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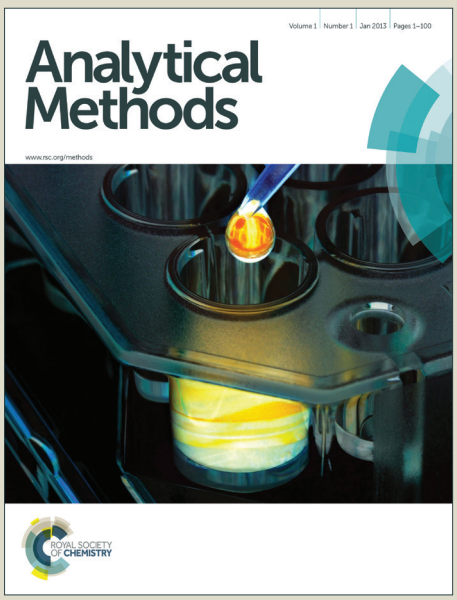

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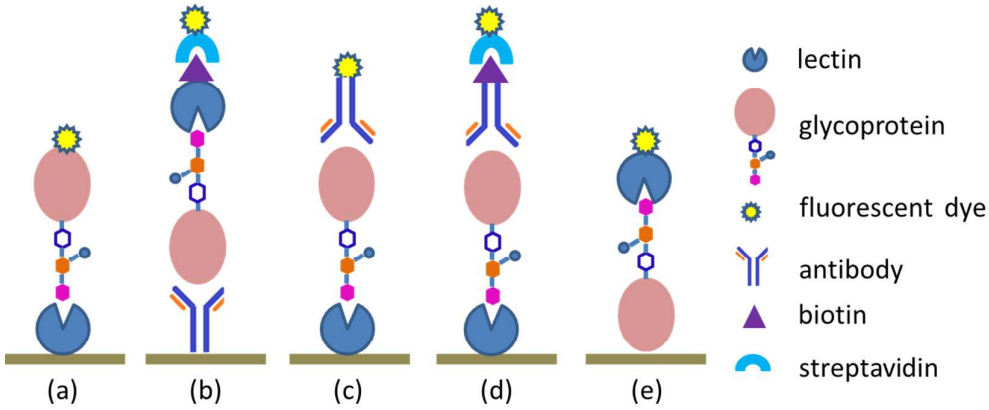

The lectin microarray technology with high-throughput and flexibility of assays might fulfill all criteria needed for direct, rapid and multiplexed monitoring of glycan profiling

Recent advances in the fabrication and detection of lectin microarray and its application in glycobiology analysis

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Abstract

Lectin microarray is increasingly used as screening tools with a high potential for glycobiology analysis, providing a high-sensitivity profiling of complex glycan features in a short time without the need for release of glycan. Application of the technology for rapid differential glycan profiling of target samples resulted in the discovery of new glycan-related biomarkers for disease detection and particular types of glycobiology studies. And the lectin microarray might offer many opportunities for further innovation, and its range of applications to the life sciences promised to be greatly expanded. Herein we describe the advances in the fabrication and detection of lectin microarray technologies and its application, which herald even more exciting breakthroughs in the coming decade.

1. Introduction

Carbohydrate modifications on proteins and lipids show a high structural diversity reflecting inherent functional roles for particular biological environments.[1] For instance, protein glycosylation plays critical roles in various biological processes ranging from cell-to-cell communication, development and differentiation to infection by viral and bacterial pathogens.[2,3] Protein glycosylation is involved in a wide range of physiological processes, such as tumor growth, cancer inflammations, immunological disorders etc. However, glycans are complex and heterogeneous biomolecules, which arise from the ability to generate regioisomers and stereoisomers using the same monosaccharide building blocks.[4] Moreover, structural diversity of glycans is further amplified by the additional modification of component saccharides (e.g. acetylation, sulfation, phosphorylation).[5,6] The highly heterogeneous and complex structures of glycan make glycan profiling a challenging task. All of the conventional methods for the glycan profiling of glycoprotein, such as high-performance liquid chromatography (HPLC), capillary electrophoresis (CE) and mass spectrometry (MS), or their combinations, generally require subsequent

labeling and glycan release of the attached glycans from their core proteins.[7,8] Therefore, these conventional techniques for glycan analysis are necessarily complicated, laborious, and time-consuming.

The lectin microarray technology with high-throughput and flexibility of assays might fulfill all criteria needed for direct, rapid and multiplexed monitoring of glycan profiling.[9] The use of lectin as a glycan profiling tool has become much more sophisticated with the introduction of microarray, in which panels of lectin with distinct glycan-binding properties are immobilized on a single chip for glycan analysis. Although the technique is not quantitative and does not allow complete determination of glycan structures as MS can, matching of identical glycan profiles can fall into the range of this technology. Since the first publication in 2005, the lectin microarray technology has attracted increasing attention and advanced rapidly. To improve the sensitivity and specificity of the lectin microarray, the discovery, purification and recombination of lectin, immobilization techniques, and detection approaches of lectin microarray have been became main directions of development. And some recent works focused on various application of the lectin microarray in the differential analysis of subtle changes in the glycosylation pattern of cell lines, in medical fields for development of disease-related biomarkers, and so on. [10-12]

A number of reviews have recently been published either fully focused on the topic of lectin microarray, or underlines a key position of lectin microarray in hot area of glycomics.[13,14] In this article, we have given an overview focused on the fabrication and detection methods in lectin microarray technology.

2. Lectin microarray platform

Lectin microarray is gaining popularity because of the high throughput capabilities, rapid and comprehensive monitoring of carbohydrate alterations, good reproducibility as well as low amounts of samples required. The technology is an attractive platform for detecting glycan patterns on intact biological structures (including whole cells), and providing glycosylation differences and quantitation by fluorescence of candidate glycoprotein biomarkers in a high throughput manner. Fig. 1 shows the schematic representation of lectin microarray strategies depending on the fabrication, detection and application, and these strategies are summarized below.

The lectin array (Fig. 1 (a)) is a direct assay format employing selected lectin

with known specificities immobilized on a suitable surface and then sample is loaded on the array. This subsequent monitoring of a binding event can be performed in several ways such as tagging the sample with a fluorescent dye like cyanine 3 bihexanoic acid dye (Cy3).[15,16] With the lectin array, differential analysis of subtle changes in glycosylation pattern of cell lines, for example, to compare normal and defective ones or to investigate the effect of various treatment conditions were reported. Recently, Etxebarria developed a high-throughput method for the rapid analysis of protein glycosylation in biofluids by combing electrophoresis protein separation with lectin-array-based glycan profiling into a single experiment. This approach can serve as a first qualitative attempt to analyze the glycome of an organism with a selection of 20 or more lectins, which can be followed up by more sophisticated mass spectrometry techniques for the structural identification of glycan biomarkers.[17]

