

The Hydroxyl-Modified Surfaces on Glass Support for Fabrication of Carbohydrate Microarrays

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Abstract: Glycan-protein interactions play important biological roles in biological processes. But there is a lack of simple high-throughput methods to elucidate recognition events between carbohydrates and protein. Although, there have been a number of glycan arrays developed in recent years utilizing different strategies and for different purposes, the method presented in this paper, a direct covalent immobilization of sugars to hydroxyl-modified glass surface, can be a very useful general method. Here, two strategies have been developed for the production of carbohydrate microarrays by the underivatized sugars that efficiently immobilized on hydroxyl-functionalized glass surface by formation of glycosidic bond with the hemiacetal group at the reducing end of the suitable carbohydrates via condensation. Firstly, untreated glass slides were amino- and epoxy-silanized, respectively. Then, they were further hydroxyl functionalized with different surface chemistries. The carbohydrate microarrays were fabricated on hydroxyl-functionalized glass by robotic arrayer. Additionally, experiments for the characterization of carbohydrates-protein interaction were performed to compare these strategies. Overall best results in terms of convenience and sensitivity were obtained with hydroxyl-functionalization on epoxysilanized surfaces. The hydroxyl-functionalized slide was used to detect the amount of mannose immobilized on the glass surface. The limit of detection of the fabricated mannose microarray was 100 nM.

Keywords: Carbohydrate microarrays, carbohydrate-protein interaction, glycosidic bond, hydroxyl-modified surface.

1. INTRODUCTION

More than 50% of all proteins carry various glycan chains. Glycans are a large group of Compounds consisting of sugars with diverse structures that are present both inside and on the surface of cells. They fulfill many different roles, but it is the way in which they interact with proteins that allows them to play an important role in a variety of biological events. Specific protein-glycan interactions underlie many aspects of important biological processes, for example, cell differentiation, cell adhesion, immune response, trafficking and tumor cell metastasis, which occurs through glycans of glycoprotein and glycolipid, as well as polysaccharide displayed on cell surfaces and glycan-binding proteins (GBPs) [1-6]. However, the functions of glycans in biology have not been extensively studied due to the more complex structures of glycans. Thus, tools for studying protein-glycan interactions are important to gain an understanding of biological functions and the roles of these interactions in biological processes. The presentation of glycans in a microarray format on solid surface can provide an efficient way to simultaneously monitor multiple binding events between immobilized glycans and GBPs as well as characteristics of glycans. However, it is difficult to find a robust, general, and controlled strategy for fabrication of the implementation of glycans microarray. An efficient slide surface for the construction of high-throughput glycan microarray has become

an attractive prospect, particularly according to the many biological activities and potential medical applications of glycans [7].

Recently, few methods have been developed to present both natural and synthetically derived glycan structures in array formats [7-9]. These methods differ in the mode of immobilization, e.g. non-covalent versus covalent, the nature of the surface upon which the immobilization takes place, and the diversity and complexity of the presented glycans. For example, polysaccharides and glycoproteins microarray have been fabricated on nitrocellulose-coated glass slides and hydrophobic black polystyrene substrate by non-covalent adsorption (i.e., passive adsorption) [10-13]. Non-covalent passive adsorption of underivatized glycans is considered simple as fabrication is relatively easy and it is possible to use a broad repertoire of naturally isolated glycans. It is important that immobilised glycans are tightly adsorbed and are able to resist repeated incubations and washings. On the other hand, oligosaccharide and monosaccharide microarrays have been covalently immobilized by using derivatized oligosaccharide and monosaccharide candidates on functionalized surface. For instance, the glycan arrays were fabricated on gold surfaces via Diels-Alder reactions between oligosaccharide- cyclopentadiene conjugates and benzoquinone functionalized surface. The results indicated that this immobilization method was highly efficient and oligo (ethylene glycol)-linked carbohydrate monolayers displayed minimal non-specific adsorption with proteins [14]. The glycan microarrays were constructed by covalent immobilization of maleimide-linked monosaccharides on glass slide functionalized with thiol groups [15]. Functionalized glycans

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can also be immobilised covalently in a more specific manner to various solid supports. Several sophisticated glycan array prototypes based on the chemical attachment of functionalized glycans onto thiol-coated, cyclopentadienyl-coated, phosphane-coated glass slides or radical-activated microchips have been reported [16-22]. However, these approaches required multi-step chemical modifications of both the glycan moieties with chemical active groups or ligands prior to immobilization and the surface itself, which are tedious and time-consuming.

To date, several methods have been developed by combined with the currently robust spotting technologies to provide a one-step direct covalent immobilization of intact glycans for glycan microarray construction. The oligosaccharides were immobilized on the aminoxyacetyl-functionalized surface by the formation of oxime bonding with the carbonyl groups at the reducing end of the oligosaccharide probes via irreversible condensation [23]. The nonderivatized oligosaccharides were directly attached to hydrazide-modified self-assembled monolayers on gold surfaces [24] and covalent immobilized on hydrazide-coated glass slides [25]. Such surface chemistry can eliminate the current tedious and laborious pretreatments.

In order to provide both excellent selectivity and quantitative performance, the development of glycan microarrays requires the attachment strategy to be simple, efficient. Herein, a method was used to prepare carbohydrate microarrays, wherein the various reducing carbohydrates could be efficiently immobilized on hydroxyl functionalized glass surfaces by formation of a glycosidic bond with the hemiacetal group at the reducing end of the carbohydrates via condensation. They were thus presented to the medium with a well-defined orientation which is similar to the orientation of glycans in natural glycoconjugates. This technology could enhance carbohydrate accessibility to protein binding while minimizing nonspecific binding.

