Glycoconj J (2012) 29:1–12
DOI 10.1007/s10719-011-9359-9

GLYCOARRAY SECTION

Design of a covalently bonded glycosphingolipid microarray

Emma Arigi · Ola Blixt · Karsten Buschard · Henrik Clausen · Steven B. Levery

Received: 14 August 2011 / Revised: 3 October 2011 / Accepted: 28 October 2011 / Published online: 22 November 2011

Abstract Glycosphingolipids (GSLs) are well known ubiquitous constituents of all eukaryotic cell membranes, yet their normal biological functions are not fully understood. As with other glycoconjugates and saccharides, solid phase display on microarrays potentially provides an effective platform for *in vitro* study of their functional interactions. However, with few exceptions, the most widely used microarray platforms display only the glycan moiety of GSLs, which not only ignores potential modulating effects of the lipid aglycone, but inherently limits the scope of application, excluding, for example, the major classes of plant and fungal GSLs. In this work, a prototype "universal" GSL-based covalent microarray has been designed, and preliminary evaluation of its potential utility in assaying protein-GSL binding interactions investigated. An essential step in development involved the enzymatic release of the fatty acyl moiety of the ceramide aglycone of selected mammalian GSLs with sphingolipid *N*-deacylase (SCDase). Derivatization of the free amino group of a typical lyso-GSL, lyso-G_{M1}, with a prototype linker assembled from succinimidyl-[(*N*-maleimidopropionamido)-diethyleneglycol] ester and 2-mercaptoethylamine, was also tested. Underivatized or linker-derivatized lyso-GSL were then immobilized on *N*-hydroxysuccinimide- or epoxide-activated glass microarray slides and probed with carbohydrate binding proteins of known or partially known specificities (*i.e.*, cholera toxin B-chain; peanut agglutinin, a monoclonal antibody to sulfatide, Sulph 1; and a polyclonal antiserum reactive to asialo-G_{M2}). Preliminary evaluation of the method indicated successful immobilization of the GSLs, and selective binding of test probes. The potential utility of this methodology for designing covalent microarrays that incorporate GSLs for serodiagnosis is discussed.

Keywords Glycosphingolipid · Glycolipid · Ganglioside · Glycan · Lectin · Antibody · Microarray · Glycan array

E. Arigi · O. Blixt · H. Clausen · S. B. Levery (✉)
Department of Cellular and Molecular Medicine,
University of Copenhagen,
Blegdamsvej 3,
2200 Copenhagen N, Denmark
e-mail: levery@sund.ku.dk

K. Buschard
Rigshospitalet, Bartholin Institute,
Blegdamsvej 9,
2100 Copenhagen OE, Denmark

Present Address:
E. Arigi
Department of Biological Sciences,
The Border Biomedical Research Center,
University of Texas at El Paso,
500 West University Avenue,
El Paso, TX 79968, USA

Introduction

Glycosphingolipids (GSLs), composed of hydrophilic glycans attached to hydrophobic ceramide (Cer) moieties, are important components of the plasma membranes of all eukaryotic cells. Although their normal biological functions are not fully understood, it has been appreciated for some time that the distribution of GSLs in neuronal and myelin membranes, and in other tissues and organs, makes them potential targets for involvement in the pathogenesis of autoimmune diseases, as well as potential diagnostic markers for autoimmune neuropathies and other disorders [1–4]. For example, sulfatide (galactosylceramide-3′-*O*-sulfate), a major GSL component of the islets of Langerhans expressed both on the surface and secretory granules of the insulin producing β-cells, has been

implicated in insulin dependent diabetes mellitus (IDDM) also known as type 1 diabetes [5–8]. In this chronic autoimmune disorder characterized by gradual destruction of insulin producing β-cells, elevated levels of immunoglobulin G (IgG) type autoantibodies against sulfatide have been detected in newly diagnosed IDDM patients, but not those with non-insulin-dependent diabetes (NIDDM) [9]. Since sulfatide is a potential target in the autoimmune process involved in development of the disease, improved screening sensitivity will aid prognosis and provide a better understanding of the role of anti-sulfatide antibodies and other autoantibodies in disease progression.