A sandwich format of assays, including lectin-overlay antibody sandwich array (Fig. 1 (b)) and antibody-overlay lectin sandwich array (Fig. 1 (c) and (d)), is based on the well characterized and highly specific antibodies used on either for capture or for detection. Haab and co-workers [18] developed the antibody-lectin sandwich array (ALSA) and applied it to the glycan analysis of MUC1 and CEA in the serum of pancreatic cancer patients (Fig. 1 (b)). In this format, to avoid interaction with the lectin probe, the carbohydrate cis-diols on the captured antibody are oxidized by sodium perixdate. The derivatized antibodies are immobilized on a nitrocellulose slides. The sample may be loaded on the array without sample processing and detection is performed with various biotinylated forms of lectin and fluorescently-labeled streptavidin. And in order to properly interpret measurements of a glycan on a protein, the amount of glycoprotein captured from the crude sample may be determined by the amount of immobilized antibody.[19] The sample can be incubated repeatedly on replicate microarray, each time probed either with a lectin, to characterize glycan levels, or with an antibody, to characterize the core protein levels. Whenever possible, multiple antibodies to different epitopes on the target protein should be used to increase the confidence of the assay. Cao and co-workers [20] performed the assay using a 96-well plate format to profile 16 different glycoforms of proteins captured by 72 different antibodies in cyst fluid from mucinous and non-mucinous pancreatic cysts (n=22), and then tested a three marker panel in 22 addition samples and 22 blinded samples. Glycan alterations were not widespread

among the proteins but were mainly confined to MUC5AC and endorepellin, which were capable of discriminating mucinous from non-mucinous pancreatic cysts. The ALSA approach has shown to be an effective approach for profiling glycan variation on multiple proteins captured directly from a crude proteome.[21,22]

The antibody-overlay lectin array was initially developed by Kuno's group and applied to glyco-biomarker discovery (Fig. 1(c) and (d)).[23,24] The specific antibody is used for immunopurification, quantitation, and detection of the target protein on the array. The assay must be obtained from a targeted glycoprotein firstly, and then the isolated glycoprotein is quantitated by immunoblotting.[25] To suppress the unoccupied binding sites on lectin array, non-labeled "human" polyclonal IgG (hpIgG) were added to the array prior to adding the target-specific antibody. The blocking procedure is an effective way of eliminating background noise without any derivatization reaction to antibody. However, the incubation time must be optimized to suppress the analyte exchange between the pre-captured target glycoprotein and an excess amount of the blocker hpIgGs. In this sandwich configuration, sample processing steps may be incurred sample losses and thereby affected the sensitivity and the specificity of the method.

The glycoprotein–lectin array (Fig. 1 (e)) is a reverse phase dot blot lectin array formed by the immobilization of the purified sample (cellular lysate, tissue lysate or serum, etc.) containing glycoproteins on array surface, subsequently treated by incubation of the array with multiple labeled lectins. The assay have been successfully used for comparative studies monitoring individual glycosylation changes within a glycoproteome representing different biological states and have identified potential biomarkers in cancer.[26] The advantage of glycoprotein–lectin array is that the glycoproteins do not require labeling, while the disadvantage is that the high effective concentration of samples within one spot increases the tendency for spurious interactions.[27,28] In present, we emphasize the fabrication and detection techniques of the normal phase lectin microarrays (Fig. 1 (a)～(d)).

3. Challenge of lectin microarray

Now, it is very well recognized that lectin microarray may be used as a quick and convenient means for profiling of glycans. However, it should be noted that there are inherent challenges to the lectin microarray attributed to the lectin-glycan interaction, which shows different characteristics with antigen-antibody interactions (Table 1).

Firstly, glycan recognition is limited to the combined substrate specificities of the arrayed lectins.[29] Lectin are carbohydrate-binding proteins which are found in a many organisms ranging from bacteria to mammals. They can bind terminal or internal residues in an oligosaccharide and provide a rich source of probes for glycan profiling. However, the specificity of glycan recognition is usually based on structural epitopes rather than the entire glycan, so the structural elucidation of glycan is often impossible. And with the limitation of epitopes recognition, lectin used in the microarray is probably not sufficient to examine the full spectrum of glycans presented in biology.[30] Therefore, the discovery of new lectin and the detail characterization of glycan binding partners of known lectin are concerned by researchers. And combined with other technology, lectin microarray has the potential to elucidation of glycan structures. Secondly, in comparison to antigen–antibody binding interactions, lectin–glycan interactions are relatively weak, i.e., in dissociation constant (Kd), $10^{-7}\sim10^{-3}$ M, and are consequently harder to detect.[31,32] Therefore, it offers more challenges for the high spatial density of lectin immobilized in an array and for the novel detection techniques. Facing with above challenges, the lectin microarray technology has obtained much development.

4. Fabrication of lectin microarray

In this section, we highlight advances in lectin sources, immobilization, detection, which contributed to novel designs and assay capabilities.

4.1 Lectin sources

Lectin is a kind of glycan-binding protein with non-enzymatic in action and non-immune in origin.[33] And it can be discovered from a variety of species, ranging from viruses and bacteria to plants and animals. The discovery and characterization of new naturally occurring lectin is very important for further development of the field, widening possible applications.

The lectin microarray has been developed in recent years essentially by utilizing natural lectin, most of which are derived from plants and are commercialized. However, plant-derived lectins do suffer from multiple disadvantages. First, they are isolated and purified from natural sources. This leads to inconsistencies in activity and availability due to seasonal changes and differences in purification. Second, many plant lectin are glycosylated, complicating glycomic evaluation of complex samples, which may contain carbohydrate-binding proteins. In addition, these natural

lectins cannot provide a fully comprehensive repertoire to profile glycome complexity.

Recombinant lectin can address several issues associated with plant lectin. Hsu et al. [34] described an efficient strategy for the systematic creation of recombinant lectin for use in microarray technology. They demonstrated this strategy by creating a small panel of bacterially-derived lectin that is easy to purify and show reliable and well-defined activity. They utilized this panel to create a recombinant lectin microarray that is able to distinguish glycopatterns for both proteins and cell samples. The use of fusion-tags in recombinant lectin could aid greatly in controlled orientation of lectin during immobilization process, which might improve sensitivity. Propheter et al.[35] initially fabricated an oriented lectin microarray utilizing the interactions between glutathione (GSH) and glutathione-S-transferase (GST). They also presented a unique method for orienting GST-fusion lectin in situ on an NHS-activated surface by the creation of localized GSH-scaffolds via a one-pot protein immobilization strategy.[36]

Furthermore, in order to overcome the limitation of natural lectin, multiple mutated lectin with new recognition patterns and tailored specificity has been constructed. Maenuma et al. constructed multiple mutated lectin, which were then used for the binding assays with various cell lines. The binding profiles of each cell line were specific and the results of cluster analysis revealed quantitative similarities in the binding profiles of cells with similar origin. These results indicated that a library of mutated MAH was useful as a tool for the profiling of various cells based on the variations of the surface glycans.[37] To develop a satisfactory repertoire of Sialic acids (Sia)-binding lectin, Yabe et al.[38] showed a rational creation of a Sia-binding lectin based on the strategy “natural evolution-mimicry”, where Sia-binding lectin are engineered by error-prone PCR from a Gal-binding lectin used as a scaffold protein. However, the derived mutant, designated Sia-recognition EW29Ch (SRC), showed relatively low affinity for a 2-6Sia compared with naturally occurring lectins, because of its monovalency. They then engineered a tandem repeat construct (SRC2) showing substantial affinity for a 2, 6-sialylated N-glycans, almost comparable to a natural a2-6Sia-specific lectin from Sambucus sieboldiana (SSA).[39] Heu et al. [40] successfully engineered a novel 6’sulfo-Gal-specific lectin for which no practical probes have been available. Thus, the lectin should be highly useful for future studies involving sulfated glycans, i.e. sulfoglycomics.