Two strategies have been developed for the production of carbohydrate microarrays based on this method that exhibited robust reproducibility and facilitated rapid and convenient fabrication of carbohydrate microarrays.

2. EXPERIMENTAL METHODS

2.1. Materials

Untreated glass slides for the fabrication of microarrays were purchased from Gold Seal (Pre-cleaned micro slides, Portsmouth). *N*-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC), succinic anhydride, 4-hydroxybenzoic acid, 16-hydroxyhexadecanoic acid, 4-hydroxybenzamide and 4-hydroxybutyric acid hydrazide were obtained from Alfa Aesar (Phentex (China) Ltd.). Orcin, (3-aminopropyl)triethoxy silane (APTES), (3-glycidyloxypropyl)trimethoxysilane (GPTS), lectin concanavalin A (Con A) and Top-Block were from Sigma-Aldrich Co. Carbohydrates were purchased from Calbiochem (Merck Biosciences GmbH, Germany). The Cy3 monofunctional dye were purchased from Amersham (Amersham Life Science, USA).

Other chemical reagents were obtained from commercial suppliers and used without further purification. All of the solutions were prepared with ultra-pure water obtained from

a Milli-Q50 SP Reagent Water System (Millipore Corporation, USA). Con A was labeled with Cy3 dye according to the manufacturer's instructions.

Laser scanning system, GenePix 4000B (Molecular Devices, Sunnyvale CA). Microarray Spotter, CapitalBio SmartArrayerTM-48 with dual systems of contact print-head and proprietary non-contact spray-head technology (Capital Bio-chip Inc., Beijing, China).

2.2. Surface Derivatization of Glass Slides

Generally, unless stated otherwise, all reactions were carried out at room temperature.

Untreated glass slides were amino- and epoxy-silanized, respectively. The slides were washed with 100% ethanol and then etched by immersion in 10% NaOH at room temperature for 1 h, followed by sonification for 15 min. After washing the slides thoroughly with water and ethanol, they were derivatized in the appropriate solution for 3 h, and again followed by a sonification step. The following derivatization solutions were used: GPTS slides: 2.5% GPTS, 10 mM acetic acid in ethanol; APTES slides: 5% APTES in 95% ethanol/water. After silanization, GPTS-treated slides were washed thoroughly with ethanol, while APTES slides were washed first with water and then twice with ethanol. All slides were spun by dry and finally baked at 37°C for 1 h in a vacuum oven. Storage of modified supports was at 4°C.

2.3. Hydroxyl Functionalization from Epoxysilanized Glass Slide (Scheme 1. A)

GPTS slides were additionally derivatized with two linkers, 4-hydroxybenzamide and 4-hydroxybutyric acid hydrazide, respectively. Prior to use, the two linkers were prepared in a final concentration of 20 mM with anhydrous DMF. The slides were covered with 50 μ L of linker. The reaction was carried out under a coverslip for 3 h. The slides were washed twice with DMF and twice with ethanol, and dried by centrifugation.

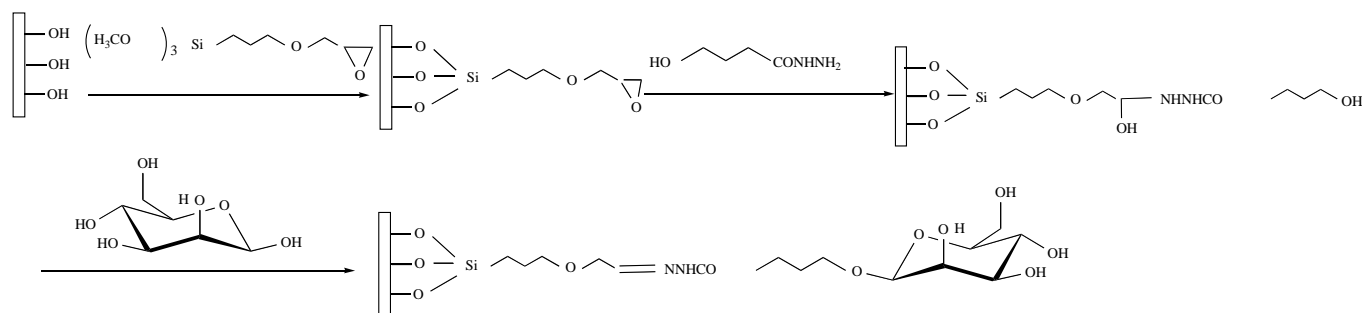
2.4. Hydroxyl Functionalization from Aminosilanized Glass Slide (Scheme 1. B)

APTES slides were additionally derivatized with two linkers, 4-hydroxybenzoic acid and 16-hydroxyhexadecanoic acid, respectively. The two linkers were dissolved in ethyl acetate and stored at a concentration of 200 mM at 4°C. 3.45 mg of NHS was dissolved in 30 mL dry ethyl acetate, 30 mmol of the linker was added to the NHS solution maintained by a nitrogen blanket over the solution. 6.18 g of DCC was dissolved in 10 mL of ethyl acetate and it was added to the NHS/linker solution to react overnight under a nitrogen blanket. The insoluble dicyclohexyl urea (DCU) was removed by filtration using a glass fiber filter pad [26]. The APTES slides were covered with 50 μ L of NHS-linker. The reaction was carried out under a coverslip at room temperature for 3 h. The slides were washed twice with DMF and twice with ethanol, and dried by centrifugation.

