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Lectin microarray

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Abstract

Lectin microarray is a new technology that utilizes a panel of lectins immobilized on well-defined substrate for high-throughput analysis of glycans and glycoproteins. In this article, we have reviewed the fabrication and detection schemes in lectin microarray and discussed its novel applications in glycomics. We have also demonstrated a lectin array on PDMS with MALDI-TOF-MS for glycoprotein analysis. This method has been demonstrated for differential analysis of serum glycoproteins in oral cancer and healthy control subjects.

Keywords

Glycan; Lectin; Oral cancer; Protein microarray

1 Introduction

Lectins are a diverse group of proteins that have specific binding activity toward carbohydrate residues of glycoproteins and glycolipids. More than 300 lectins have been discovered from a variety of species, ranging from viruses and bacteria to plants and animals. These sugar-binding proteins are generally classified into five groups, according to the monosaccharide for which they exhibit the highest affinity: mannose, galactose/*N*-acetylgalactosamine, *N*-acetylglucosamine, fucose, and sialic acid. Lectins exhibit relatively high affinity and specificity for oligosaccharides of glycoproteins and glycolipids. These proteins, which function as recognition molecules in cell-molecule and cell-cell interactions, have been implicated in a number of essential biological processes including cell proliferation, cell arrest, apoptosis, tumor cell metastasis, leukocyte homing, and trafficking, and especially microbial (viral and bacterial) infection [1,2].

In addition to the clinical application in blood group typing, lectins (*e.g.*, fluorescent-tagged lectins) are employed as histochemical and cytochemical reagents for detection of glycoconjugates in tissue sections, on cells and subcellular organelles, and in investigations of intracellular pathways of protein glycosylation. Immobilized lectins, such as those that are covalently bound to agarose, silica, or other polymer stationary phases, are often used for the separation and purification of glycoproteins, glycopeptides, and oligosaccharides by affinity chromatography. Recently, lectin microarray has been developed for rapid and sensitive analysis of glycans in a high-throughput fashion [3–16]. These microarrays consist of a collection of, mostly plant-derived, lectins immobilized onto a solid support at a high

spatial density. Display of lectins in a microarray format enables multiple, distinct binding interactions to be observed simultaneously and therefore provide a novel method for the rapid characterization of carbohydrates on glycoproteins or glycolipids. In this article, we have given an overview on lectin micro-array technology, with a focus on fabrication and detection strategies of these arrays. We have also discussed a lectin array on PDMS with MALDI-TOF MS for analysis of serum glycoproteins.

2 Fabrication of lectin microarray

A schematic diagram for lectin array-base assay is shown in Fig. 1. The arrays have been fabricated on well-defined substrate through the means of either covalent bonding or physical adsorption for lectin immobilization. Commercially available array platforms such as PhotoChips and OptoDexbiotin in slide formats were initially used for preparation of lectin microarrays. PhotoChips is a thin-film slide coated with the dextran-based polymer OptoDex derivatized with aryltrifluoromethyl-diazirine (ATFMD) groups. On illumination, the ATFMD groups form reactive carbenes, which eventually react with any vicinal molecule to form covalent bonds and lead to covalent linkage of any molecule of interest to any surface of interest. Once lectins printed on the PhotoChips slide, a 4-min illumination with high-power UV lamp (1000 W) at 365 nm was sufficient to photo-activate the covalent bonding between lectins and the dextran polymer. As for the OptoDex-biotin coating, biotin is linked to the dextran polymer. Therefore stepwise affinity binding of neutravidin followed by microprinting of biotinylated lectins successfully yielded the desired lectin array on the OptoDexbiotin platform [3]. Recently, a lectin array consisting of a set of over 20 lectins, the Qiagen Qproteome™ GlycoArray kits, has been commercialized. The array-based assay has been demonstrated for rapid analysis of glycosylation profiles of multiple glycoproteins including the cancer biomarker prostate-specific antigen and recombinant human erythropoietin, which is used in replacement therapy for renal failure patients as well as for cancer therapy-induced anemia [13].

