrates on the underivatized surface, 2) site-specific and covalent immobilization of chemically conjugated carbohydrates on the modified surface, 3) site-specific but noncovalent immobilization of chemically conjugated carbohydrates on the underivatized surface, and 4) site-specific and covalent immobilization of chemically unmodified carbohydrates on the modified surface. The first three techniques have already been developed, while the last is still under investigation.

First, chemically unconjugated glycans are nonspecifically and noncovalently adsorbed on an underivatized surface (Figure 2). This method needs neither a modified surface

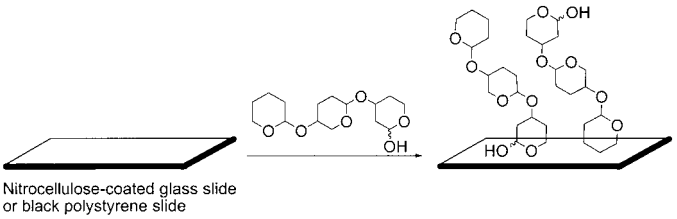

Figure 2. Nonspecific and noncovalent immobilization of chemically unmodified carbohydrates on a solid surface.

nor chemical-linking techniques, making it simple for the fabrication of carbohydrate microarrays.[8] However, the immobilized carbohydrates should be large enough for tight adsorption on the surface. For example, Wang et al. immobilized a variety of chemically unconjugated microbial polysaccharides on nitrocellulose-coated glass slides by physical adsorption, mainly through hydrophobic interactions, to prepare microbial polysaccharide microarrays.[8a] The immobilization efficiency of this method was significantly affected by size of carbohydrates; polysaccharides of 3.3–2000 kDa were efficiently adsorbed on the solid surface, but smaller carbohydrates were less retained on the surface after extensive washing. However, the recognition properties of noncovalently immobilized polysaccharides were preserved, based on the binding experiments with monoclonal antibodies. Willats et al. developed sugar-coated microarrays using a new microarray slide surface.[8b] They immobilized polysaccharides, proteoglycans, neoglycoproteins and plant cell extracts on the black polystyrene slides, prepared by injection-molding of black polystyrene and an oxidative surface modification. In this case, samples were attached to the slides by ionic bonding, hydrogen bonding, and hydrophobic interactions. Since the carbohydrate microarrays were fabricated with a black hydrophobic resin, a relatively high signal-to-noise ratio was observed after probing with dye-labeled proteins.

Second, chemically conjugated carbohydrates are site-specifically and covalently attached to the properly modified surface (Figure 3). This method requires both a modified surface and chemical-linking techniques. Preparation of chemically conjugated carbohydrates is time-consuming and sometimes difficult for nonexperts. However, simple carbohydrates and oligosaccharides are both efficiently immobilized on the surface in a site-specific manner, enhancing protein binding to the immobilized carbohydrates. For this