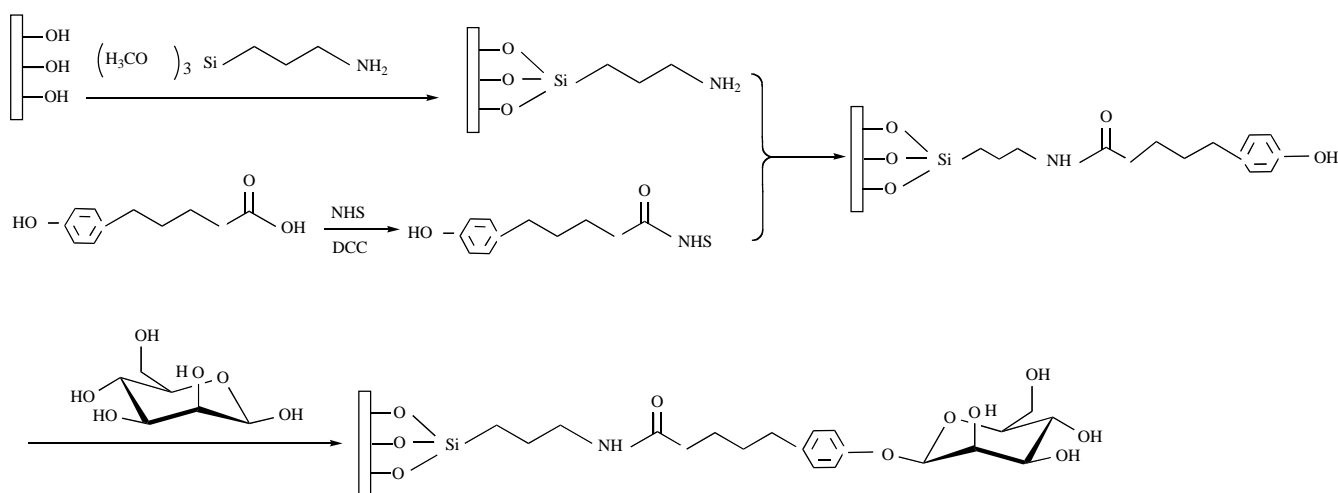
2.5. Detection of Carbohydrates Immobilized on Hydroxyl Modified Glass Slide

The mannose, trehalose and saccharose were dissolved and diluted to 5 mg/mL in water. 2 μ L of carbohydrate solu-

A)



B)



Scheme (1). Scheme of two chemical approaches for the preparation of hydroxyl functionalized glass slide and the immobilization of carbohydrate (mannose). The underivatized carbohydrate was efficiently immobilized on hydroxyl functionalized glass surfaces by formation of a glycosidic bond with the hemiacetal group at the reducing end of the carbohydrates via condensation. **A.** Hydroxyl functionalization from epoxy-silane glass slide; **B.** Hydroxyl functionalization from aminosilane glass slide.

tions were spotted on hydroxyl-activated carriers with a pipette. Subsequently, they were incubated at 60% relative humidity for 2 h. Then, the slides were incubated continually in a desiccator for 12 h so that the carbohydrates were immobilized to the surface of slides by the glycosidic bond via condensation. Unbound carbohydrates were removed from glass by washing for 5 min in 10 mmol/L PBS containing 0.05% Tween 20 (PBST, pH7.0), then centrifuged at 1000 rpm for 1 min to dry. The immobilized sugar on the hydroxyl-modified surface of glass slide was determined by 45% sulfuric acid containing 0.16% orcin [27].

2.6. Fabrication of Carbohydrate Microarrays

The carbohydrates were dissolved and diluted in different spotting buffers. 5 nL of the carbohydrates solution from a 384-well plate were sprayed onto a hydroxyl-functionalized glass slide with a distance of 600 μm between the centers of adjacent spots by using a Microarray Spotter in 60% relative humidity followed by incubation for 2 h. Subsequently, the slides were incubated continually in a desiccator for 12 h so that the carbohydrates were coupled to the solid phase by the glycosidic bond via condensation. Finally, they were kept in 4°C in a desiccator to be ready for lectin binding assays.

2.7. Lectin Binding Assays

Unbound carbohydrates were removed from glass by washing for 2 min in PBST and subsequently blocked for 45 min at 37°C in 3% TopBlock solution. After blocking, the slides were washed once with PBST for 2 min and once with PBS for 2 min, then centrifuged at 1000 rpm for 1 min to dry. Incubation of the microarrays with fluorescent labeled lectin occurred at 4°C for 1 h in a humid chamber, respectively. After incubation, the slides were washed once with PBST for 10 min and twice with PBS, each for 10 min. Then, spun to dry.

2.8. Scanning and Evaluation

The microarrays were scanned at 70% photomultiplier tube and 100% laser power settings with a Genepix 4000B confocal scanner (Axon Instruments, Inc. USA). The acquired images were analyzed at 532 nm for Cy3 detection by Genepix 3.0 software. Average background was subtracted from each data point. The slides were first scanned at conditions optimal for each individual slide and additionally analysed at a setting identical to all slides of the respective experimental series. Each data point presented in this report

represents the means of median \pm 1 S.D. of 5-9 replicated measurements obtained from at least three slides.

3. RESULTS AND DISCUSSION

3.1. Preparation of Hydroxyl-functionalized Slide Surface and Detection of Immobilized Carbohydrates

Two strategies have been utilized for preparation of hydroxyl-functionalized surface, self-assembled monolayers containing hydroxyl groups on glass slide as a platform for immobilizing an array of carbohydrates. The use of hydroxyl-terminated self-assembled monolayers for the immobilization of the underivatized carbohydrates takes advantage of the glycosidic bonding formation reaction between a hydroxyl group with the hemiacetal group at the reducing end of the suitable carbohydrates via condensation. The efficiency of the method relies on the formation of the glycosidic bond under slightly acidic condition and on the good stability of the glycosidic bonding under neutral or alkaline condition [28, 29]. To the best of our knowledge this article constitutes the first report of such a strategy.

