Published in final edited form as:

Biosens Bioelectron. 2011 October 15; 28(1): 355–361. doi:10.1016/j.bios.2011.07.044.

Novel 3-Dimensional Dendrimer Platform for Glycolipid Microarray

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Abstract

Glycolipids are important biological molecules that modulate cellular recognitions and pathogen adhesions. In this paper, we report a sensitive glycolipid microarray for non-covalently immobilizing glycolipids on a microarray substrate and we perform a set of immunoassays to explore glycolipid-protein interactions. This substrate utilizes a three-dimensional hydrazide-functionalized dendrimer monolayer attached onto a microscopic glass surface, which possesses the characteristics to adsorb glycolipids non-covalently and facilitates multivalent attributes on the substrate surface. In the proof-of-concept experiments, gangliosides such as GM1, FucGM1, GM3, GD1b, GT1b, and GQ1b, and a lipoarabinomannan were tested on the substrate and interrogated with toxins and antibodies. The resulting glycolipid microarrays exhibited hypersensitivity and specificity for detection of glycolipid-protein interactions. In particular, a robust and specific binding of a pentameric cholera toxin B subunit to the GM1 glycolipid spotted on the array has demonstrated its superiority in sensitivity and specificity. In addition, this glycolipid microarray substrate was used to detect lipoarabinomannan in buffer within a limit-of-detection of 125 ng/mL. Furthermore, *Mycobacterium tuberculosis* (*Mtb*) Lipoarabinomannan was tested in human urine specimens on this platform, which can effectively identify urine samples either infected or not infected with *Mtb*. The results of this work suggest the possibility of using this glycolipid microarray platform to fabricate glycoconjugate microarrays, which includes free glycans and glycolipids and potential application in detection of pathogen and toxin.

Keywords

Glycolipids; microarray; GM1; dendrimer; hydrazide; lipoarabinomannan

1. Introduction

Glycolipids are important biological molecules located on the exoplasmic surface of the cell membrane. The carbohydrate chain of the glycolipid is covalently attached to a lipid moiety that is anchored to the cell membrane. Glycolipids play many functional roles in biological processes. For example, glycosphingolipids are becoming relevant in our minds due to their understood involvements in autoimmunity (Adar and Ilan 2008), neurologic disorders

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(Mocchetti 2005), and cell signal transduction events (Boggs et al. 2008; Kasahara and Sanai 2000). Numerous bacterial toxins and lectins recognize carbohydrate-derivatized lipids on the cell surface, often with high affinity, and operate as virulent factors for pathogen adhesion to host cells (Hooper and Gordon 2001; Spear 2004). These lipids are glycosylated on their ceramides at the reducing end and can be classified into glyceroglycolipids, glycosphingolipids, and glycosylphosphatidylinositols (GPI). Gangliosides, a kind of glycosphingolipids, are composed with one or more sialic acids and comprise 5-10% of the lipid composition of the plasma membrane on neuronal and glial cells (Derry and Wolfe 1967). One of the best examples for toxin-ganglioside interactions is the binding of the cholera toxin B subunit (CTB) which is produced by *Vibrio cholerae* to the ganglioside GM1 (Angstrom et al. 1994). The GM1 ganglioside contains a pentasaccharide glycan motif being structured as $Gal\beta 1-3GalNAc\beta 1-4(NeuAca2-3)Gal\beta 1-4Glc\beta 1-$ (Fig. 1A). The binding epitope of GM1 to the CTB includes the internal Galactose (Gal) and most of the terminal Gal and N-acetylneuraminic acid (NeuAc) motifs (Angstrom et al. 1994). Another important class of glycolipid molecules is that of sizable lipoglycans (which have molecular weights that are usually more than 10 kDa), such as lipoarabinomannan (LAM) that is present on the cell wall of *Mycobacterium tuberculosis* (*Mtb*), a gram-positive bacterium. In addition to serving as a major cell wall component, the lipoglycan is thought to serve as a virulent factor in bacteria.