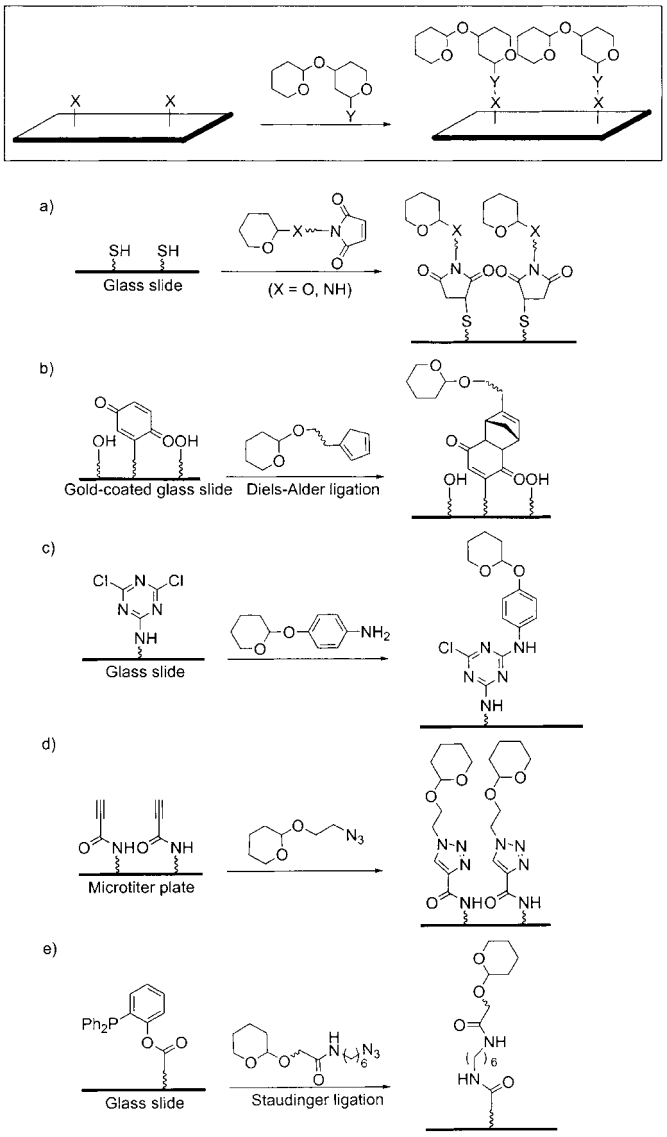

Figure 3. Site-specific and covalent immobilization of chemically modified carbohydrates on the derivatized solid surface; a) attachment of maleimide-linked carbohydrates to thiol-coated glass slides, b) attachment of cyclopentadiene-linked carbohydrates to benzophenone-coated gold surfaces through Diels–Alder reactions, c) attachment of *p*-aminophenyl glycosides to glass slides coated with cyanuric chloride, d) attachment of azide-linked carbohydrates to alkynylated lipid-coated microtiter plates, and e) attachment of azide-linked carbohydrates to phosphane-coated glass slides by means of Staudinger reactions.

reason, this technique is more suitable for the fabrication of simple carbohydrate and oligosaccharide microarrays than the first one. For instance, our group exploited carbohydrate microarrays by attaching maleimide-connected carbohydrates to SH-coated glass slides (Figure 3a).[9] To reduce steric hindrance during protein binding to carbohydrates on the surface, tethers of proper lengths were inserted between maleimide groups and carbohydrate moieties. Protein-binding experiments indicated that carbohydrates connected with long tethers interacted more strongly with proteins relative to those linked by short tethers. Alternatively, thiol-linked carbohydrates were immobilized on either maleimide-functionalized self-assembled monolayers or glass slides coated with bovine serum albumin.[10]

Mrksich and co-workers prepared carbohydrate chips by immobilizing cyclopentadiene-containing carbohydrates on a benzoquinone-coated gold surface by menas of a Diels–Alder reaction (Figure 3b).[11] Modification of the gold surface was initiated by immersing gold-coated glass slides into a mixture of alkanethiols with (1%) and without (99%) appended hydroquinone groups to produce self-assembled monolayers of hydroquinone and penta(ethylene glycol) groups. Chemical or electrochemical oxidation was then performed to convert hydroquinone to benzoquinone groups. Finally, the monosaccharides tethered to cyclopentadiene groups were covalently immobilized on the gold surface through the Diels–Alder reaction. This reaction was found to be highly efficient and selective for the immobilization of carbohydrates on the surface.

Schwarz et al. developed glycol arrays by covalently immobilizing a variety of *p*-aminophenyl glycosides on glass slides modified with cyanuric chloride and patterned with a hydrophobic Teflon mask (Figure 3c).[12] Wong and co-workers explored a cycloaddition reaction between azide-containing sugars and alkynylated lipids noncovalently attached to the microtiter plates for the preparation of a microtiter-plate-type carbohydrate array (Figure 3d).[13] The alkyne, which contained a 14 carbon lipid component, was first noncovalently adsorbed on the plate by hydrophobic interactions, and then azido sugars were covalently immobilized by copper(I)-accelerated regiospecific 1,3-dipolar cycloaddition reactions between the alkyne and azide groups. Waldmann et al. have fabricated small molecule microarrays that include carbohydrates by using Staudinger reactions between azide-containing substances and phosphane-derivatized glass slides (Figure 3e).[14] The glass surface was modified with fourth-generation polyamidoamine (PAMAM) dendrimers to increase reactive sites on the surface. The required azide-linked carbohydrates were facilely synthesized by solid-phase synthesis by using the safety-catch linker strategy.