A parallel approach for glycan profiling based on artificial recognition elements, such as DNA/RNA aptamers or modified DNA /RNA aptamers (amine or boronate derivatives of nucleotides), borolectins, peptide borolectins and small recognition element based on boronate can be a useful alternative to complement natural lectin and their mutants.[41-45] The Lavigne lab [42-43] reported the design, synthesis and utility of boronic acid functionalized peptide-based synthetic lectins (SLs) binding to glycoproteins and highlighted efforts in library design optimization and peptide sequencing (Fig. 2). In a more recent study, they described the use of boronic acid functionalized SLs in an array format for the differentiation of structurally similar cancer associated glycans and cancer cell lines. They also demonstrated the utility of SLs in recognizing glycoproteins with up to 50-fold selectivity, even in 95% human serum.[42] The incorporation of the boronic acid moiety into DNA would allow the selection to gravitate toward the glycosylation site and therefore for the specific recognition of the glycosylation site. Li and coworkers developed a platform method for the selection of DNA aptamers for glycoproteins with the ability to differentiate glycosylation variations. This was the first time for the selection of boronic acid-modified aptamers that allows for carbohydrate substructure focused selection of aptamers for a glycoprotein.[45]

4.2 Lectin immobilization

To improve the specificity and sensitivity of lectin microarray, the immobilization of lectin in the direct assay format has several implications. In a physiologically relevant context, multi-metric lectin often interacts with multiple glycans contributing to a high apparent binding affinity.[46] Qrientation of lectin on solid surfaces is a key element for accessibility of recognition domains to the solution phase affecting stability of immobilized lectin as well. Therefore, the high spatial density and orientation of lectin immobilized in an array format provides an ideal platform for the rapid and sensitive detection of glycan epitopes. Toward this end, lectin microarrays were developed wherein several lectins were immobilized to a suitable solid phase (microscopic slide), maintaining the conformation and the functionality of the lectin, and achieving maximal binding capacity. Lectin microarray basically relys on non-covalent and covalent attachment of lectin to some sort of surface. In immobilization methods mentioned above, individual lectin is immobilized mainly in a random fashion and in an oriented manner, respectively. Table 2 illustrates the number of studies performed to date along with methods of

immobilization, detection, and the nature of analytes.

Generally, non-covalent immobilized methods are thought to allow more molecular flexibility on the slides to more easily align binding sites. To date, the typical, simple and low cost technique is prepared by immobilizing lectin onto nitrocellulose using a contact spotter or non-contact printer.[47,48] Nagaraj and coworkers [49]developed a new technology (Lectin NanoProbe Array) based on piezoelectric liquid dispensing for non-contact printing and probing of a lectin array. In this technology, lectin was dispensed on to the nitrocellulose slide surface. Upon printing, they were irreversibly adsorbed. With piezoelectric lectin printing, the sensitivity of glycoprotein detection was improved due to the high density of lectin per spot on the array. Meanwhile equipment and procedures developed for DNA or protein microarrays are easily adaptable to the development of lectin microarray. Therefore, this physical immobilized method has become commonly applied for the fabrication of lectin microarray. Other different planar surfaces can be also used for lectin microarray, such as gel slide and glass slide.[50] To sustain the natural activities of lectin in the internal aqueous phases, Koshi et al.[51] introduced a new lectin microarray using a supramolecular hydrogel for fixing fluorescent lectin. Under the semi-wet conditions, lectin denaturation was effectively suppressed so that the embedded lectin acted as a talented molecular recognition scaffold toward specific saccharides. The specific recognition reactions between biotin and avidin system were also effectively used for lectin microarray fabrication. Angeloni et al. [52] immobilized lectin on Opto-Dex-biotin plates through biotin-neutravidin-biotin bridging. The Opto-Dex-biotin platform was based on dextran coated glass slides, which was derivatized with aryl-trifluoromethyl-diazirine groups. On illumination, aryl-trifluoromethyl-diazirine groups formed reactive carbenes, which eventually reacted with any vicinal molecule to form covalent bonds and lead to covalent linkage of any molecule of interest to the surface. As for the OptoDex-biotin coating, biotin was linked to the dextran polymer. Four biotinylated lectins were then successfully immobilized on Opto-Dex–biotin plates using neutravidin as a bridge. In another early example of lectin microarray fabrication, Carlsson et al. [53] linked amino-biotin on the gold wafer derivatized with N-hydroxysuccinimide (NHS) ester monolayer. The biotinylated surface was then incubated with Streptavidin followed by biotinylated lectin. DNA hybridization method has also been applied to the immobilization of lectin to the planar surface. Fromell et al. employed this method

coupling the model lectin ConA, to polystyrene nanoparticles via a poly(ethyleneoxide) linker which protected the lectin from denaturation and prevented unspecific protein adsorption. The Con A-coated particles were then attached to the analytical surface via hybridization of complementary oligonucleotide pairs. Since the oligonucleotide sequence can be varied in a large number of ways, oligonucleotides-driven immobilization gave the possibility to generate a nearly infinite number of specific binding sites and to offer minimal steric hindrance to glycoprotein/cell binding.[54]

In the above-mentioned methods, there is a lack of absolute control in guaranteeing the optimal orientation, native multimeric quarternary structure, optimal multivalent clustering of carbohydrate recognition domains of lectin and their metal ion requirements, such as Ca^{2+}, Mg^{2+}, etc. A recent innovation in lectin microarray technology arises from the oriented deposition of recombinant bacterial lectin containing a glutathione- S-transferase–fusion domain. As shown in Figure 3, Propheter et al. [35] initially fabricated an oriented lectin microarray utilizing the interactions between glutathione (GSH) and glutathione-S-transferase (GST). This approach can increase the accessibility of carbohydrate-binding site, thus improving the sensitivity of lectin microarray technology. In order to overcome the need for a premade GSH-surface and print both oriented GST-tagged lectin and native lectin on the same slide chemistry, Propheter et al. presented a unique method for orienting GST-fusion proteins in situ on an NHS-activated surface by the creation of localized GSH-scaffolds via a one-pot protein immobilization strategy. This oriented lectin microarray improves overall sensitivity and limits of detection by site-specific orientation of recombined lectin.[36]

Until now, preferred immobilization protocol involves covalent attachment of lectin to appropriately derivatized surface. A series of lectins are immobilized on glass slides modified by various active functional groups (epoxy or NHS-ester-modified) through the amine functional group of lysine side chains of the protein-backbone of lectin.[55-64] Pilobello and co-workers [55] initially used a manual arrayer to print lectin microarrays on either aldehyde- or epoxide-derivatized glass slides, which yielded spots of 700 μm in diameter. In order to attachment of lectins on the glass slides, Kuno et al. [57] chose 3-glycidoxypropyltrimethoxysilane as a silane-coupling reagent to prepare epoxy-coated glass slides for lectin fabrication. The commercial Nexterion H slide was widely used in the fabrication of lectin microarray.[60] This slide was coated

with a cross-linked, multi-component polymer layer activated with NHS esters to provide covalent immobilization of amine groups. The permeable, 3-D hydrogel coating on the Nexterion slides can preserve the native 3-D structure of lectin thereby maintaining lectin stability and functionality. Several studies employed gold surfaces promoting self-assembling of thiols bearing functional groups for lectin microarray fabrication.[63-65] Dai et al. [65] used mercaptoundecanoic acid and mercaptoethanol to form a self-assembled monolayer with amine reactive surface functionalities on gold thin film substrates for lectin immobilization. These substrates were chosen due to their high degree of chemical homogeneity and amenability to a wide variety of chemical modifications. Poly-dimethylsiloxane (PDMS), as an inexpensive, flexible and good compatible biological material, can be used as an immobilization platform for covalent grafting of lectins on slides surfaces or within the channels of microfluidic biochips.[66,67] Hu et al.[66] fabricated the multivial PDMS slide by polymerizing PDMS elastomer on a polystyrene mold. Lectins were then covalently immobilized on PDMS surface through the use of silanization with aminopropyltrimethoxysilane and cross-linking with glutraldehyde.