The conventional method for glycolipid microarray is to use the membrane-based surface (e.g. nitrocellulose, polyvinylidene difluoride) for the non-covalent adsorption of glycolipids on the surface (Liu et al. 2009; Rinaldi et al. 2009). The disadvantage of using membrane surfaces is the instinct auto-fluorescence background, which makes it difficult to achieve ideal signal-to-noise ratio (SNR) for differentiating the specific binding of corresponding toxins, lectins, antibodies, or other glycan-binding proteins (GBPs) to glycolipids. Another approach to glycolipid microarray is to synthesize the oligosaccharide motifs containing the linkers and covalently immobilize the oligosaccharides to functionalized glass substrates (Millen et al. 2010). This approach requires tedious and time-consuming chemical synthesis of the oligosaccharides and therefore is not a feasible method for fabricating a glycolipid microarray in a high-throughput setting. Moreover, the binding affinity of carbohydrate to GBP has been found to be greatly affected by the nature of the chemical linkers that were installed for fabrication of glycolipid microarray (Ngundi et al. 2007). Therefore, retaining the native structure of glycolipids on a low-background substrate, such as the glass-based slides used in this work, will be beneficial for the studies on glycolipid-protein interactions.

Herein we report a simple yet effective approach to fabricating highly sensitive glycolipid microarrays. As illustrated in Fig. 1A, the microscopic glass substrate was coated with a hydrazide-functionalized 3-dimensional (3-D) dendrimer film, where the glycolipid probes are spotted. In our previous studies, 3-D dendrimer coating has proven to be an ideal platform for immobilization of mono-, di-, oligo-, and poly-saccharides, and has demonstrated a multivalent effect for carbohydrate-proteins interactions (Zhou et al. 2009). We hypothesize that the lipid tails in glycolipids could be inserted into multiple hydrophobic branches on the surface of the dendrimer molecules. The hydrophobic and non-covalent adsorption of the lipid portion of glycolipids into a dendrimer molecule directs the glycan portion of glycolipids outside of the dendrimer monolayer. Also, the amide bonds in the poly (amido amine) (PAMAM) dendrimer and the terminal hydrazide groups introduce two new hydrogen-bonding contacts, including a hydrogen bond donor and hydrogen bond acceptor (Fig. 1A)(Vaught et al. 2010). These amide groups, which are intermittently arranged on the substrate, have significant electronic and adhesive effects on glycolipids. To prove the hypothesis, several gangliosides, such as GM1, GM3, FucGM1, GD1b, GT1b, and GQ1b, and a GPI-like lipoglycan (LAM) as shown in **Fig. 1B** and **1C**, were applied on this 3-D thin-film substrate. Subsequently, the resultant glycolipid microarrays were interrogated

with corresponding antibodies, including both primary and fluorescence-labeled secondary antibodies, and fluorescence-labeled toxins (Fig. 1A). This ready-to-use microarray platform can be used to extensively study the characterization of the binding epitopes of glycolipids.

2. Materials and Methods

2.1 Materials and Reagents

All chemicals, unless specifically stated, were purchased from Sigma-Aldrich Chemical Corp. CTB labeled with AlexaFluo555 and secondary antibodies were products from Invitrogen® Inc. The gangliosides came from Matreya, LLC. BEI Resources provided the *Mtb* lipoarabinomannan (LAM) and its corresponding antibodies, such as CS-35 monoclonal antibody (mAb) and anti-LAM polyclonal antibody (pAb). The urine specimens used for testing the microarray substrates were obtained from the World Health Organization (WHO) TB Specimen Bank. The UriSub™ artificial urine used as negative control was obtained from CST Technologies, Inc. 75×25mm standard microscope slides were purchased from Corning Inc. (Cat. No. 2947). The FAST substrates (nitrocellulose surface) used in the comparative studies were purchased from Whatman.

2.2 Preparation of hydrazide-dendrimer-coated substrate

The hydrazide-dendrimer-coated substrate was prepared according to our established procedure (Zhou et al. 2009) with minor modification. In brief, the microscopy slides were immersed for 30 minutes in a toluene solution containing 2% of (3-Glycidyloxypropyl)-trimethoxysilane (GPTS) to allow the formation of a monolayer on the glass surface with epoxy functional groups toward the outside of the surface. The GPTS functionalized substrates were then incubated at room temperature overnight in a solution containing 0.1% PAMAM dendrimer (ethylenediamine core, generation 4) and methanol. The substrates were washed with methanol and de-ionized water and the dendrimer-coated substrates were dried in a nitrogen stream. Then, the dendrimer on the glass surface was treated with a solution of succinic anhydride (10 mM; 1 mg/mL) in DMF for 3 hours and subsequently washed with DMF. The carboxyl-functionalized substrate can be activated by N-hydroxysulfosuccinimide (NHS; 10 mM) and 1-Ethyl-3-[3-dimethylaminopropyl]-carbodiimide (EDC; 10 mM) in DMF for 1 hour, followed by treatment with a solution of 10 mg/mL aqueous of adipic acid dihydrazide (ADHZ). The substrate was incubated in this solution for 2.5 hours and then washed with water and dried with dry N₂ to achieve a hydrazide-dendrimer-coated substrate.