The surface with hydroxyl groups was prepared in just one-step from the GPTS-modified glass slides. The epoxy group of the GPTS monolayer was treated with 4-

hydroxybenzamide and 4-hydroxybutyric acid hydrazide that resulted in a monolayer with hydroxyl as most outer groups on glass slide, respectively.

Similar to the above strategy, the surface with hydroxyl groups was also prepared from the APTES-modified glass slides. The amino group of the APTES monolayer was treated with 4-hydroxybenzoic acid and 16-hydroxyhexadecanoic acid that resulted in a monolayer with hydroxyl as most outer groups on glass slide, respectively.

The immobilized sugars on the glass slides were determined by orcin sulfuric acid after the glass slides were washed. The result showed the carbohydrates (trehalose and saccharose) without the reducing end could not be immobilized. However, the carbohydrate (mannose) with the reducing end could be immobilized on the hydroxyl-modified surface of glass slide (Fig. 1). These results implied that the underivatized sugar was immobilized on hydroxyl-functionalized glass surface via the reducing end other than the hydroxyl groups of carbohydrates themselves.

3.2. Direct Comparison of Different Strategies

Steric hindrance could influence the efficiency of glycan-protein interactions thus causing a reduction of signal inten-

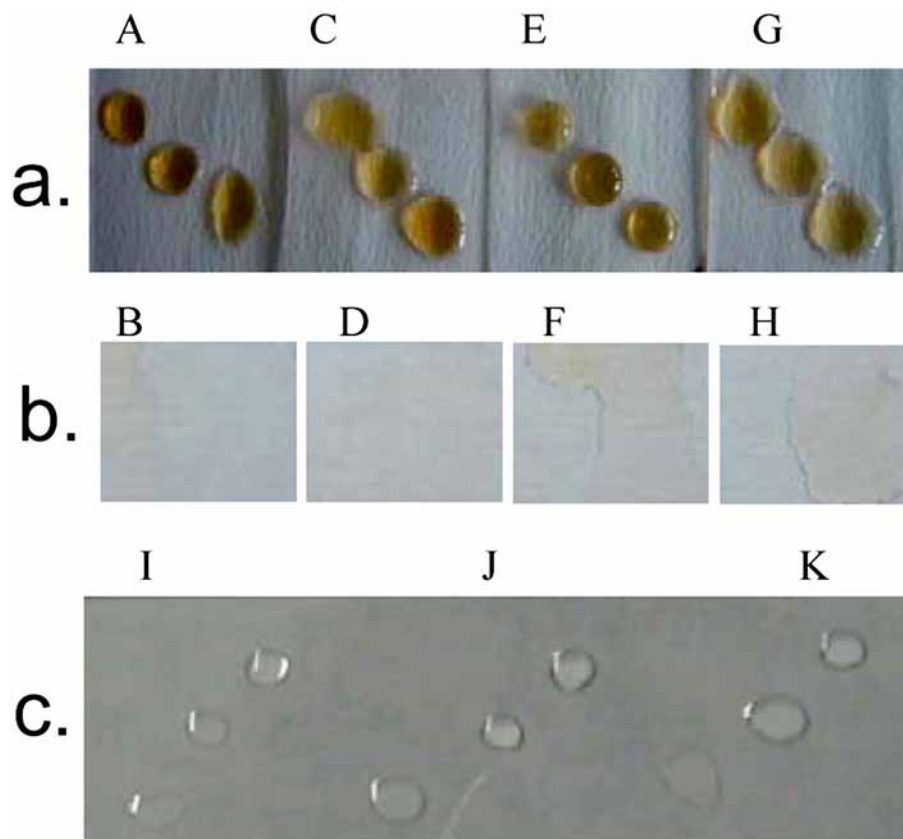


Fig. (1). The immobilized sugar on the glass slides was determined by 45% sulfuric acid containing 0.16% orcin after the glass slides were washed. **a.** Mannose was immobilized on the glass slides activated by 4-hydroxybenzamide (**A**), 4-hydroxybutyric acid hydrazide (**C**), 4-hydroxybenzoic acid (**E**), 16-hydroxyhexadecanoic acid (**G**), respectively. **b.** The glass slides were activated by the above four chemicals without mannose immobilization (**B**), (**D**), (**F**) and (**H**), respectively. **c.** The non-reduced carbohydrates were spotted on 4-hydroxybutyric acid hydrazide functionalization glass slides. After incubation and washing, they could not be detected by orcin sulfuric acid. (**I**) trehalose. (**J**) saccharose. (**K**) negative control.

sities. To examine if absolute signal intensities depend on the length of a linker has a positive influence. GPTS surfaces were additionally derivatized with two linkers, 4-hydroxybenzamide and 4-hydroxybutyric acid hydrazide, respectively. APTES slides were also additionally derivatized with two linkers, 4-hydroxybenzoic acid and 16-hydroxyhexadecanoic acid, respectively. Consequently, the signal intensities obtained on the various surfaces were normalized against the corresponding signal intensities on the slides. The result showed the fluorescent intensity was the highest when GPTS surface was additionally modified with the linker, 4-hydroxybutyric acid hydrazide (Fig. 2). The hydroxyl functionalization from 4-hydroxybutyric acid hydrazide modified GPTS surface slides were used to further experiment.