Chen [68] developed a novel boronic acids- modified glass slide for the oriented and covalent fabrication of Fc-fused lectin microarrays. Boronic acids are known to form a stable but reversible cyclic ester (boronate) with the cis diol of a saccharide in aqueous media at room temperature. This fabrication method provided higher target sensitivity due to improved surface exposure of the carbohydrate-binding site. However, this format restricted the diversity of the lectin panel, thus requiring the use of eukaryotic expression systems to produce lectin glycosylated at a unique site, which is a modification that increases the potential for false positives during analysis. Further development in controlling both density and orientation of lectins on the surface can be achieved by peptide borono lectins or using nanotechnological tools.[69]

5. Detection of lectin microarray

Due to the weak interactions between lectin and glycan, and low abundance of biomarkers in biological sample, the high sensitivity, specificity and stability of lectin microarray platforms are in high demand. There are two functional components to meet: recognition elements for binding with target glycoproteins and a transduction process to signal the binding event. Thus, the detection systems of lectin microarray technology have been significantly improved. There are two major classes of

detection schemes commonly available for construction of array type biorecognition: label-based (especially fluorescence labeling) or label-free techniques.

5.1 Label-based techniques

Label-based techniques have been dominantly employed in lectin microarray detection due to their ease of use, common availability of reagents and simple instrument requirements. They mainly rely on the use of fluorescent dye labels, such as cyanines, nucleic acid dye SYTO 85 and Cell-Tracker CMRA and other nanoparticles.[70,71] Cyanine-3 or -5 are the most commonly used fluorophores for microarray detection because of their decrease dye interaction, brightness and ability to easily label purified glycoproteins, specific antibodies, protein lysates, or body fluids samples on the amine of proteins via NHS-coupling chemistry. Nucleic acid dye SYTO 85 and Cell-Tracker CMRA are stained for intact cells and then incubated with the lectin array to profile cell-surface glycans.

Detection by label-based techniques can be carried out either by direct labeling or indirect labeling. In the direct labeling method, the glycoprotein samples or lectins are labeled directly with fluorescent dye, which are captured by the lectin array (Fig.1 (a)) or the glycoprotein–lectin array (Fig. 1 (e)) and subsequently extensively washed and measured using a confocal-type fluorescence scanner. In the direct labeling method, single-color lectin microarray experiments have been used to study differential glycosylation patterns in glycoproteins, demonstrate cell type– and developmental stage–specific glycosylation, and distinguish between pathogenic and nonpathogenic bacteria.[72] Furthermore, based on the quenching of fluorescence resonance energy transfer, Koshi et al. developed a bimolecular fluorescence quenching and recovery (BFQR) method for saccharide detection in lectin microarray.[51] As shown in Fig. 4, the noncovalently fixed fluorescent lectin acted as a talented molecular recognition scaffold toward specific saccharides, which competed with the quenchers and recovered the fluorescence for detection. A series of saccharides-lectin interaction can be read-out without tedious washing processes and without labeling the target saccharides. However, there were several disadvantages. The detection limit was suppressed because of the competitive quencher. With BFQR, the LOD was determined as 0.5 μg/μL (30 μM) for ribonuclease B. And, to extend the arrayed lectin, synthesis of the corresponding quenchers for each lectin and the subsequent optimization of the lectin/quencher

pair were required. Although a perfect one-to-one type of discrimination was always ideal in analyzing a target molecule, it was difficult for the recognition of structurally complicated and diverse biological glycoconjugates. In differential analysisusing lectin microarray, Pilobello et al.[73] presented a dual-color ratiometric detection approach for the comparison of whole mammalian glycomes. In this approach, cellular micellae were labeled by coupling of either Cy3-NHS or Cy5-NHS with the lysines on protein. Equivalent amounts of the Cy3- and Cy5-labeled samples were mixed and hybridized to each lectin microarray. They demonstrated the accuracy and reproducibility of this lectin microarray system and successfully applied this system to the examination of dynamic glycosylation changes upon cell differentiation. However, in this glycomic dual color method, "competition'' between immobilized lectin toward a set of various glycans exists. Therefore, quantitative comparison of lectin signals needs careful consideration.[74,75] However, the drawbacks of direct labeling include low sensitivity, chemical modification of the sample or lectin, and the disruption of interactions between them.

In sandwich format of assays (Fig. 1(b), (c) and d)), the indirect labeling methods are generally used. This technique offers higher specificity and high sensitivity because of low background labeling and signal amplification. Biotin labeling, together with fluorescent dye-conjugated streptavidin, has been used for effective detection of fibrosis-associated glycosylation change alteration of AGP.[76] To avoid extensively washing process of the weakly bound glycan or glycoproteins, Hirabayashi et al.[15] demonstrated evanescent-field fluorescence detection (EFFD) in lectin microarray. It allowed relatively weak interactions to be analyzed on the array under equilibrium conditions without washing procedures. And another advantage of the evanescent-field fluorescence scanning is the feasibility of real-time detection, as the interaction can be observed in liquid phase under equilibrium conditions. In fact, the EFFD assay shows high sensitivity, for example, the limit of detection (LOD) for asialofetuin (ASF) is 100 pg mL^{-1}.[77] Therefore, the EFFD method offers huge potential for glycobiology analysis. To enhance the sensitivity of glycan profiling, Meany et al.[62] described a novel tyramide signal amplification (TSA) method for the antibody-overlay lectin microarray procedure shown in Fig. 5. In this method, after interacted with lectin microarray, the target glycoprotein was subsequently binding with the biotinylated detection antibody. And the loaded streptavidin-horseradish peroxidase (HRP) catalyzed the localized deposition of biotin tyramide, resulting in

high level of biotin which might be detected by streptavidin-labeled Cy3 with increasing the sensitivity of glycan profiling. Meany et al. applied this signal amplification method to the detection of lectin microarray and showed that TSA increased the sensitivity of the microarray over 100 times using the model protein prostate specific antigen (PSA). Recently, novel lectin multimerization method was evaluated by Cao in the lectin-overlay antibody array assay.[78] In the conventional method, biological sample are loaded on antibody arrays to capture the targeted glycoprotein. And various biotinylated lectin and fluorescently-labeled streptavidin is added sequentially. But, in the novel multimer method, the perfomed multimer complex, pre-incubated by biotinylated lectins with streptavidin, is added in a single step, potentially achieving enhanced binding through multivalent interactions. The LOD of fibronectin in plasma was reduced from ～40 to ～1 μg/mL with this multimerization method. This strategy can lead to the more sensitive and informative detection of glycans in biological samples and a broader spectrum of lectins that are useful as analytical reagents.