Third, chemically conjugated carbohydrates are site-specifically and noncovalently immobilized on the unmodified surface (Figure 4). As an example of this method, Feizi et al. developed oligosaccharide microarrays by noncovalently immobilizing neoglycolipids (NGLs) on nitrocellulose.[15] The required NGLs were prepared by reductive amination of oligosaccharides with an amino lipid (1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine). The oligosaccharides were obtained by chemical or enzymatic methods by using glycoproteins, glycolipids, proteoglycans, polysaccharides and whole organs, or from chemical synthesis. The immobilization efficiency of the NGLs on nitrocellulose was found to be high irrespective of the size of carbohydrates.

The last immobilization strategy is to site-specifically and covalently attach free carbohydrates irrespective of their size on the modified surface (Figure 5). Our group is developing a method to immobilize various free carbohydrates including mono, di-, oligo-, and polysaccharides on the amino-

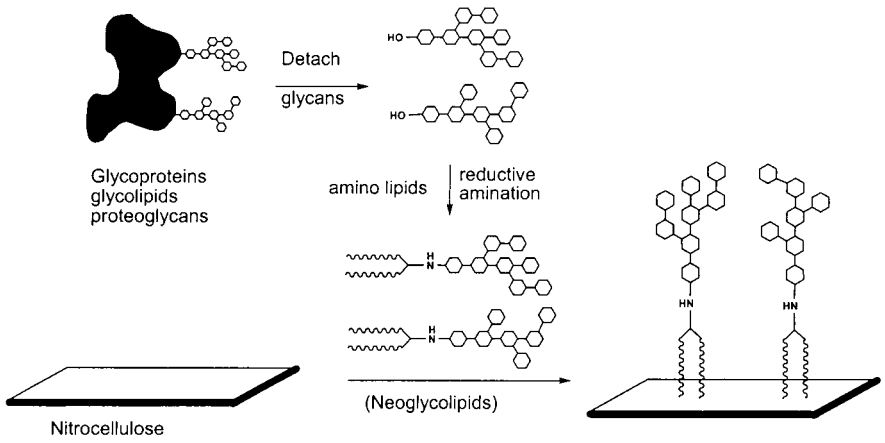

Figure 4. Site-specific and noncovalent immobilization of chemically modified carbohydrates on the underivatized solid surface.

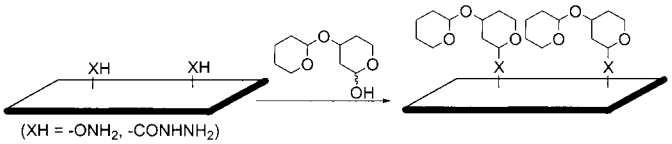

Figure 5. Site-specific and covalent immobilization of free carbohydrates on the derivatized solid surface.

oxy- or hydrazide-derivatized glass slides.[16] Preliminary protein-binding experiments show that carbohydrate microarrays prepared by this method are suitable for the high-throughput analysis of carbohydrate–protein interactions.

Applications of Carbohydrate Microarrays

YDuring last a few years, carbohydrate microarrays have been demonstrated to be an advanced technology for biological research and potential biomedical applications. These include analysis of carbohydrate–protein interactions and identification of novel carbohydrate-binding proteins, characterization of carbohydrate-processing enzymes, profiling of the binding specificity of antibodies, studies on carbohydrate-mediated cell recognition events and detection of pathogens for diagnosis, deciphering the sugar code (structures of glycans), and discovery of novel inhibitors of protein–carbohydrate interactions and carbohydrate-processing enzymes (Figure 6).