2.3 Immobilization of the glycolipids on the hydrazide-dendrimer-coated substrates

The glycolipid probes were dissolved in printing buffers of (1) phosphate-buffered saline (PBS; pH 7.4) containing 50% DMSO (v/v) for immobilizing gangliosides, and (2) carbonate-bicarbonate buffer (pH 9.6) for immobilizing LAM. The gangliosides and the other free glycans were spotted onto the glass surface at concentrations of 100 µM and 10 µM. The LAM was spotted in individual concentrations from 0.5 mg/mL to 0.005 µg/mL in two- or ten-fold serial dilutions. A given concentration of each glycolipid preparation was repeated at least three times to support statistically significant analysis. One nanoliter (1 nL) of the carbohydrate solution from individual wells of a 384-well microplate containing 20 µL of glycolipid solution was spotted onto the hydrazide-dendrimer-coated substrate in 60% relative humidity with a spot spacing of 500 µm through the use of an ArrayIt SpotBot®2 microarray printer (ArrayIt, Sunnyvale, CA) and SMP4 pin (Arrayit). The other printing parameters included 20 pre-spots, 3.0s dwell times (sample loading), and 5 wash cycles. The spot size was approximately 200 µm and multiple sub-arrays were applied on the substrates. The glycolipids-spotted substrates will be allowed to incubate in a reaction chamber (75%

humidity) overnight at room temperature. The substrates were then washed briefly with PBS buffer to remove the unbounded glycolipids.

2.4 Interrogation of antibodies or toxins with glycolipid microarrays

A 16-well silicon grid was used to separate different assays into individual wells. Then, the substrate was processed as follows: (1) blocking: the glass substrates spotted with the glycolipids were treated with a blocking buffer of PBS containing 1% BSA or 1% glyceraldehyde aqueous solution for 1 hour, and then rinsed with PBS buffer before assays; (2) assays: (a) the gangliosides on the array were directly interrogated with 1 µg/mL of AlexaFluo555-CTB in PBS; (b) the LAM on the array was indirectly stained with a primary CS-35 mAb or anti-LAM pAb (1:1,000 dilution in PBS) followed by a secondary Cy3-labeled anti-mouse IgG (H+L) antibody or anti-rabbit IgG (H+L) antibody (1:1,000 dilution in PBS). There was a washing step between the two steps of antibody interrogation. Following the immunoassays, the substrates were washed with PBS 5 times for 5 minutes each and de-ionized water prior to being spin dried.

2.5 Detection of LAM in urine using hydrazide-dendrimer-coated substrates

Urine samples were processed at 70°C for 20 minutes to denature the urinary proteins (urinary LAM is stable) and centrifuged at 10,000 rpm. Then, 20 µL of each urine sample was placed in a 384-well plate and 1 nL of each sample was spotted onto the hydrazide-dendrimer substrate as previously described. Each specimen was printed at least three times. Negative controls, such as urine samples from artificial urine, were included for comparative studies. The LAM obtained from *Mtb* H37Rv strain (ManLAM) in a concentration range from 50 µg/mL to 0.05 µg/mL was spotted on the substrates for positive controls. The urine-spotted substrates were processed by microwave treatment (50% power) for 10 minutes to enhance the LAM immobilization. Then, the unbounded components in the urine samples were washed off the substrates by DI water for 5 minutes. Each sub-array on the substrate was treated with a blocking buffer (PBS containing 1% BSA or 1% glyceraldehyde) and probed individually with the CS-35 mAb (1:1,000 dilution in PBS). After washing the substrates with PBS, the assays were traceable through the process of staining with the Cy3-labeled anti-mouse IgG (H+L) antibody (1:1,000 dilution in PBS). Following the immunoassays, the substrates were washed with PBS 5 times for 5 minutes each and de-ionized water prior to being spin dried.