The label-based detection methods described above rely on conventional fluorescence labeling, which has several potential drawbacks, such as the lack of sensitivity and photo-instability of the dyes employed.[79,80] To overcome these disadvantages, many novel labels such as gold nanoparticles, dye-doped silica nanoparticles and quantum dot (Qdot) have then been utilized for label-based detection. Gao et al.[81] developed a gold nanoparticle labeled lectin microarray-based assay for screening glycoconjugates on the microbial surfaces. They demonstrated that the developed gold-nanoparticle-labeled array was suitable for identifying the binding affinity of lectin with bacterium, as well as determining the bacterium with high sensitivity. In glycoprotein-lectin array, Jeong et al. [82] presented a novel probe of Qdot-lectin nanoconjugates to interrogate the surface glycans of tissues and patterned cells. The approach allowed highly sensitive in situ monitoring of specific lectin–glycan interactions and quantitative information on surface glycans for each examined cell line and tissue. It may be applicable in cancer diagnostics. Recently, Wang et al. [83] used dye-doped silica nanoparticles to label the glycans to study glycan-lectin interactions in the lectin microarray platform. The employed nanoparticles acted as efficient multivalent scaffolds that can enhance the affinities of glycans to the lectin. 4～7 orders of magnitude increase in affinity over the free glycans with the corresponding lectins was observed in their results. Thus, the new

developed lectin microarray platform enabled the identification and analysis of glycan epitope structures having weaker affinity than the parent glycans.

5.2 Label-free techniques

Label-based approaches require additional labeling procedure, which can possibly introduce an unwanted change of the bio-recognition pattern. Therefore, several label-free approaches that measure an inherent property of the query molecule itself, such as mass, dielectric or optical properties have been employed for the detection of lectin microarray, such as surface plasmon resonance (SPR), scanning ellipsometry (imaging), single optical microscopy and MALDI-TOF MS. SPR is based on the creation of surface plasmons, that is, oscillations of free electrons that radiate parallel to a metal or dielectric interface, and measures change in refractive index very close to the surface.[84] It is a powerful optical technique for monitoring of binding events between molecules in real time, e. g. antibody-antigen interactions, lectin-saccharide or glycoprotein interactions. 8-lectin panels together with SPR have been applied in differentiation studies of human serum glycoproteins from healthy individuals and patients with various bacterial infections.[85] Based on light being reflected at a solid interface, scanning imaging ellipsometry has also been used for making images of lectin-glycoprotein interactions at gold-coated wafers.[86] Due to its sensitivity and non-destructive nature, optical methods are advantageous in recording lectin-carboxyhydrate interactions at solid surface. Chen et al. [87] described the characterization of differences in carbohydrate expression patterns on normal and tumorigenic human breast cell lines by incubation cell suspensions with the lectin arrays. In this study, the bound cells were observed by microscopy. As mentioned before, lectin microarrays do not provide in-depth structural information on the glycans compared to mass spectrometry. Therefore, the combination of lectin array with mass spectrometry was reported for analysis of glycoprotein. Hu et al. [88] demonstrated a lectin array on PDMS with MALDI-TOF-MS for the glycosylation analysis of healthy and oral cancer sera. After incubation and washing steps, 2, 5-dihydroxybenzoic acid matrix was directly layered on each vial. The array was subsequently attached to an MALDI plate for MALDI-TOF MS analysis. The detection method can provide accurate mass measurement and resolve the multiple glycoproteins bound to each immobilized lectin on slide. For high-throughput glycoprotein biomarker screening, an antibody–glycoprotein sandwich assay together with a fluorescent lectin and MALDI-MS was applied to quantitatively measure

glycosylation levels and identify analytes captured on the antibody arrays, respectively. The MALDI-MS detection of the tryptic products of the captured protein on the array serves as a complementary technique to verify the identity of the target of the antibody and a means to monitor the nonspecific binding. For profiling of a relatively small amount and obtaining detail structural information of a target glycoprotein, the detection sensitivity and complementary protocol of MALDI-TOF MS combined with lectin array needs to be improved.

These novel detection techniques have greatly promoted the development of lectin microarray. With improved sensitivity of label-based techniques, they continue to be the preferred method of lectin microarray detection. Besides continuous advancements and increasing applications of label-based strategies, the label-free techniques may find widespread use in lectin microarray platform due to the benefits they offer in future. Further progress in detection techniques will provide enhanced sensitivity and specificity, and further miniaturized array formats.

6. Lectin microarray applications

As a rapid, sensitive and high-throughput platform for glycan analysis, lectin microarray has been widely used in glycobiology analysis. A distinct application of lectin microarray lie in rapid evaluation of purified glycoproteins, such as glycoprotein drugs.[89] Based on the binding of an intact glycoprotein drugs to an array of lectins, the result of the characteristic fingerprint, which is tightly associated with the glycan composition of protein, has been demonstrated. With the development of the ratiometric two-colour lectin microarray method, lectin array has also been used for rapid differential glycan profiling, such as bacterial cells, fungal cell or different types of mammalian cells (species and states). Heu K.L. et al. [60] applied the lectin microarray technology to the examination of bacterial glycans. The observed glycosylation patterns can successfully distinguish pathogenic from non-pathogenic Escherichia coli strains. And they also showed that lectin microarray technology was a powerful method to quickly evaluate dynamic changes in surface glycosylation of bacteria in response to environmental stimuli. Accordingly, the other manifestation was made by Ebe et al. using CHO and its LEC mutant strains, which applied detergent solubilized cell membrane fractions as a glycoprotein source.[90] The procedure of lectin microarray technology is simple and proves to be applicable in glycome diagnosis of HIV virus. With the lectin microarray, Krishnamoorthy L. et al. [16]

revealed that the glycome signatures of intact HIV-1 virions and host cell microvesicles were almost identical, which explains well the origin of the virus particles. This study provided important support for the 'exosome' hypothesis of viral release. Maeda et. al.[91] also reported a lectin microarray study of glycoantigens in neonatal porcine islet-like cell clusters (NPCCs), which contained useful information for the future production of immunomodified pigs with less antigenicity than a-Gal transferase knockout (GalT-KO) pig toward clinical applications of NPCCs. Another example of the successful application of the lectin microarray is glycome diagnosis of a series of stem cells. Using high density lectin microarray, Tateno et al [92] performed a more comprehensive analysis using 114 types of human induced pluripotent stem cells (iPSCs) generated from five different somatic cells (SCs) and compared their glycome profiles with those of human embryonic stem cells (ESCs). Thirty-eight lectins discriminating between SCs and iPSCs/ESCs were statistically selected, and characteristic features of the pluripotent state were then obtained at the level of the cellular glycome. At last, rBC2LCN was found to detect only undifferentiated iPSCs/ESCs and not differentiated SCs. Hence, the high density lectin microarray has proved to be valid for not only comprehensive analysis of glycans but also diagnosis of stem cells under the concept of the cellular glycome.