One approach to deciphering the interactions of glycans and glycoconjugates with proteins is to incorporate them into microarrays [10–20]. Modern glycan microarray technology currently provides a robust platform for investigation of a wide variety of carbohydrate-protein interactions, including discovery and clinical exploitation of natural anti-carbohydrate antibodies [4, 14, 20–23]. GSLs, however, comprise a somewhat special subset of compounds at the intersection of an organism's glycome and lipidome, wherein the lipid moiety may (or may not) have a significant influence, either direct or indirect, on the binding of the glycan to other molecules. Therefore, arrays displaying only GSL-derived glycans could miss important interactions. The extent to which this is true remains to be fully explored. It is of particular interest to explore the possibility that the ceramide moieties of GSLs can act as an extended binding epitope for antibodies, similar to what has been observed with certain O-glycopeptide epitopes, such as the tandem repeat of the human mucin-1 (MUC1), where reactivity of some monoclonal antibodies (MAbs) appears to be specific for both glycan and peptide backbone [24–26]. For example, in the characterization of the binding epitope of the anti-sulfatide MAb Sulph 1, it has been suggested that the lipid moiety forms an essential component for antibody binding [27]. However, further experiments could clarify the role of the ceramide moiety in binding.

Conceptually, the practice of arraying GSLs in a planar configuration for discovery and exploitation of their interactions has its roots in thin layer chromatography (TLC) overlay staining, in which complex GSL mixtures separated on a TLC plate were probed with glycan binding proteins (GBPs) [28, 29]. Since the original description of TLC overlay methodology, it has evolved and diversified over the years, in parallel with ELISA techniques [30, 31], to include a wide variety of GSL array designs based on non-covalent and covalent immobilization of GSLs and GSL analogues [10, 16, 32–36]. In one interesting case, the ganglioside G_{M1} was incorporated into an immobilized fluid supported lipid bilayer approximating display in a cell membrane [37]. Multidimensional approaches that combine mass spectrometry and immunoassays *in situ* have also been developed [10, 38–40]. With few exceptions to date, the covalent array platforms prepared wholly or in part for the study of GSL interactions have relied on synthetic procedures that append amine functionalized linkers directly to the reducing end of GSL-derived carbohydrates [41–43], eliminating all features of the lipid aglycone. These structures may not fully mimic the presentation of sphingolipid-linked glycans. Moreover, enzymatic methods for release of GSL glycans from their ceramide aglycones are not applicable to the major classes of fungal and plant GSLs, which are in both cases glycosylinositol phosphorylceramides (GIPCs) [44]; chemical methods for removal of the ceramide moiety from these compounds yield a glycosylinositol, not a convenient form for functional immobilization. Covalent GSL arrays incorporating all or part of the sphingolipid ceramide moiety have also been developed [33, 36], but appear to require at least some facility with synthetic organic chemistry, and studies so far have been confined to a very limited repertoire of compounds. Most recently, some of the problems with fabrication of covalent GSL arrays were solved by a chemical method based on oxidation of the sphingoid *E*-4 unsaturation, enabling appendage of a bifunctional fluorescent linker for attachment to an activated surface [45]. While this development facilitated generation of a broad spectrum GSL microarray, with retention of the ceramide *N*-acyl moiety and the proximal portion of the sphingoid, it is still not universally applicable, since many ceramide forms are not unsaturated at C-4/C-5 of the sphingoid.

We sought to develop a sensitive, universally applicable, high throughput (HTP) screening platform on a microarray format that utilizes minimal sample, while retaining a desirable characteristic of traditional methods such as ELISA and membrane arrays, displaying the GSLs with partial or complete immunological presentation of their ceramide components. The microarray design relies on covalently coupling lyso-glycosphingolipids (lyso-GSLs) onto commercially available amine-reactive *N*-hydroxysuccinimide (NHS)-ester or epoxide activated glass slides via the primary amino group of the sphingoid base, either directly or through a prototype linker. The first essential step in the microarray fabrication involved the enzymatic deacylation of the ceramide moiety of GSLs using the enzyme sphingolipid ceramide *N*-deacylase (SCDase) [46–49]. Previously, we used this strategy to generate fluorocarbon derivatives of gangliosides and other GSLs [50], including a fluorocarbon-labeled G_{M1} derivative reactive with cholera toxin B chain (CTX-B) when displayed on a fluorous surface. Our preliminary results herein demonstrate the efficacy of a versatile covalent microarray format in displaying lyso-GSLs in recognizable conformations, as shown by the selective binding of G_{M1} ganglioside by CTX-B, and of asialo-G_{M1} by peanut agglutinin (PNA). Demonstrations of immunological recognition were successfully carried out with an anti-

sulfatide monoclonal antibody, Sulph 1, and with a polyclonal antibody reactive to asialo-G_{M2}.