2.6 Microarray Imaging and Data analysis

Microarrays were scanned at 10 µm resolution with the scanning laser confocal fluorescence microscope (Alpha Innotech, Inc., San Leandro, CA). The emitted fluorescent signal was detected by a photomultiplier tube (PMT) at 570 nm for Cy3. For all microarray experiments, the laser power was 75% and the PMT gain was 70%. The fluorescent images were analyzed using the Image Acquisition & Analysis software from AlphaScan™ in the following procedures: (1) the signal-to-noise ratio (SNR) of each spot on the glycolipid microarray was calculated by dividing the mean fluorescence intensity by the mean local background intensity; (2) The fluorescence intensity can be reflected by a average SNR (AvSNR) of at least three replicates for an individual assay; (3) in order to normalize the substrate-to-substrate variations (e.g., the hydrazide-dendrimer substrate versus the FAST substrate), the SNRs for a substrate will be normalized against the SNR of a negative control spotted only with the printing buffer, which results in a signal-to-background ratio (S/B).

3. Results and Discussion

3.1 Fabrication of the 3-D hydrazide-dendrimer microarray substrate

The fabrication of the hydrazide-dendrimer microarray substrate was performed according to our established procedure (Fig. S1 in supplemental information) (Zhou et al. 2009). In brief, glass-substrate silanol groups react with GPTS to form an epoxy terminated monolayer. Then, the GPTS coated substrate was incubated with PAMAM dendrimer to achieve 3-D dendrimer-coated-glass substrates displaying amino terminal groups. Further modifications of the amino group with succinic anhydride, EDC/NHS, and adipic acid dihydrazide, continuously, yielded the hydrazide-dendrimer-coated substrate. In the initial tests to compare different functionalized substrates used for spotting glycolipids, the hydrazide-functionalized substrate was found to possess better glycolipid immobilization capability and spot uniformity more accurately than the substrate coated only with dendrimer (Fig. S2 in supplemental information). This phenomenon may be attributed to the fact that the hydrazide termini having the H-bond acceptor and H-bond donor, which facilitates the insertion of lipid tails, and thus immobilizes the glycolipids (as shown in Fig. 1A) (Vaught et al. 2010). Therefore, in the latter tests, the hydrazide-functionalized substrate was applied for fabrication of the glycolipid microarrays and was also utilized in the subsequent immunoassays.

3.2 Toxin detections on the glycolipid microarray

In the initial tests, the gangliosides were spotted onto the glass substrates coated with the hydrazide-dendrimer in a printing buffer containing PBS, pH 7.4, and 50% of DMSO to improve the solubility and homogeneity of the gangliosides in the printing buffer. The resulting glycolipid arrays were interrogated with the CTB toxin. As shown in Fig. 2A, the GM1 exhibited high specificity to recognize the CTB that was fluorescently labeled with AlexaFluor555. The AvSNR was 86.4 ± 10.8 and 50.9 ± 9.7 when its spotting concentration is 100 μM and 10 μM , respectively. However, the fucosylated GM1 (FucGM1) exhibited a faint affinity to CTB (AvSNR is 10.0 ± 0.65 at spotting concentration of 100 μM), which demonstrates the “shielding” (or steric hindrance) effect of the fucosylation on the binding site of GM1. Additionally, other gangliosides (e.g. GM3, GD1b, GT1b and GQ1b) showed low affinity to CTB (Fig. 2A).

In order to compare covalent and non-covalent immobilization on this 3-D surface, the oligosaccharides (free glycans), which contain a reducing end that allows covalent attachment to the hydrazide-dendrimer surface (Zhou et al. 2009), were used in the comparison studies. As shown in Fig. 2B (the fluorescent image and the carbohydrate list are shown in Table S1 in supplemental information), the glycans (covalent immobilization) exhibit similar immobilization characteristics compared to the glycolipids (non-covalent immobilization). GM1 showed extraordinary binding capability to CTB compared to other carbohydrates. Two carbohydrates, a Blood Group A trisaccharide containing a terminal GalNAc and a lacto-N-tetraose (LNT) containing a terminal Gal, were found to have a weak affinity to cholera toxin as well as FucGM1 (AvSNR = 4-10 at spotting concentration of 100 μM). The universal platform was a combination of glycan microarray and glycolipid, and therefore referred to by us as the “glycoconjugate microarray”. This result demonstrated that the 3-D hydrazide-dendrimer surface can be used to immobilize both free glycans covalently and glycolipids non-covalently.