Altered protein glycosylation occurs along with various biological phenomena, including development, tumorigenesis and metastasis. It has long been established that distinct glycan structures are associated with specific forms of disease. Apparently, the disease related glycome has great potential as a source of biomarkers. For the rapid and multiplexed analysis of protein glycosylation, the lectin microarray technology has been most intensively applied in the discovery of disease-related glycoprotein biomarkers. He et al.[93] used a method combining lectin microarray and LC -MS/MS to discover the cell surface glycoprotein markers of a glioblastoma-derived stem-like cell line. Two lectins *Trichosanthes kirilowii* agglutinin (TKA) and Peanut agglutinin (PNA) which can distinguish the two cell lines were used to capture the glycoproteins by affinity chromatography. The LC-MS/MS analysis and label-free quantification resulted in the identification of 12 and 11 potential glycoprotein markers from the TKA and PNA captured fractions, respectively. Western blotting analysis of 6 selected proteins confirmed the differential expression On the basis of the glycosylation profiling results of α1-acid glycoprotein (AGP), Kuno A. et al [76] developed a compatible multiple lectin-antibody sandwich immunoassay for

monitoring liver fibrosis. They locked AGP, glycosylation change of which has been reported to be closely associated with liver fibrosis. This established lectin-antibody sandwich immunoassay was subsequently automated to achieve rapid measurement within 20 min.[94] Chen et al. [95] developed a novel lectin-overlay antibody sandwich array with additional desialylated procedure for profiling aberrant O-glycosylation of CA125 in the diagnosis and management of primary invasive epithelial ovarian/tubal cancer (iEOC). They demonstrated that the Neu5Acα2, 6GalNAc (STn) and GalNAc (Tn) glycoforms on CA125 and MUC1 from primary iEOC patients could be detected in patients with a moderately elevated CA125 and a pelvic mass, improved discrimination of benign and borderline masses from primary iEOC. Fry et al. [96] applied lectin microarray technology to the discovery of specific lectin-binding signatures associated with metastatic breast cancer. A single 50 μm section of a primary breast tumor or <1 μL of breast cancer patient serum or urine was found to be sufficient to detect glycosylation alterations associated with metastatic breast cancer, as inferred from lectin-binding patterns. Ahuja et al. [97] reported the comparative lectin microarray profiles of the glycome associated with proteins of neonatal wild type (*wt*) and retinal degeneration 1 (*rd1*) mice retinae. Their results showed that the nature of glycans in postnatal days 2 (PN2), PN7, and PN14 *wt* and *rd1* retinal proteins was diverse, dynamic, retina-specific, and correlated quantitatively with neonatal retinal development and, to a limited extent, with retinal degeneration; the quantification of retinal glycans, namely Siaα2-3Galβ1-4GlcNAc, could be used as a diagnostic marker for evaluating the retinal electrophysiologic integrity during transplantation and therapeutic studies. Glycan profiling analysis by lectin microarray was performed by Inoue et al. [98] to identify the urine biomarkers for diabetic nephropathy. The increased signals of urine samples were observed in Sia α2-6Gal/GalNAc-binding lectins (SNA, SSA, TJA-I) during the progression of diabetic nephropathy. They also demonstrated that fetuin-A was a candidate to predict the progression of diabetic nephropathy.

In a more recent study, Kuno A. et al. [99] developed a new rapid and simple glycan-based immunoassay — FastLec-Hepa, which could detect unique fibrosis-related glyco-alteration in serum hyperglycosylated Mac-2 binding protein (M2BP) within 20 min. The serum FastLec-Hepa counts increased with advancing fibrosis and illustrated significant differences in medians between all fibrosis stages. This developed ‘‘on-site diagnosis’’ system enabled the direct measurement of the

disease-related glycoprotein without pre-treatment of serum. It will revolutionize the use of glycol-diagnosis in clinical medicine.

7. Outlooks and perspectives

In summary, lectin microarray is a promising technology in glycomics and glycoproteomics, providing fast, sensitive, and high-throughput profiling of the glycan structures. Although there have been many kinds of lectins from a variety of species, such as viruses, bacteria, plants, and animals, the lectins currently used in the microarray are probably not sufficient to examine the full spectrum of glycans present in biology. And certain patterns of glycans, such as those with specific sulfation patterns, O-manno, sialylated, O-fucosylated, and a considerable percentage of glycolipids are not yet represented on current lectin microarrays. Thus, the discovery of new lectin and the detail characterization of glycan binding partners of the known lectins are the long-term development field. Due to the weak interactions of lectin–glycan, the advancement of various fabrication and detection technologies has been spurred for lectin microarray technology. Novel modes of attachment to solid support using the glycan residues of the lectin are anticipated to provide optimal orientation while hiding away the interfering glycan components. As to the detection technology, due to rapid, simple and cost-effective assays, label-based techniques are continuous to be the preferred method of detection. However, label-free systems are carrying out multiplexed detection without the interference of any tagging agent. Some of the emerging label-free techniques include SPRi-based systems, silicon nanowire field-effect transistors and quantum dots and gold nanoparticles. Further progress in detection techniques will provide enhanced sensitivity and specificity, and further miniaturized lectin array formats.

Lectin microarrays have been proved a valuable and viable platform for biomarker discovery in early stage cancer. To further understanding of the biosynthetic pathways involved in glycan alterations, lectin microarray combined with other analytical tools (mass spectrometry, qPCR, and so on) is now routinely applied in the complex and challenging field of glycomics. At last, as the level of throughput continues to increase, bioinformatics takes on an ever greater importance in the organization and use of experimental data. Thus, the development of bioinformatics tools will lead to better data-mining and interpretation. As progress continues toward fully automated, high-throughput methodology for glycomic studies, we expect that

the many techniques used to study glycosylation as a system will unify into a single approach.

Table 1 Characteristics of lectin-glycan interaction and antigen-antibody interaction

	Lectin-glycan interaction	**antigen-antibody interaction**	**Refs.**
specificity of recognition	structural epitopes	antigen epitopes	29,30
affinity binding sites	diverse protein structural frameworks	a complementary surface (epitope) on the antigen	29,30
dissociation constant (Kd)	10^{-7}～10^{-3}	10^{-8}～10^{-12}	29,32
binding force	hydrogen bonding, vander Waals force, hydrophobic interactional force	electrostatic force, hydrogen bonding, vander Waals force, hydrophobic interactional force	29,30

Table 2 Summary of numbers of lectin, methods of immobilization, and detection method of lectin microarray

Mode of attachment		Coupling and slides	No. of lectins	Labling/detection	Analytes	Refs.
Non-covalent	Random	Absorbed in nitrocellulose slides	24	Cy3,NHS-Fluos and FITC-labeled/FS	Glycoproteins, glycoform mixtures derived from the glycoproteins	47
			30	Cy3/FS	Recombinant CTLY4-IgG fusion glycoprotein	48
			3	AnaTag HiLyte Fluor 555 dye/FS	Commercial glycoproteins	49
		Absorbed in gel-slides	13	Cy3/FS	Commercial glycoproteins	50
		Absorbed in a hydrogel	6	Fluorescein-quencher pair,recovery with sample/FS	Glucose, glycoproteins,oligosaccharides,	51
		Dextran glass slides via biotin	4	Cy3 and Cy5/FS	Commercial glycoproteins	52
		Golden glass slides via biotin	8	Unlabeled samples/imaging elipsometer	Sera from human, pig, sheep,and guinea pig	53
		Polystyrene nanoparticles via DNA	1	Alexa dyes/FS	Commercial glycoproteins	54
	Oriented	NHS coated slides via interaction between GSH and GST	7	Cy5 and phycoerthryin/FS	Commercial glycoproteins	35
			7	Cy3/FS	Commercial glycoproteins	36
Covalent	Random	Aldehyde and epoxy glass slides	9	Cy3/FS	Commercial glycoproteins	55
		Aldehyde slides	5	Anthranilic acid/Epifluorescence microscope	Heteroglucan from Pleurotus ostreatus mycelia	56
		Epoxy glass slides	39	Cy3/EFFS	Commercial glycoproteins	57
			43	Fluorescent dye/EFFS	Pathological AGC cells with or without IFCC	58