Mahal *et al.* initially used a manual arrayer to print lectin arrays on either aldehyde- or epoxide-derivatized glass slides, which yielded spots of 700 μm in diameter. Importantly, the use of glycerol in the spotting buffers must be reduced for protein-array printing because glycerol appeared to inhibit lectin binding to the glass surface [4]. Subsequently they prepared lectin microarrays by directly spotting the lectins on Nexterion H slides (50–60% humidity, 25–277°C) using a personal MicroArrayer. The permeable, 3-D hydrogel coating on the Nexterion slides can preserve the native 3-D structure of proteins thereby maintaining protein stability and functionality. After printing, the slides were allowed to incubate at room temperature for 1 h to ensure maximum coupling efficiency to the activated surface. The slides were then submerged in blocking solution (50 mM ethanolamine, pH 8.0) for 1 h, washed 3 times in PBST (PBS + 0.05% Tween 20) and finally washed with PBS [7,8].

Zheng *et al.* used *N*-hydroxy succinimidyl ester alkyl disulfide 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. Once spotted onto the gold film substrate, the lectins were allowed to react for 3 h in a humid chamber. The array was then incubated for 30 min in a solution of 1% BSA in PBS to minimize nonspecific binding [5]. In order to immobilize lectins on the glass slide, Kuno *et al.* chose 3-glycidioxypropyltrimethoxysilane as a silane-coupling reagent to prepare epoxy-coated glass slides. Lectins (at a final concentration of 0.25–1 mg/mL) were then spotted onto the epoxy-coated glass slide and incubated in a humid chamber (>80% humidity) at 257°C for 3 h for lectin immobilization. After spotting, excess nonimmobilized lectins were removed by

washing with TBS, pH 7.4 containing 1% Triton-X (TBST) and treated with blocking solution (1% BSA in PBS) for 1 h [6,12].

In contrast to the covalent bonding method for lectin immobilization, Koshi *et al.* introduced a new lectin micro-array using a supramolecular hydrogel matrix for embedding fluorescent lectins. A suspension of 0.25% gelator 1 W/V was heated to form a homogeneous solution. A 10- μ L portion of the hot solution was spotted on a glass plate and incubated to complete gel gelation under the high humidity at room temperature for 1 h. Mixed solution (1 μ L) of fluorescent lectins and quenchers was then dropped onto each resultant hydrogel spot. This allows fluorescent lectins noncovalently embedded in a hydrogel substrate. Under the semiwet conditions provided by the supramolecular hydrogel and protein denaturation was effectively suppressed so that the embedded lectin acts as a talented molecular recognition scaffold toward specific saccharides [9].

3 Detection in lectin microarray

Fluorescence detection has been the dominant detection scheme in lectin array-based assays. Therefore the labeling of biological samples with fluorescent probes is almost inevitably required prior to the analysis. In fact, fluorescent-tagged standard glycoproteins are often used to evaluate the linearity and LOD of the lectin array methods. For instance, the array signals were found to be linear for Cy3-labeled ovalbumin within the range of 50–300 μ g/mL, with an LOD of 10 μ g/mL [4]. The fluorescent detection of a known glycoprotein on the array can also be realized by the means of initial incubation with a biotinylated antibody (to the glycoprotein) followed by incubation with fluorescent-labeled streptavidin [15]. In order to profile cell-surface glycans, intact cells were stained with fluorescent probes (*e.g.*, nucleic acid dye SYTO 85 or Cell-Tracker CMRA) and then incubated with the lectin array. This allowed fluorescent detection of those cells bound to various immobilized lectins on the array [7,8,12].

In contrast to antibody-antigen interactions, lectins generally have low affinity to their ligands ($K_d > 10^{-6}$ M) and may not be able to survive from the extensive washing steps to remove unbound proteins. For this reason, Hirabayashi *et al.* demonstrated evanescent-field fluorescence detection (EFFD) in lectin microarray for weak interactions between lectins and carbohydrates in a liquid phase [6,10,16]. EFFD has been used extensively for biosensors to study real-time binding events on the glass slide surfaces. This method has an advantage to analyze relatively weak interactions (*e.g.*, between lectins and glycoproteins) because it does not require the washing step after a probing reaction. A second advantage of evanescent-field fluorescence scanning is the feasibility of real-time detection, as the binding reaction can proceed under equilibrium conditions. Therefore, lectin microarray with EFFD is useful for real-time evaluation of the binding between glycoprotein and immobilized lectins. This system was found sufficient for the detection of 50 ng/mL ($\sim$ 1 nM) of Cy3-labeled asialofetuin [6].