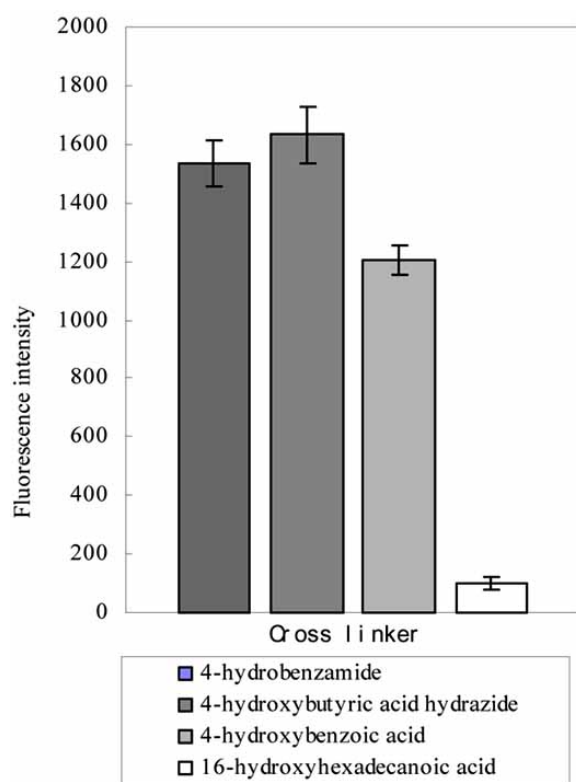


Fig. (2). Direct comparison of various surfaces. All experiments were performed with an identical sample at the conditions optimal to the respective system. The data presented here was the means of median \pm 1S.D. from the five signal intensities obtained on mannose at a spotting concentration of 1 mM.

3.3. Spotting Buffers

Spotting buffer composition can influence the glycan binding capacity of a surface, the stability of glycans and the quality of the spots produced. Several spotting buffers (200 mM acetate buffer, pH 5.5; PBS buffer, pH 7.0; 100 mM carbonate buffer, pH 8.5) were tested on the hydroxyl-coated surfaces. In addition, the effect of the supplements 0.5 % trehalose and 0.5 % glycerol in PBS was also investigated, respectively. The result showed the fluorescent intensity was the highest when spotting buffer was acetate buffer. Moreover, when spotting buffer was PBS supplemented with 0.5 % glycerol, the fluorescent intensity was the weakest (Fig. 3).

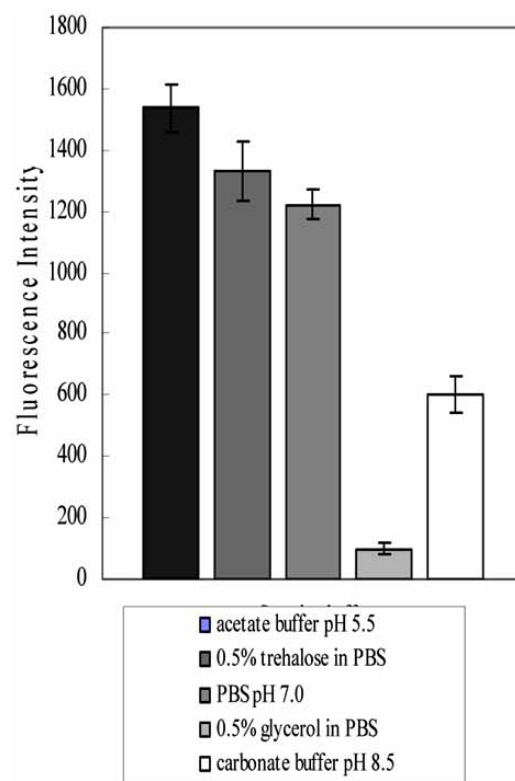


Fig. (3). Effect of spotting buffer. Data are represented for mannose in the spotting solution of 1 mM with an identical Con A concentration on 4-hydroxybutyric acid hydrazide functionalization glass slides.

3.4. Blocking

Unspecific background signal (GBP binding in the absence of carbohydrate) is one of the most serious problems encountered in glycan microarray like protein microarray technology. Because the hydroxyl group on the surface of glass slides could be much less reactive with proteins, the hydroxyl-modified slides can prevent from unwanted protein adsorption and other non-specific interactions at the surface. For improving blocking efficiency, we also used BSA (~60 kDa) in concentrations of 1% and 3% in 1 \times PBS, 4% milk powder and TopBlock, a mixture of small proteins (~3 kDa), at the same concentrations. TopBlock is more soluble than BSA [30]. The carbohydrate-containing slides were incubated at 37°C for 45 min in the respective solution. Typical result for 4-hydroxybutyric acid hydrazide modified GPTS surface slides was shown in Fig. (4). On all surfaces analyzed, background blocking was superior with TopBlock. While TopBlock did not affect the specific signal intensities obtained in the subsequent analyses. The strong inhibitory effect on signal intensities was also observed with 4% milk powder, a classical blocking reagent. Therefore, 3% TopBlock solution was used as blocking reagent in all experiments reported here.

3.5. Determination of Optimal Conditions for Studies of Incubation Temperature, Time and pH of Incubation Buffer

In order to assess the most suitable conditions, the different incubation temperature and incubation time of the carbo-

hydrate microarrays with fluorescent labeled Con A were tested, respectively. The results showed when incubation temperature was 4°C and incubation time was 1 h, the fluorescent intensity was the highest.