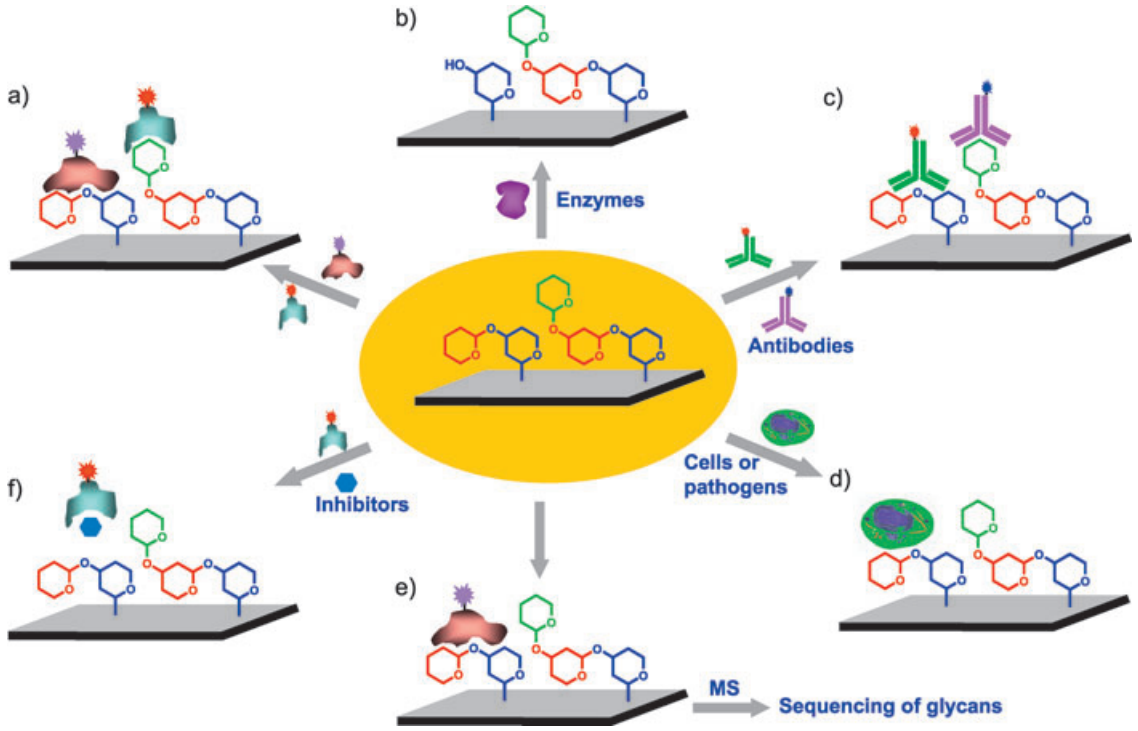

Figure 6. Applications of carbohydrate microarrays; a) high-throughput analysis of carbohydrate–protein interactions and rapid identification/characterization of novel carbohydrate-binding proteins, b) rapid determination of substrate specificity or enzymatic activity of carbohydrate-processing enzymes, c) profiling of carbohydrate–antibody interactions and detection of specific carbohydrate-binding antibodies for the diagnosis of diseases, d) characterization of carbohydrate-mediated cell recognition events, e) deciphering oligosaccharide code in a glycome, and f) high-throughput screening of inhibitors of carbohydrate-processing enzymes and modulators to prevent carbohydrate-protein interactions for drug discovery.