Materials and methods

Glycosphingolipids and reagents

Glycosphingolipids (GSLs), *i.e.*, monosialogangliosides G_{M1} (**1**), G_{M2} (**2**), and G_{M3} (**3**); and lyso-GSLs galactopsychosine (**Gal-Sph**), glucopsychosine (**Glc-Sph**), lyso-lactosylceramide (Lac-Sph), lyso-sulfatide (**S3Gal-Sph**), and lyso-G_{M1} (**G_{M1}-Sph**) were purchased from Matreya LLC (Pleasant Gap, PA, USA). Asialo forms of gangliosides, G_{A1} (**8**) and G_{A2} (**9**), were generated by mild acid treatment of the parent gangliosides, G_{M1} and G_{M2}, respectively. Disialogangliosides G_{D1a} (**10a**), G_{D1b} (**10b**), G_{D3} (**11**), G_{T1b} (**12**), and G_{Q1b} (**13**) were obtained from Biocarb (Lund, Sweden). Synthetic oligosaccharides (G_{M1}-ose, G_{M3}-ose, lactose, and G_{D3}-ose) with azide terminated reducing end spacers [42] were obtained from the Consortium for Functional Glycomics (CFG, La Jolla, CA). Before printing, they were converted to amino-terminated forms by reduction, as previously described. Sphingolipid ceramide *N*-deacylase (SCDase) from *Pseudomonas* sp. was purchased from Sigma (St. Louis MO, USA).

Alexa Fluor 555 conjugated recombinant cholera toxin B (AF555-CTX-B) and Alexa Fluor 488 conjugated PNA lectin (AF488-PNA) were purchased from Molecular Probes Inc. (Eugene, OR, USA). A 1.0 mg/mL AF555-CTX-B solution was prepared by dissolving 100 μg of the reagent in 0.1 mL of phosphate buffered saline (PBS, pH 7.4), and the solution stored at 2-5°C. It was diluted 1/5000 in assay buffer (0.0015 mol/L KH_2PO_4, 0.0065 mol/L Na_2HPO_4, 0.5 mol/L NaCl, 0.003 mol/L KCl, 10% w/v BSA, and 10% v/v Triton-X-100; pH 7.4) prior to use. AF488-PNA was dissolved in water to make a stock solution of 1 mg/mL and diluted to 20 μg/mL in assay buffer prior to use.

The anti-sulfatide monoclonal antibody (MAb) Sulph 1 was obtained from the laboratory of Karsten Buschard (Bartholin Institute, Copenhagen, Denmark; originally kindly donated by Dr. Jan-Eric Månsson, Neurochemistry, Sahlgren's University Hospital,Göteborg, Sweden.). Anti-G_{A2} polyclonal antibody (pAb) was purchased from Matreya LLC (Pleasant Gap, PA, USA).

The heterobifunctional linker NHS-PEO_2-maleimide (succinimidyl-[(*N*-maleimidopropionamido)-diethyleneglycol] ester; 2-mercaptoethylamine (2-MEA), and analytical grade dimethylsulfoxide (DMSO) were purchased from Pierce Biotechnology, Inc., (Rockford, IL). A 250 mM stock solution of NHS-PEO_2-maleimide was prepared by dissolving 100 mg in 940 μL of DMSO. 2-MEA (6 mg) was dissolved in 100 μL of the conjugation buffer (PBS-EDTA: 20 mM Na_2HPO_4, 150 mM NaCl, 5 mM EDTA, pH 7.5).