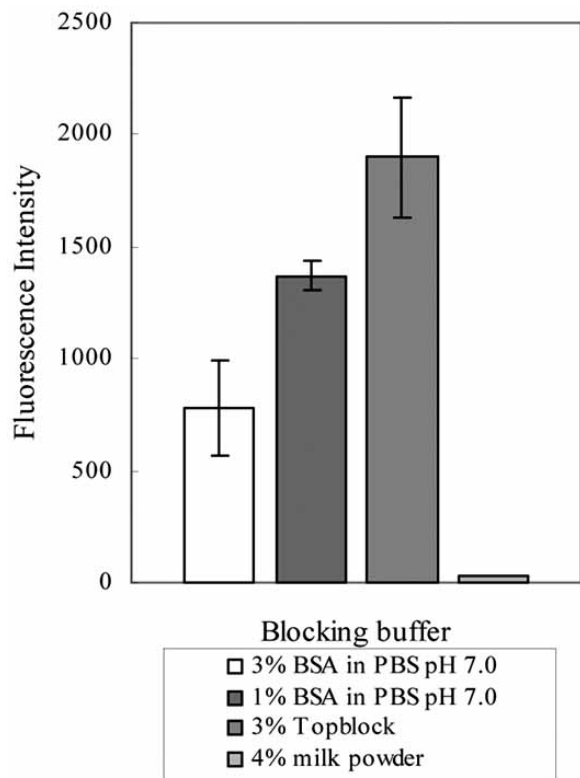


Fig. (4). Blocking with 3% and 1% of BSA, 3% of TopBlock and 4% of milk powder, respectively. Data are represented for mannose in the spotting solution of 1 mM with an identical Con A concentration on 4-hydroxybutyric acid hydrazide functionalization glass slides.

Because the use of hydroxyl-terminated self-assembled monolayers on the glass for the immobilization of the underivatized oligosaccharides utilizes the glycosidic bonding formation reaction under slightly acidic condition, the effect of incubation in phosphate buffer of different pH (6.0, 6.5, 7.0, 7.5 and 8.0) for the stability of the glycosidic bond on the glass was assessed. The result showed the fluorescent intensities were higher under neutral and alkaline condition (Fig. 5A and 5B) and was consistent with the good stability of the glycosidic bond under the same condition.

3.6. Optimal Carbohydrate Concentration and Binding Capacity

The hydroxyl-functionalized slide could also be used to detect the amount of carbohydrates immobilized on the glass surface. To determine the detection limit of carbohydrates binding to the hydroxyl-functionalized surface and the optimal carbohydrate concentration in the spotting solution, mannose was sprayed on the glass slide in concentrations ranging from 100 mM to 1.0 fM (Fig. 6A) and incubated with Cy3-labeled Con A. Fig. (6B) showed the representative example for the detection of the amount of mannose that

was immobilized on the slide surface. The calculated limit of detection (LOD, the concentration which gives fluorescent signal higher than the negative control +3 S.D.) of the fabricated carbohydrate microarray was found to be in the nanomolar (100 nM) spotting concentration, herein, the spotting buffer (SB) was to be negative control. As shown in Fig. (6B), as low as 100 nM of mannose was detected by spotting the mannose solution on the hydroxyl-functionalized surface and binding with Con A. The ability to detect very low levels of glycan materials arrayed at high density is a significant advantage of microarrays compared to existing methods of glycan analysis, such as ELISA and immunodot assay [31, 32]. This result demonstrated that microarrays require only a very small quantity of carbohydrate, in our assay the loading of the spot was 5 nL, and therefore the minimum amount for the detection was 0.5 femtomole per spot. Assay miniaturization through the construction of high-density microarrays is thus well suited for the investigation of carbohydrate-protein interaction.

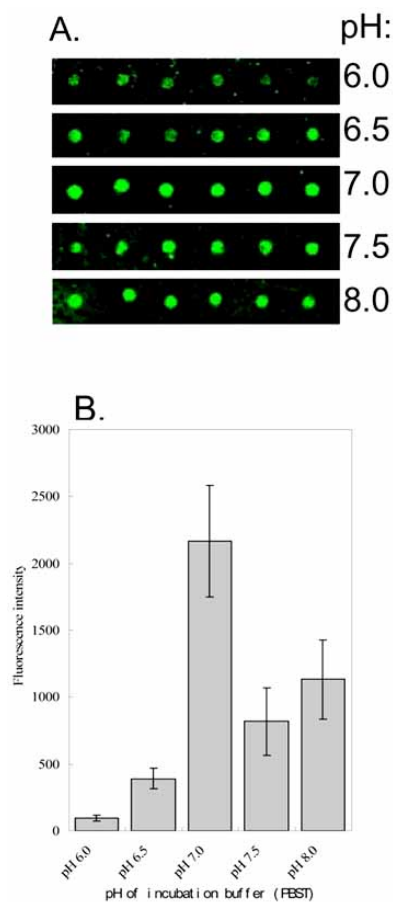


Fig. (5). The effect of incubation in phosphate buffer of different pH. A. Images of detection in phosphate buffer of different pH. B. Data are represented for mannose in the spotting solution of 1 mM with an identical Con A concentration in phosphate buffer of different pH on 4-hydroxybutyric acid hydrazide functionalization glass slides.