			45	Cy3/EFFS	Cell membrane extract of endometrial cancer cell and tissues	59
		Nexterion H slides	21	SYTO 85(nucleic-acid dye)/FS	whole cell bacteria	60
			68	Cy3 or Cy5/FS	Cell membrane, microvesicles and HIV virions	61
			38	Cy3,Tyramide Signal Amplification/FS	PSA enriched fromL LNCAP cells and commercial glycoproteins	62
		Gold–thiol–lipid–NHS-derived covalent linking-SAM	6	Fluorescein/FS and Unlabeled cells/optical microscope	Commercial glycoproteins and Whole mammalian cells	63
			7	Unlabeled cells/microscope	Normal and tumorigenic human breast cell	65
		PDMS slides	3	Unlabled samples/MALDI-TOF MS	Glycoproteins extracted from oral cancer and control sera	66
	Oriented	Boronic acids-modified glass slides	1	F3-biotin and streptavidin-Cy3/FS	Sample of biotinylated F3(glycoprotein from *Ganoderma lucidum*)	68

FS, fluorescence scanner
NHS, N-hydroxysuccinimide
GST, glutathione-S-transferase
EFFS, evanescent-field fluorescence scanners
AGC, advanced gastric cancer
IFCC, intraperitoneal free cancer cell
PSA, prostate-specific antigen(prostate cancer biomarker)
SAM, selfassembledmonolayer
MS, mass spectroscopy

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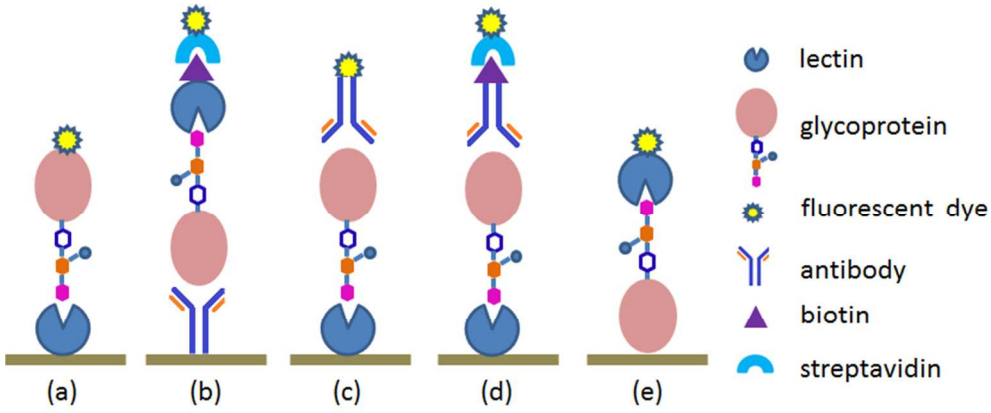
Fig. 1 Different platforms of lectin microarray. (a) lectin array, (b) lectin-overlay antibody sandwich array, (c) and (d) antibody-overlay lectin sandwich array, and (e) glycoprotein-lectin array.

Fig. 2 (a) Schematic representation of a phenylboronic acid substituted peptide (PBL, sequence chosen at random) binding to a glycan or glycoprotein. (b) Biased split-and-pool method used to generate the "low" diversity PBL library. (Reproduced from ref. 40, *Chem. Bio. Chem.*, 2007, 8, 2048–2051 with permission from Wiley-VCH.)

Fig. 3 Schematic for fabricating an oriented lectin microarray utilizing the interactions between glutathione (GSH) and glutathione-S-transferase (GST). a) 50 mm GSH, 100 mm $NaHCO_3$, pH 9.3. (Reproduced from ref. 33, *Chem. Bio. Chem.*, 2010, 11, 1203-1207 with permission from Wiley-VCH.)

Fig. 4 Schematic illustration of BFQR fluorescent detection system on hydrogel chip. (Reproduced from ref. 49. *J. Am. Chem. Soc.*, 2006, 128, 10413-10422 with permission from American Chemical Society. ª 2006 American Chemical Society.)

Fig. 5 Principle of detection of a target glycoprotein on antibody overlay lectin microarray using TSA. (Reproduced from ref. 60. *J. Proteome. Res.*, 2011, 10, 1425-1431 with permission from American Chemical Society. ª 2011 American Chemical Society.)



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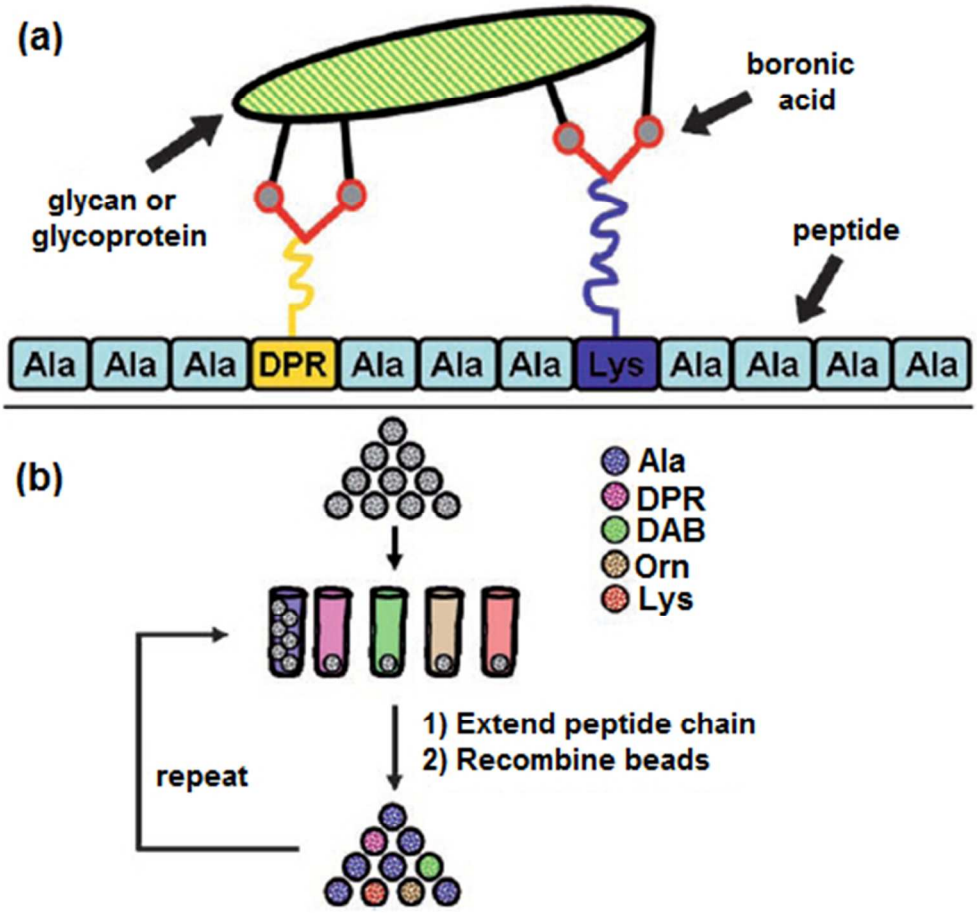