First, carbohydrate microarrays can be used for the high-throughput analysis of carbohydrate–protein interactions and the rapid identification/characterization of novel carbohydrate-binding proteins (Figure 6a). To date, several research groups have investigated glycan–protein interactions with these microarrays by probing with fluorophore-labeled proteins.[9–11] Protein-binding studies with carbohydrate microarrays showed that the relative binding affinities of lectins to the immobilized carbohydrates were well consistent with those obtained from solution-based assays (e.g., hemagglutination inhibition assay or isothermal titration calorimetry). Furthermore, quantitative binding affinities of lectins to carbohydrates on the surface were also analyzed by determining IC_{50} values of soluble carbohydrates with these microarrays.[9,11] One of the most challenging biological fields in the post-genomic era is to identify and characterize tens of thousands of proteins encoded by a genome. This microarray-based technology can be further applied for identification and characterization of new carbohydrate-binding proteins from a proteome in a high-throughput fashion.

Second, glycan microarrays can be utilized to rapidly determine the substrate specificity or enzymatic activity of carbohydrate-processing enzymes (Figure 6b). As a model study for this, the microarray containing GlcNAc and fucose was treated with β-1,4-galactosyltransferase (GalT) and UDP-Gal, and then probed with fluorophore-labeled *A. aurantia* and *E. cristagalli*.[9a] Fluorescence images exhibited that GlcNAc was selectively converted to LacNAc by GalT. These studies suggest that the carbohydrate microarrays are useful tools for characterizing novel carbohydrate-processing enzymes in the post-genomic era.

Third, glycan microarrays can be applied to profile carbohydrate–antibody interactions and to detect specific carbohydrate-binding antibodies for the diagnosis of diseases (Figure 6c). Willats et al. assessed the binding specificities of monoclonal antibodies with microarrays composed of polysaccharides, proteoglycans, neoglycoproteins, and plant cell extracts.[8b] The carbohydrate epitopes on the solid surface were specifically recognized by the corresponding antibodies. It was also found that the detection limit (ca. 80 fg) of this microarray assay was superior to that using the conventional ELISAs and immunodot assay methods (~5 pg and ~10 ng, respectively). Wang and co-workers more extensively investigated the binding specificity of human antibodies with carbohydrate microarrays containing 48 microbial polysaccharide probes. For this study, they used only a limited amount of human serum (1 μL).[8a] The antibody-binding experiments demonstrated that antibodies interacted specifically with the corresponding polysaccharides. In addition, by using this technology, unexpected antibody specificities were found and previously unknown cellular markers (Dex-Ids) were also discovered. Most pathogens contain specific polysaccharides on the cell surface. Pathogen-infected humans provide antibodies that bind to the pathogenic polysaccharides. The microbial polysaccharide microarray can be further used for the diagnosis of pathogen infection by using human serum samples. Another possible application for diagnosis is to detect tumor cells in human bodies because they frequently express tumor-associated carbohydrates.

Fourth, carbohydrate microarrays can be exploited to characterize carbohydrate-mediated cell recognition events (Figure 6d). Nimrichter et al. incubated glycol arrays with dye-labeled primary chicken hepatocytes that expressed GlcNAc-specific lectin on their surface.[17] Cell-binding experiments showed that intact cells adhered to the GlcNAc but not the Gal on the solid surface. They also applied this technology to examine adhesion of human $CD4^+$ T-cell to carbohydrates on the surface. $CD4^+$ cells recognized sialyl Le^x, perhaps through cell surface L-selectin, but rarely adhered to the nonfucosylated form. These experiments suggest that carbohydrate microarrays can be used for detecting pathogens which express specific carbohydrate-binding proteins on their cell surface.

Fifth, microarray technology can be used for deciphering the oligosaccharide code in a glycome (Figure 6e). One representative example is to determine structure of oligosaccharide chains by using neoglycolipid microarrays and mass spectrometry.[6b,15] The required neoglycolipids (NGLs) were prepared by coupling of oligosaccharides obtained from natural sources or chemical synthesis to an aminolipid by reductive amination. NGL mixtures were then separated by high-performance thin-layer chromatography. NGL microarrays containing the purified NGLs were probed with proteins and sequences of recognized oligosaccharides were analyzed by mass spectrometry (MS). This method can be extended for glycomic research to map carbohydrate structures in glycoproteins or glycolipids that are recognized by specific proteins.