On the other hand, on the assessment of glycan-protein interaction, the immobilized carbohydrates on the glycan microarrays participate in biospecific interactions with pro-

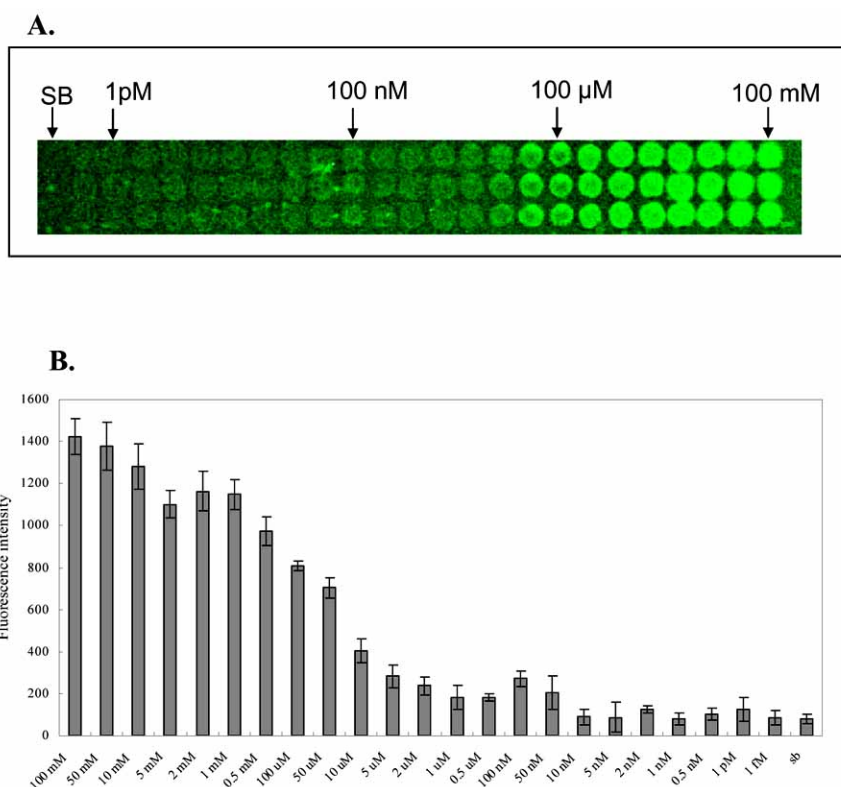


Fig. (6). **A.** Glycan microarray fabrication and detection. The 4-hydroxybutyric acid hydrazide activated GPTS slide was printed with mannose at a range of concentrations between 100 mM and 1 fM. Fluorescent images were then probed with Cy3-labeled Con A. The arrow refers to the printing concentration. **B.** Quantification of raw data was presented here and result was the means of 3 replicate medians \pm IS.D. The detection limit was determined to be at 100 nM printing concentration.

teins and enzymes, whereas the hydroxyl groups on the monolayers provide essentially complete resistance to unwanted protein adsorption and other non-specific interactions at the surface. Fig. (7A) showed the multiple-analyte characterization conducted on the prepared carbohydrate microarray. The carbohydrates were found to bind to their specific lectin proteins. For example, the carbohydrate microarray probed with Con A showed significant fluorescence intensity in the spots arrayed with mannose and glucose. Analysis of the fluorescent intensity further revealed the binding of Con A to the oligosaccharides is in the order of mannose > glucose (Fig. 7B). This affinity difference is consistent with previous report in which cyclopentadiene modified oligosaccharides were arrayed on benzoquinone functionalized gold surface [18]. Weak signal was obtained in the spots that arrayed with cellobiose which consists of two glucose units. In addition, scarcely any signal was observed in the spots arrayed with methyl- α -mannoside, which the C1 position was substituted with methyl group. This result indicated that methyl- α -mannoside could be not immobilized on the hydroxyl-modified glass surface and validated the selectivity of this surface chemistry for glycosidic bond formation at the reducing end of carbohydrates.

3.7. Storage of Hydroxyl-Functionalized Slides (Blank Slides)

The ability to store slides prior to spotting is an important time- and material-saving element in microarray technology, since it allows bulk production of slides and their subsequent

continuous consumption in experimentation. Here, binding of linker on GPTS slides was carried out immediately, and then freshly prepared hydroxyl-functionalized slides were kept either at 4 °C or room temperature (RT) in a desiccator, respectively. They were used for carbohydrate spotting within a day after preparation or after 2, 4, 6 and 8 weeks. All slides were scanned at identical scanner adjustments in order to assure that there was no difference in the sensitivity of the scanner during time. Linker-modified GPTS slides in RT only slightly improved their performance during the eight week period of storage. However, they showed a strong increase in signal intensities after four weeks of storage under 4 °C.

3.8. Storage of Carbohydrate-containing Slides

Parallel to the above experiment, linker-modified GPTS slides were used for spotting carbohydrates the day after the coating had taken place and stored at 4 °C and RT in a desiccator, respectively. All slides were scanned at identical scanner adjustments in order to assure that there was no difference in the sensitivity of the scanner during time. All microarrays in RT could be used for at least two months without any apparent deterioration of the performance parameters. As a matter of fact, an increase of signal intensities was observed after two weeks of storage under 4 °C.

Glycan microarrays are valuable addition to the glycome analysis toolbox. The potential use of glycan microarrays is very broad, and includes, for example, receptor ligand char-