In order to achieve accurate comparisons between samples, Pilobello *et al.* demonstrated a ratiometric lectin array approach based on two-color labeling for differential profiling of cellular glycomes (Fig. 2A). In this approach, cells are lysed and membranes are isolated. Two isolated membrane samples are then labeled by coupling of either Cy3-NHS or Cy5-NHS with the lysines on proteins. Equivalent amounts of the appropriate Cy3- and Cy5-labeled samples are mixed and hybridized to each lectin microarray. After washing, the arrays are detected by using a fluorescent scanner. The resultant data are then analyzed, and annotated by using known lectin specificities, deconvolving the array data into carbohydrate patterns. This ratiometric lectin microarray approach was reproducible and successfully applied to the examination of dynamic glycosylation changes upon cell differentiation [11].

As for the lectin array prepared with the supramolecular hydrogel matrix, Koshi *et al.* demonstrated that a bimolecular fluorescence quenching and recovery (BFQR) method for detection of diverse glycoconjugates on lectin arrays [9]. A schematic illustration of the BFQR detection principle is presented in Fig. 2B. In this approach, a mixture of fluorescent lectins and quenchers are noncovalently embedded in a supramolecular hydrogel substrate. The lectins act as a talented molecular recognition scaffold toward specific saccharides, which compete with the quenchers and recover the fluorescence for detection. This enables one to fluorescently read-out a series of saccharides using the recognition selectivity and affinity of the immobilized lectin without labeling of the target saccharides. In addition, tedious washing processes are not necessary in this detection mechanism. However, the BFQR method has limitations. First, the sensitivity is suppressed because of the competitive quencher. With BFQR, the LOD was determined as 0.5 $\mu\text{g}/\mu\text{L}$ (30 μM) for ribonuclease B. Furthermore, synthesis of the corresponding quenchers for each lectin and the subsequent optimization of the lectin/quencher pair are required for extending the arrayed lectins. Although, a perfect one-to-one type of discrimination is always ideal in analyzing a target molecule, it is difficult for the recognition of structurally complicated and diverse biological glycoconjugates [9].

4 Applications

As a high-throughput platform for glycan analysis, lectin microarray has become a promising technology in glycomics and glycoproteomics. A distinct application of lectin microarray lies in rapid analysis of glycosylation profiles of glycoproteins. The method, as demonstrated in multiple studies, is based on binding of an intact glycoprotein to an array of lectins, resulting in a characteristic fingerprint that is tightly associated with the protein's glycan composition [13,15]. Lectin microarray is also a useful tool for quantitative analysis of lectin-glycoprotein interactions. For instance, by applying various concentrations of a fluorescent-tagged glycoprotein to interact with various lectins on the array, the K_{d} s between immobilized lectins and glycoprotein can be determined according to the binding isotherms [6].

Lectin microarray may prove to be a valid method for the discovery of glycan-related biomarkers of human cancers. By using a 43-lectin protein array for differential glycan analysis of formalin-fixed paraffin-embedded tissue sections of adenocarcinoma and normal epithelia of the colon, wisteria floribunda agglutinin (WFA) was found to clearly differentiate cancerous from normal epithelia with $p < 0.0001$. The obtained results correlated well with the subsequent histochemical study using biotinylated WFA. This approach was sensitive enough for glycan profiling of a tissue section consisting of approximately 500 cells [14].

Lectin microarray was also reported for profiling of bacterial cell-surface glycans. Intact bacteria labeled with a fluorescent dye were hybridized to a panel of lectins immobilized on the array. Analysis of the binding pattern and correlation with the known carbohydrate-binding specificities of the lectins allowed a fast assessment of glycans on the cell membrane. The glycan pattern observed clearly distinguished related *Escherichia coli* strains from one another, providing a fast and efficient means of bacteria typing. In addition, dynamic alterations in the cell-surface glycans of a pathogenic *E. coli* strain were readily monitored by using the lectin array. These indicate that lectin array-based assay is useful for not only bacterial typing but also real-time examination of the dynamic role of bacterial sugars in response to external stimuli [7,8].