The applications of this sensitive glycolipid microarray has the potential to address an emerging need for detecting toxin levels for food safety, military, and Homeland Security applications (Ngundi et al. 2006; Taitt et al. 2008). It is known that food poisoning is caused by toxins, such as botulinum toxins, which are secreted into contaminated food through

bacterial growth. There are a variety of methods to detect biologically-derived toxins, including high pressure liquid chromatography (HPLC) (Haubl et al. 2007), mass spectrometry (van Baar et al. 1999), and Enzyme-linked Immunosorbent Assays (ELISA) (Hochel et al. 2004). These methods are time consuming, labor intensive, costly, and usually only test for one individual component at a time. Alternately, the glycolipid microarray technology based on the 3-D hydrazide-dendrimer surface can provide a multiplexed platform to detect different types of toxins simultaneously with heightened sensitivity and rapidity while using a small sample size. We are continuing our effort to develop the array biosensors which determine signatures of different biological specimens using toxin-binding glycolipids that are covalently or non-covalently immobilized in patterns on the 3-D array substrates.

3.3 LAM detection on microarray

In a latter case, the 3-D dendrimer substrate was applied to immobilize LAM, which is a large-molecule glycolipid containing more than 100 monosaccharide units (molecular weight is 17 kDa). A printing buffer of 50 mM carbonate-bicarbonate (pH 9.6) facilitates a satisfied immobilization of LAM onto the 3-D hydrazide-dendrimer substrate. LAM can be specifically probed by a CS-35 mAb, which is a specific antibody to recognize the arabinan epitope in LAM (Kaur et al. 2002). As shown in Fig. 3, the increase of concentration of LAM on the surface can achieve a linear increase of fluorescence intensity when LAM was interrogated with CS-35 mAb (primary antibody) and a Cy3 labeled anti-mouse IgG antibody (secondary antibody). LAM at the printing concentration of 250 nanogram (ng)/mL still achieved a visible increment of fluorescent signal compared to the negative control that was spotted only with the printing buffer (Fig. 3A).

In a comparative experiment, the LAM was simultaneously immobilized on the FAST substrates (nitrocellulose-supported) and the hydrazide-dendrimer substrates. The hydrazide-dendrimer substrates exhibited higher sensitivity and lower background compared with FAST nitrocellulose substrates (Fig. 3B). The limit of detection (LOD) of the LAM on hydrazide-dendrimer substrate was 125 ng/mL (Fig. S4 in supplemental information), which was equal to 0.125 picogram (pg) of LAM for each spot when 1 nL of LAM solution was spotted onto the glass surface.

Then, we tested the microarrays interrogated with individual antibodies – CS-35 mAb and anti-LAM pAb – to compare the binding epitopes and affinities of different anti-LAM antibodies against the LAM immobilized on the substrates. The printing concentration of LAM ranged from 50 $\mu\text{g/mL}$ to 0.005 $\mu\text{g/mL}$ in a series of 10-fold dilution. As shown in Fig. 4, the CS-35 mAb (panel A) exhibited heightened sensitivity for the recognition of LAM on the surface compared to the anti-LAM pAb (panel B). The fluorescent intensity became saturated at the spots printed with 5 $\mu\text{g/mL}$ of LAM on both arrays that were interrogated with CS-35 mAb and anti-LAM pAb (Fig. S5 in supplemental information). The maximum AvSNR on the array interrogated with CS-35 mAb was 116.35 ± 10.01 , while the maximum AvSNR value for anti-LAM pAb was 41.85 ± 7.00 . The array absent of interrogation with CS-35 mAb or anti-LAM pAb did not reveal positive fluorescence (Fig. 4, panel C). Additionally, other mannose-containing molecules, such as 6-Mannobiose, 2-Mannobiose and Mannan, did not show affinity to both of the antibodies. The results implied that the binding epitope of the two anti-LAM antibodies are not on the mannan portions of LAM which consist of α -(1 $\rightarrow$ 6)-Man and α -(1 $\rightarrow$ 2)-Man fragments (designated with blue hexagon in Fig. 1C) (Chatterjee et al. 1992). It also confirmed the previous studies which showed that epitopes of CS-35 mAb are on arabinan portions of the LAM (designated with yellow pentagon in Fig. 1C), which locate at the non-reducing termini of LAM such as the arabinosyl hexasaccharide and tetrasaccharide (Kaur et al. 2002; Rademacher et al. 2007). Although an α -(1 $\rightarrow$ 2)-Man dimer or trimer is occasionally linked to the non-

reducing end of LAM in pathogenic mycobacterial strains, such as the *Mtb* LAM used in this study, it cannot affect the binding of anti-LAM antibodies to arabinan motifs on the glycolipid microarray.