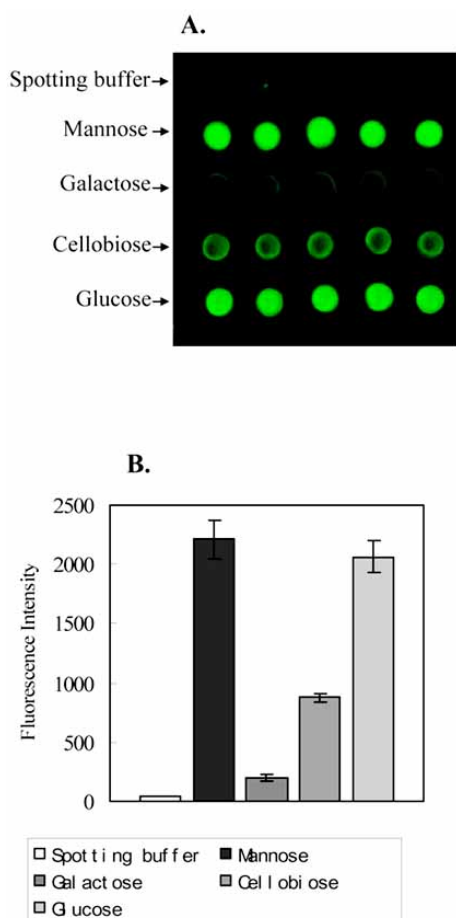


Fig. (7). **A.** Fluorescence image of glycan microarrays spotted with 4 oligosaccharides were incubated with Cy3-labeled Con A (20 $\mu\text{g}/\text{mL}$ in PBST). **B.** Quantification of raw data was presented here and result was the means of 5 replicate medians \pm 1S.D.

acterisation, antibody specificity profiling, bacterial and viral adhesion as well as enzyme characterisation. Currently, the glycan microarrays are used as a high-throughput screening tool for proteins of interest in complex biological samples. For preparation of glycan microarrays, ideally, no modification or minor modification of the glycan species is required for the attachment, and the immobilized glycans are presented in a regular and homogeneous environment so that all underivatized glycans are immobilized on functionalized surface of solid support. We report here a convenient approach for constructing carbohydrate microarray by direct immobilizing underivatized carbohydrates on hydroxyl-functionalized surface of glass support. Two strategies have been developed for the production of carbohydrate microarrays based on mono- and disaccharides covalently linked to the surface via a linker at their reducing ends and exhibited robust reproducibility and facilitates rapid using the fluorescent ConA as the detection system (the production and application of glycan microarrays with mono-, di- and oligosaccharides based on this method have been performed, the results will be published in another paper). We studied, compared and optimized strategies for the production of carbohydrate microarrays with the aim of creating a platform that can present the non-reducing end of the carbohydrate to the solution

and retain their anomericity as well as mimic the orientation of glycans in natural glycoconjugates suitable to the various kinds of GBPs present in a complex protein mixture.

From the two options of carbohydrate immobilisation on glass slides that we tested, the epoxysilane (GPTS) surface derivatised additionally with 4-hydroxybutyric acid hydrazide linker performed best overall. It is simple to produce, showed very high sensitivity, very good signal-to-background ratios and good spot quality. The reactive hydroxyl surface is able to react easily with the hemiacetal group at the reducing end of the suitable carbohydrates by the glycosidic bonding formation formed under slightly acidic condition and stabilized under neutral and alkaline condition. The hydroxyl surface can avoid from unwanted protein adsorption and other non-specific interactions at the surface.

Optimal experimental conditions for the blocking process are mostly the result of empirical optimisation as solid-phase immunoassays [33] and might require adaptation to specific preconditions. However, a too high protein concentration always leads on a decrease in signal intensity on microarrays. Small soluble proteins seemed to be more efficient reagents for blocking, but should note that a mixed protein, for example, milk powder always caused significantly a decrease in signal intensity on glycan microarrays, possible reason was that some proteins in milk powder could bind with carbohydrates on microarrays, consequently, these protein-glycan conjugates could not bind with other GBP again.

The four linkers of different length were used to prepare the hydroxyl modified surfaces that exhibited differences with respect to the binding of GBP. The hydroxyl functionalization surfaces from 4-hydroxybenzamide, 4-hydroxybutyric acid hydrazide modified GPTS slides and 4-hydroxybenzoic acid modified APTES slides exhibited relatively high signal intensities, however, from 16-hydroxyhexadecanoic acid modified APTES slides exhibited relatively low signal intensities. This means there is not a positive influence for absolute signal intensities that depend on the length of a linker according to the tested result of the length of the longest linker.

Since 90% of the mass of any proteome is contributed by approximately 10% of the proteins [34-36], analyses by glycan microarrays should be highly sensitive in order to detect the interesting GBPs of low abundance. Despite the rather good sensitivity values reported here for the simplest attachment strategies, more development is needed in this direction. One important and challenging field is the labelling and detection of complex protein mixtures. Signal amplification by rolling circle amplification [37] or biotin-avidin / streptavidin based methods [23], for example, or the use of reporter molecules other than fluorescence, such as radioactive labelling, could be instrumental in improving assay sensitivity and accuracy further.

4. CONCLUSIONS

In summary, we have developed a simple and efficient method to prepare glycan microarray on hydroxyl-functionalized glass surface by formation of glycosidic bond with the hemiacetal group at the reducing end of suitable

carbohydrates via condensation, moreover the results reported here showed its simplicity and robustness. Importantly, carbohydrates are immobilized on array format directly without the need for the modification to generate reactive groups and can mimic the orientation of glycans in natural glycoconjugates. This methodology offers the advantages of including simplicity, the use of undervatized glycans, inert surfaces, and the capability of quantitative analysis. The hydroxyl-functionalized slide described here also has the advantage that they are compatible with all of the principal techniques used for analyzing microarrays.

ACKNOWLEDGMENTS

This work is supported by NFSC (Grant No.30870549) and the National "863" Project (Grant No. 2007AA02Z413) from the Chinese Ministry of Science and Technology.

ABBREVIATIONS

- APTES = (3-Aminopropyl) triethoxy silane
 GPTS = (3-Glycidylpropyl) trimethoxysilane
 GBP = Glycan-binding protein
 S.D. = Standard deviation
 NHS = N-Hydroxy-succinimide
 DCC = Dicyclohexyl carbodiimide

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