Similar approach has been used for direct profiling of the mammalian cell-surface glycome (CHO cells). On the lectin array, CHO cells bound to both asialo complex-type *N*-glycan

binding lectins and to high mannose-type *N*-glycan binding lectins, but to lesser extent. This observation suggests that CHO cells predominantly express complex-type *N*-glycans. The glycan profile obtained for CHO cells was compared with those of their glycosylation-defective mutants, Lec2, Lec8, and Lec1. Lec2 having a CMP-sialic acid transporter-deficient mutation is known to express lower amounts of sialylated glycans. In fact, the signals for sialic acid-binding lectins were decreased, and enhanced signals were observed instead for Gal/GalNAc-specific *O*-glycan binding lectins likely due to the reduced incorporation of sialic acid. Lec8 has a deletion mutation in the Golgi UDP-Gal transporter, and hence, expresses much reduced galactosylated glycoconjugates. As expected, Lec8 showed a significant decrease in the signals for β -Gal binding lectins, whereas compensated signals were observed for an agalactosylated glycan (GlcNAc) and Tn (GalNAc) binding lectins. Lec1 is a β 1-2-*N*-acetylglucosaminyltransferase I-deficient mutant, which lacks both complex and hybrid type *N*-glycans whereas expresses increased level of high-mannose type *N*-glycans. Therefore, a significant increase was observed for the signals of mannose-binding lectins, whereas the signals for asialo binding lectins decreased substantially. The signals for a series of *O*-glycan binding lectins also increased, possibly because of total reduction of complex-type *N*-glycans. Similar approach was used to compare cell surface glycans of K562 cells before and after differentiation and a significant increase in the expression of *O*-glycans was found on differentiated cells. These studies clearly demonstrated that lectin array-based assay is useful for global profiling mammalian cell-surface glycans and can distinguish the glycosylation phenotypes of mammalian cells [12].

5 Lectin array on PDMS

We have recently demonstrated a lectin array on PDMS with MALDI-TOF MS for analysis of glycoproteins [17]. In our approach, we fabricated a multivial PDMS slide and then immobilized lectins on the surface of PDMS vials through the use of chemical modification (Fig. 3). PDMS was chosen as a substrate due to their high degree of chemical homogeneity and amenability to a wide variety of chemical modifications. PDMS as a material is also inexpensive, flexible, and compatible with biological applications. As shown in Fig. 3A, the multivial slide was prepared by polymerizing PDMS elastomer with a polystyrene mold. The PDMS slide was then oxidized and sequentially treated with 3-aminopropyltriethoxysilane (silanization), glutaraldehyde (cross-linking), and lectins for immobilization of lectins on PDMS surface (Fig. 3C).

Three lectins, Erythrina Cristagalli lectin (ECL), Jacalin, and peanut agglutinin (PNA) were immobilized on the surface of fabricated PDMS vials and evaluated for analysis of glycoproteins in both oral cancer and control subjects' sera. To allow for MALDI-TOF MS analysis, 2, 5-dihydroxybenzoic acid matrix (20 mg/mL in 0.2% TFA and 50% ACN) was directly layered on each PDMS vials. The slide was subsequently attached to an MALDI plate for MALDI-MS measurement. Figure 4 depicts the MALDI-MS spectra for the serum glycoproteins bound to Jacalin between oral cancer and matched control subjects. The cancer and control samples exhibited similar glycoprotein patterns, however two proteins of *m/z* 8027 and 35 358 were only observed in the cancer sample. Several other proteins also showed substantially higher levels in the cancer than in the control subject. The MALDI-MS spectra for the same cancer sample obtained from two different PDMS vials immobilized with Jacalin are shown in Fig. 4C. The almost identical spectra observed suggested the array-based assay is reproducible. Similarly, both ECL and PNA revealed more glycoproteins in the cancer than the control sample. These results indicate that lectin array is useful for harnessing the differentially expressed serum glycoproteins between cancer and healthy control subjects. MALDI-MS was used as the readout mechanism in our study because it provides accurate mass measurement and can resolve the multiple glycoproteins bound to each mobilized lectin on the array. However, the detection sensitivity needs to be

improved, especially for large glycoproteins. Sample prefractionation may help enrich proteins within certain mass ranges for a better sensitivity. In addition, depletion of high-abundance serum proteins may allow probing low-abundance glycoproteins on the lectin array.