In this study, LAM was applied as a model for testing bacterial antigenic lipoglycan on the 3-D array substrate. LAM has been indentified as a novel antigenic biomarker for the diagnosis of Tuberculosis (TB), an infectious disease, caused by *Mtb*, which infects approximately one-third of the world population (Lawn et al. 2009; Sada et al. 1992). Studies have shown that the use of anti-LAM antibodies in the diagnosis of active TB can lead to inaccurate and misleading results (Tessema et al. 2002). In contrast, the platform for detecting LAM antigen provides a sensitive approach for diagnosing TB infection (Boehme et al. 2005). An appropriate microarray surface that could efficiently immobilize LAM would serve as a high-throughput analysis of TB-infected bodily fluids (e.g., serum, urine, saliva). In a proof-of-concept test, we spotted heat-processed urine specimens originated from *Mtb* infected individuals (TB positive; $n = 10$) and non-infected individuals (TB negative; $n = 10$) to the hydrazide-dendrimer substrates and detected the urinary LAM by interrogation with the CS-35 mAb. As shown in Fig. 5, the urine samples from TB patients ($n = 10$) displayed greater reactivity to CS-35 mAb ($P = 0.028$) than the urine samples from healthy individuals ($n = 7$). We ruled out three TB negative samples from individuals with diabetes who could not be considered “healthy”. The UriSub™ artificial urine exhibited lower reactivity on the substrate than that of the healthy individuals (Fig. 5). The healthy urine samples were from groups with an increased exposure to mycobacterial environments, such as avirulent mycobacteria in water and soil. It is also possible that some healthy individuals reside in TB highly or moderately endemic regions and that these individuals have latent TB infection (LTBI). In the TB diagnosis using TB LAM as biomarker, the artificial urine has been reliably applied as a negative control to evaluate the sensitivity and specificity of the tests (Boehme et al. 2005; Lawn et al. 2009). In this test, if the cutoff was calculated as two times of the SNR for the artificial urine sample, seven of ten samples were considered positive (70% sensitivity) and 6 of 7 healthy samples were considered negative in the assay (86% specificity). In addition, as shown in Fig. 5, the ManLAM was able to be strongly reacted with CS-35 mAb on the hydrazide-dendrimer substrate at concentration of 50 $\mu\text{g/mL}$ (SNR was in a range of 70 to 130 for individual tests). This spot on the array was set as a positive control to examine the validation of individual tests.

Although the current LOD of LAM on the hydrazide-dendrimer substrate is about 125 ng/mL, which could be at the edge of the concentration of LAM in bodily fluids (Hamasur et al. 2001), the initial tests on the “real world” TB urine samples have demonstrated the feasibility for the use of this sensing platform for pathogen detection. Of course, more improvement on the hydrazide substrate (sensor) and consistency on the manufacturing process could potentially enhance the sensitivity and specificity of the detection.

4. Conclusion

This paper addressed an important substrate for fabricating glycolipids microarray. This substrate possessed 3-D hydrazide-dendrimer modification and exhibited superior binding to glycolipids molecules via electronic adsorption. The proof-of-concept tests on the gangliosides and the lipoarabinomannan have revealed the sensitivity and specificity of this platform for analyzing glycolipid-protein interactions. In particular, the CTB having pentameric binding sites to GM1 showed significantly increased binding affinity to GM1 on this substrate when compared to other ~80 glycoconjugates, including free glycans and glycolipids. Also, the lipoarabinomannan could be sensitively detected by antibodies on this substrate surface at printing concentration of 125 ng/mL, which is the approximate concentration of this antigenic lipoglycan in bodily fluids in tuberculosis patients. In

addition, the initial proof-of-concept has demonstrated the feasibility for *Mtb* LAM detection in urine specimens. Therefore, this novel glycolipid microarray platform could serve as a glycobiology research tool for studying glycolipid-protein interactions, as well as serve as a simple prototype of biosensors for sensitive detection of toxins and diagnosis of infectious diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH/NCRR Grant 5R44RR02763 (J.Z. and X.Z). The authors gratefully acknowledge NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH, to provide *Mycobacterium tuberculosis* LAM and anti-LAM antibodies for this work, and the WHO/TDR TB Specimen Bank to provide TB urine specimens. The authors also thank Dr. Denong Wang of SRI International who contributed to technical discussions.