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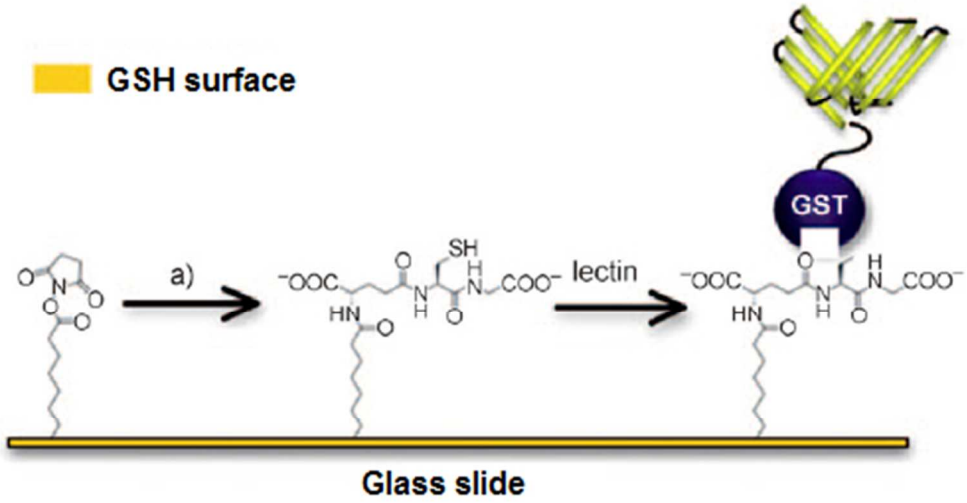
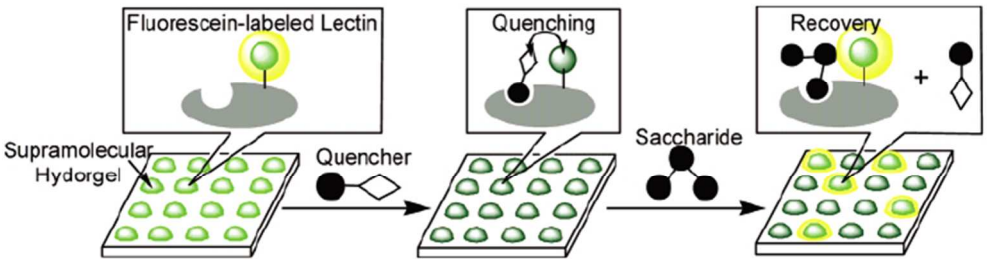


Schematic for fabricating an oriented lectin microarray utilizing the interactions between glutathione (GSH) and glutathione-S-transferase (GST). a) 50 mm GSH, 100 mm NaHCO3, pH 9.3. (Reproduced from ref. 33, Chem. Bio. Chem., 2010, 11, 1203-1207 with permission from Wiley-VCH.)
134x71mm (96 x 96 DPI)



Schematic illustration of BFQR fluorescent detection system on hydrogel chip. (Reproduced from ref. 49. J. Am. Chem. Soc., 2006, 128, 10413-10422 with permission from American Chemical Society. ª 2006 American Chemical Society.)
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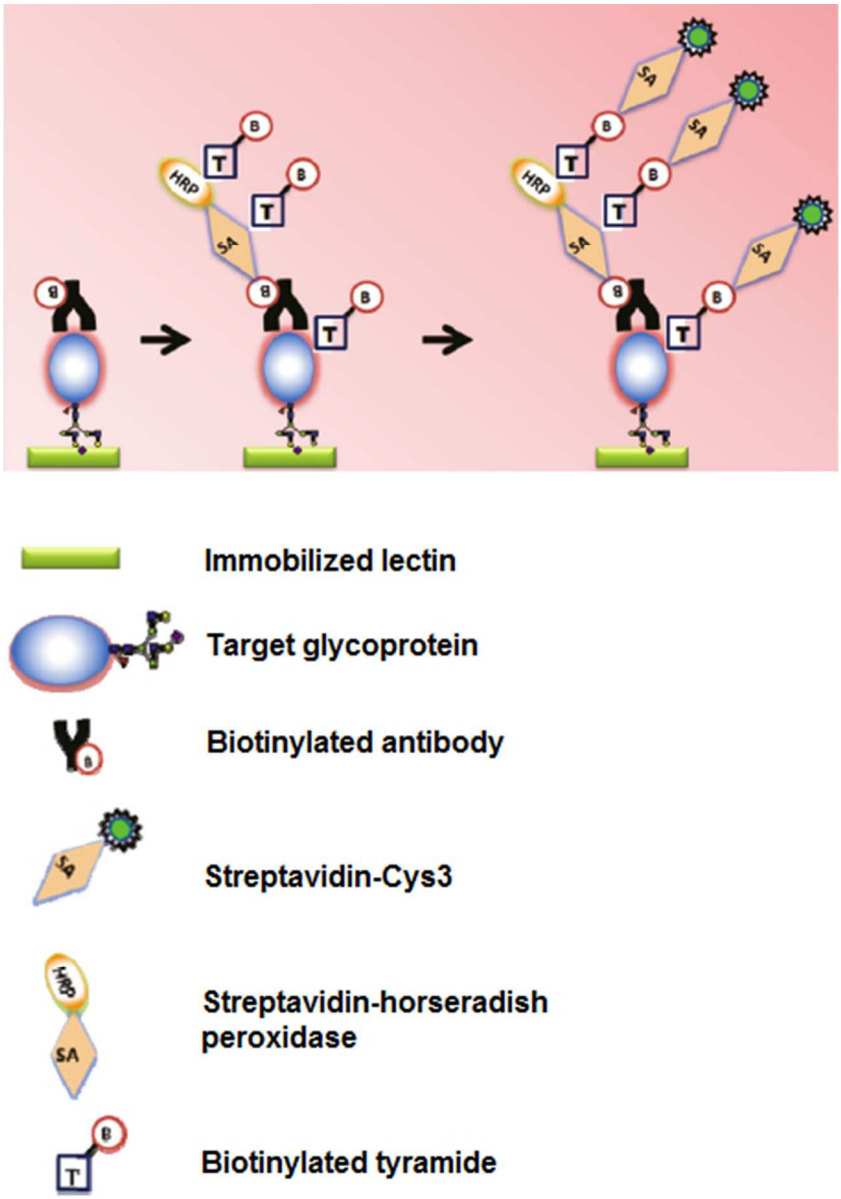

Principle of detection of a target glycoprotein on antibody overlay lectin microarray using TSA. (Reproduced from ref. 60. J. Proteome. Res., 2011, 10, 1425-1431 with permission from American Chemical Society. [a] 2011 American Chemical Society.)
120x172mm (96 x 96 DPI)