Another important application of this technology is high-throughput screening of new inhibitors of carbohydrate-processing enzymes that are involved in the biosynthesis of the disease-related carbohydrates, as well as novel modulators that disrupt carbohydrate-protein interactions for drug discovery (Figure 6f). Earlier, there was no example for this application with carbohydrate microarrays. However, very recently, it was reported that fucosyltransferase (FucT) inhibitors were screened with microtiter-type carbohydrate arrays.[18] LacNAc, a substrate for FucT, immobilized on the microtiter was incubated with 85 synthetic compounds in the presence of FucT and GDP-Fuc, and then probed with peroxidase-coupled *T. purpureas* (a fucose specific lectin). Four inhibitors with nanomolar K_i's were discovered. Although the reported screening method should be further improved to apply carbohydrate microarrays for drug discovery, this technology has a potential for development of new inhibitors.

Conclusions and Perspective

As described above, carbohydrate microarrays can be used in various research fields such as glycomics, drug discovery, and diagnosis. However, a few limitations need to be overcome for wide applications of this state-of-the-art technolo-

gy. First, glycan microarrays should be improved for detecting proteins with weak binding affinities. Although many examples exhibited multivalent interactions between the immobilized carbohydrate probes on the solid surface and proteins, these interactions may be not enough to detect weakly binding proteins. One possible solution is to fabricate carbohydrate microarrays containing multivalent carbohydrate probes on the surface to enhance their binding affinity with proteins. Another method is to prepare chip bases that contain functional groups which crosslink proteins upon their binding to the immobilized carbohydrates.

Second, efficient detection methods should be developed for the optimal use of carbohydrate microarrays. Fluorescence detection with fluorophore-labeled proteins has been most widely used, because of its high sensitivity. However, protein labeling often results in protein denaturation and/or interference with carbohydrate ligand binding. Recently, a method to prevent denaturation of proteins during their labeling with fluorophores was developed.[19] Proteins labeled by fluorophores at the C termini were isolated by in vitro expression of proteins in the presence of fluorophore-containing puromycin derivatives. As fluorophore labeling proceeds during protein expression, an extra protein-labeling procedure can be omitted. Furthermore, C-terminal modification of proteins may rarely interfere with their binding to carbohydrates. However, detection techniques that do not require protein labeling are still more useful. Surface plasmon resonance (SPR) spectroscopy is suitable for this purpose. This method does not need labeled proteins and its high sensitivity allows the detection of low-affinity binding. However, SPR cannot be applied to characterizing protein–carbohydrate interactions in a high-throughput manner. Recently, SPR imaging technology has been developed to overcome this limitation.[20] This technology was applied to the detection of RNA–DNA and protein–DNA interactions with DNA arrays. Mass spectrometry can also be used for detecting the modification of carbohydrates on glycan microarrays.[21] For example, enzymatic reactions by carbohydrate-processing enzymes on carbohydrate microarrays were characterized by using MALDI-TOF MS.[21b] This technique was also applied to the determination of the time-dependence of enzymatic glycosylation.

Third, usefulness of carbohydrate microarrays depends on how the diverse carbohydrate probes are immobilized on the surface. Natural carbohydrate ligands are more useful for studies of carbohydrate–protein interactions than simple synthetic ones. However, isolation of carbohydrates from cells or glycoconjugates produces only a limited amount of natural glycans. Moreover, the isolated glycans should be modified for immobilization on the surface. Synthetic methods are more convenient for obtaining a sufficient amount of diverse and modified carbohydrates. Over a decade, new synthetic methods (e.g., automated oligosaccharide synthesis, programmable one-pot synthesis of carbohydrates, and combinatorial synthesis of carbohydrates) have continuously emerged to prepare complex oligosaccharides, and the improved methods will certainly provide structurally diverse and complex carbohydrates.[22] Once various carbohydrates are obtained, systematic studies of carbohydrate-recognition events with carbohydrate microarrays can be performed. If the limitations described above are overcome in the near future, carbohydrate microarrays will be more applicable for biological studies and biomedical research.

Acknowledgements

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