References

- Adar T, Ilan Y. J Immunotoxicol. 2008; 5(2):209–220. [PubMed: 18569392]
- Angstrom J, Teneberg S, Karlsson KA. Proc Natl Acad Sci U S A. 1994; 91(25):11859–11863. [PubMed: 7527546]
- Boehme C, Molokova E, Minja F, Geis S, Loscher T, Maboko L, Koulchin V, Hoelscher M. Trans R Soc Trop Med Hyg. 2005; 99(12):893–900. [PubMed: 16139316]
- Boggs JM, Gao W, Hirahara Y. J Neurosci Res. 2008; 86(7):1448–1458. [PubMed: 18189317]
- Chatterjee D, Lowell K, Rivoire B, McNeil MR, Brennan PJ. J Biol Chem. 1992; 267(9):6234–6239. [PubMed: 1556132]
- Derry DM, Wolfe LS. Science. 1967; 158(807):1450–1452. [PubMed: 4862383]
- Hamasur B, Bruchfeld J, Haile M, Pawlowski A, Bjorvatn B, Kallenius G, Svenson SB. J Microbiol Methods. 2001; 45(1):41–52. [PubMed: 11295196]
- Haubl G, Berthiller F, Hametner C, Rechthaler J, Jaunecker G, Freudenschuss M, Krska R, Schuhmacher R. Anal Bioanal Chem. 2007; 389(3):931–940. [PubMed: 17668191]
- Hochel I, Viochna D, Skvor J, Musil M. Folia Microbiol (Praha). 2004; 49(5):579–586. [PubMed: 15702549]
- Hooper LV, Gordon JI. Glycobiology. 2001; 11(2):1R–10R. [PubMed: 11181556]
- Kasahara K, Sanai Y. Glycoconj J. 2000; 17(3-4):153–162. [PubMed: 11201786]
- Kaur D, Lowary TL, Vissa VD, Crick DC, Brennan PJ. Microbiology. 2002; 148(Pt 10):3049–3057. [PubMed: 12368438]
- Lawn SD, Edwards DJ, Kranzer K, Vogt M, Bekker LG, Wood R. Aids. 2009; 23(14):1875–1880. [PubMed: 20108382]
- Liu Y, Palma AS, Feizi T. Biol Chem. 2009; 390(7):647–656. [PubMed: 19426131]
- Millen SH, Lewallen DM, Herr AB, Iyer SS, Weiss AA. Biochemistry. 2010; 49(28):5954–5967. [PubMed: 20515023]
- Mocchetti I. Cell Mol Life Sci. 2005; 62(19-20):2283–2294. [PubMed: 16158191]
- Ngundi MM, Taitt CR, Ligler FS. Sensor Letters. 2007; 5:621–624.
- Ngundi MM, Taitt CR, McMurphy SA, Kahne D, Ligler FS. Biosens Bioelectron. 2006; 21(7):1195–1201. [PubMed: 15946840]
- Rademacher C, Shoemaker GK, Kim HS, Zheng RB, Taha H, Liu C, Nacario RC, Schriemer DC, Klassen JS, Peters T, Lowary TL. J Am Chem Soc. 2007; 129(34):10489–10502. [PubMed: 17672460]
- Rinaldi S, Brennan KM, Goodyear CS, O’Leary C, Schiavo G, Crocker PR, Willison HJ. Glycobiology. 2009; 19(7):789–796. [PubMed: 19349623]