6 Conclusion

In summary, lectin microarray is a promising technology in glycomics and glycoproteomics. The display of lectins in a microarray format enables a fast, sensitive and high-throughput profiling of the sugar structures with subtle differences. A well-demonstrated application of lectin micro-array is microbial typing based on profiling of cell-surface glycans. Pathogenic bacterial strains had distinct binding characteristics to lectins and therefore could be differentiated from the nonpathogenic strains based on lectin binding patterns on the array [7]. Lectin array-based assay also represents a novel method for the quality control during the bio-technological production of recombinant human protein (*e.g.*, antibody or peptibody) drugs, which are glycoproteins often produced from mammalian cell systems. The lectin array-based assay is useful as a screening tool for multiple samples in parallel, making it possible to monitor glycosylation profiles of the protein drugs throughout the stages of clone selection and optimization, process development, and manufacturing [13]. Lectin microarray may also have great potential in disease biomarker studies. Protein glycosylation has been shown as a key event in human disease progression such as cancer cell invasion and metastasis and many glycosyl epitopes actually constitute tumor-associated antigens. Many clinical biomarkers and therapeutic targets are glycoproteins such as prostate-specific antigen in prostate cancer, Her2 (ErbB-2) in breast cancer, cancer antigen 125 in ovarian cancer, and cancer antigen 19-9 in pancreatic cancer. Lectin microarray may turn out to be a promising tool for high-throughput analysis of minute clinical samples towards the identification of glycoprotein biomarkers for cancer detection.

However, this technology has limitations that remain to be addressed. First, the purpose of lectin array analysis is not to identify accurate glycan structures, but rather to obtain information of functional glycans that are recognized by a panel of lectins. Often a more sophisticated approach, *e.g.*, lectin affinity chromatography with tandem MS, is required for identification of glycoproteins or glycan structures [18]. A second limitation is the lack of commercial availability of lectins or other sugar-binding proteins that diversely recognize unique sugar structures. Since, cellular glycome is dynamic and complex, we anticipate to produce high-density arrays with a more diverse panel of immobilized lectins in order to achieve a more distinct and characteristic analysis of glycome [7,12].

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Abbreviations

BFQR	bimolecular fluorescence quenching and recovery
EFFD	evanescent-field fluorescence detection

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Figure 1.
Schematic diagram of lectin array-based assay. Adapted with permission from ref. [12].

Figure 2.

(A) Two-color ratiometric detection. In this approach, cells are lysed and membranes are isolated. Two isolated samples are then labeled by coupling of either Cy3-NHS or Cy5-NHS with the lysines on proteins. Equivalent amounts of the appropriate Cy3- and Cy5-labeled samples are mixed and hybridized to each lectin microarray. After washing, the arrays are scanned by using a fluorescent scanner. Reproduced with permission from ref. [11], Copyright 2007 National Academy of Sciences, USA. (B) BFQR detection. In this approach, fluorescent lectins and quenchers are noncovalently embedded in a supramolecular hydrogel substrate. The lectins act as a talented molecular recognition scaffold toward specific saccharides, which compete with the quenchers and recover the fluorescence for detection. Reproduced with permission from ref. [9].

Figure 3.

Fabrication of a lectin array on PDMS surface. (A) Fabrication of the multivial PDMS slide by polymerizing PDMS elastomer on a polystyrene mold. (B) A 16-vial PDMS slide. (C) Covalent immobilization of lectins on PDMS surface through the use of silanization with aminopropyltrimethoxysilane and cross-linking with glutaraldehyde.

Figure 4.

(A) MALDI-MS spectra for the serum glycoproteins bound to Jacalin between oral cancer and matched control subjects. (B) A zoom-in view of MALDI-MS spectra within m/z of 3000–11 000. (C) MALDI-MS spectra for the same cancer sample obtained from two different PDMS vials immobilized with Jacalin.