- Sada E, Aguilar D, Torres M, Herrera T. J Clin Microbiol. 1992; 30(9):2415–2418. [PubMed: 1401008]
- Spear PG. Cell Microbiol. 2004; 6(5):401–410. [PubMed: 15056211]
- Taitt CR, Shriver-Lake LC, Ngundi MM, Ligler FS. Sensors. 2008; 8:8361–8377.
- Tessema TA, Bjune G, Hamasur B, Svenson S, Syre H, Bjorvatn B. Scand J Infect Dis. 2002; 34(2): 97–103. [PubMed: 11928861]
- van Baar BL, Hulst AG, Wils ER. Toxicon. 1999; 37(1):85–108. [PubMed: 9920483]
- Vaught JD, Bock C, Carter J, Fitzwater T, Otis M, Schneider D, Rolando J, Waugh S, Wilcox SK, Eaton BE. J Am Chem Soc. 2010; 132(12):4141–4151. [PubMed: 20201573]
- Zhou X, Turchi C, Wang D. J Proteome Res. 2009; 8(11):5031–5040. [PubMed: 19791771]

Fig. 1. (A) A schematic representation of the 3-dimensional (3-D) surface that is coated with hydrazide modified PAMAM dendrimer on glass substrate. The experimental procedure to fabricate the surface modification has been described in the previous publication (Zhou et al. 2009). The glycolipids immobilized on the surface were able to bind to GBPs or antibodies through direct or indirect interrogations. The hydrazide groups on the termini of the dendrimer possess the attributes of being H-bond acceptor and H-bond donor simultaneously. (B) The chemical structures of the gangliosides used in this study. The structural distinctions of the gangliosides were denoted based on GM1 structure. (C) The structure of LAM, a GPI-like lipoglycan existing on the cell wall of mycobacterium.

Fig. 2. Glycolipid microarray (hydrazide-dendrimer substrate) that was stained with CTB. (A) The substrates were printed with the gangliosides in two concentrations (100 μ M and 10 μ M) and stained with AlexaFluor555 labeled CTB (1:1,000 dilution). The microarray scanner was set in a rainbow palette and the standard rainbow scale of fluorescence intensity is shown; (B) The fluorescence intensity (AvSNR) of the glycoconjugate microarray on the hydrazide-dendrimer substrate that was spotted with 84 individual free glycans and glycolipids (the array image and the carbohydrate list are shown in supplemental information Fig. S3 and Table S1). The free glycans were spotted in concentrations of 100 μ M and 10 μ M and the AvSNRs were exhibited side by side for the two concentrations.

Fig. 3.

(A) Reactivity of CS-35 mAb with the ManLAM that was immobilized on a hydrazide-dendrimer substrate. Each spot on the substrate was coated with 1 nL of two-fold serial dilutions of LAM in carbonate-bicarbonate buffer (pH 9.6) ranging from 1 μ g/mL to 62.5 ng/mL. The LAM on each spot was interrogated with CS-35 mAb (mouse) at 1:1,000 dilution. A secondary antibody, Cy3-labeled anti-mouse IgG was used to detect the intensity of the immunoassay. The microarray scanner was set in a green palette. (B) Comparison of reactivity of LAM to CS-35 mAb on a hydrazide-dendrimer glass substrate to a FAST nitrocellulose substrate. The fluorescence intensity was normalized against the intensity of a negative control (printing buffer) and the data were evaluated by the resulting S/B values.

Fig. 4.

The comparison of the activity of two anti-LAM antibodies against the LAM immobilized on 3-D hydrazide-dendrimer substrates (LAM titer assay). The glass substrates were immobilized with LAM and mannose-containing molecules, and stained with CS-35 mAb (1:1,000; mouse) (**A**), anti-LAM pAb (1:1,000; rabbit) (**B**) as primary antibody. Cy3-labeled goat anti-mouse IgG and goat anti-rabbit IgG were applied as secondary antibodies in panel A and B, respectively. Panel C was the assay without applying primary antibodies, however used Cy3-labeled anti-mouse IgG as a secondary antibody (a negative control). Each assay was arrayed as 8 replicates to ensure reproducibility of the results. The microarray scanner was set in a green palette.

Fig. 5. Reactivity of urine specimens from clinically diagnosed healthy individuals ($n = 7$) and TB infected patients ($n = 10$) toward CS-35 mAb on the hydrazide-dendrimer substrate. Statistical differences between groups were calculated using one-way ANOVA test (2-quartile). The cutoff (dash line) for TB positivity was considered to be two times the background SNR for the artificial urine control. The inserted image shows immobilization of 50 µg/mL ManLAM (positive control) on the substrate.