HPTLC analysis

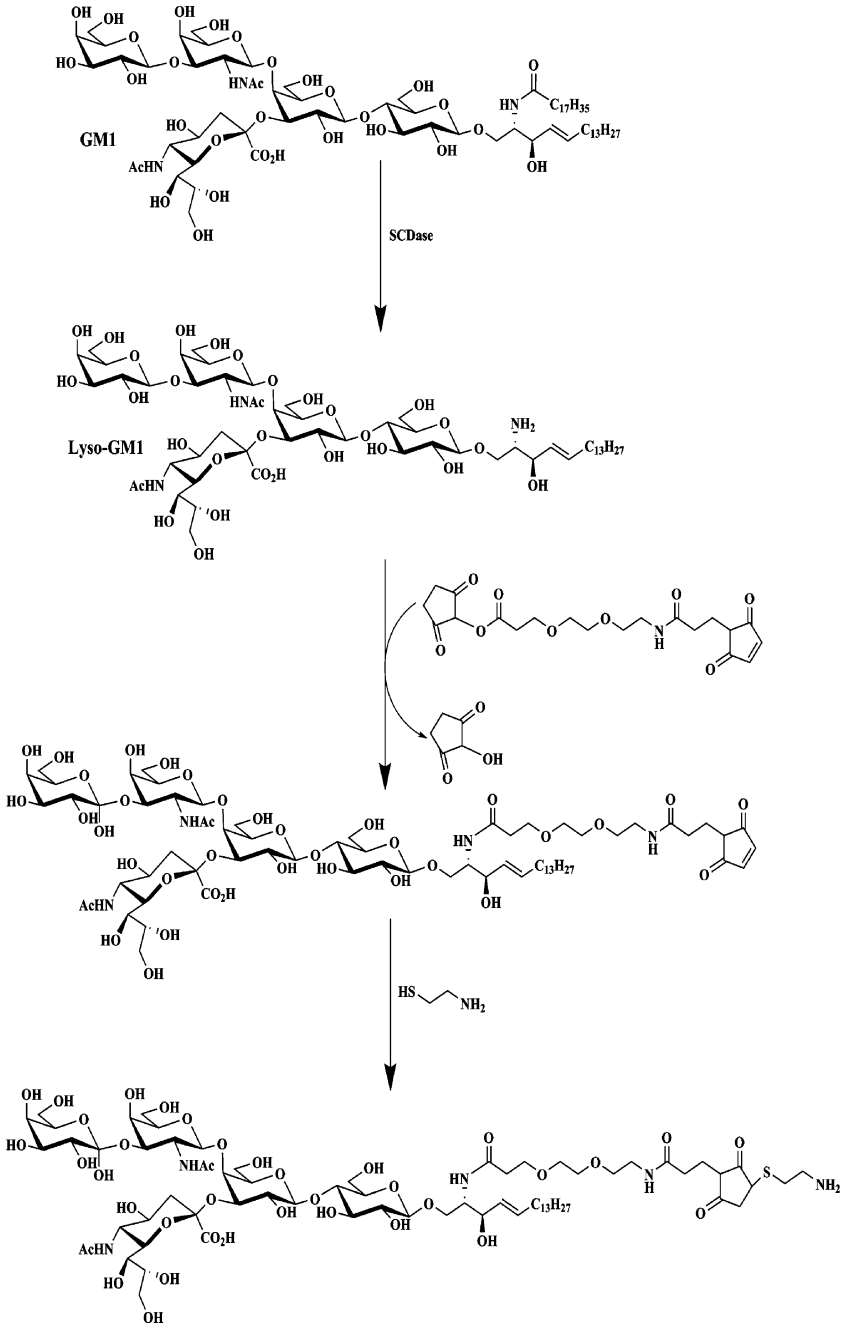
HPTLC was performed on silica gel 60 plates (200 μm layer thickness, 60 Å particle size; E. Merck, Darmstadt, Germany) using chloroform/methanol/water (50:47:14 v/v/v, containing 0.038% w/v $CaCl_2$) as mobile phase. GSLs and their derivatives were solubilized in isopropanol/hexane/water (55:25:20, v/v/v, upper phase discarded; solvent C) and applied by streaking from 5 μL Micro-caps (Drummond, Broomall, PA). Detection was made by Bial's orcinol reagent (orcinol 0.55% [w/v] and H_2SO_4 5.5% [v/v] in ethanol/water 9:1 [v/v]; the plate was sprayed and heated briefly to ~200–250°C).

De-*N*-acylation of GSLs

GSL de-*N*-acylation (to generate a free sphingoid primary amine group) was carried out with *Pseudomonas* sphingolipid ceramide de-*N*-acylase (SCDase; see Scheme 1) [46, 47]. In general, 20 mU of enzyme was added to the sample (~50 μg) dispersed in a biphasic environment containing 10 μL buffer (0.8% taurodeoxycholate sodium salt [TDC] in sodium acetate, pH 5.5) and 100 μL n-decane (to partition the fatty acids away from the aqueous phase since the enzyme has reverse activity) [48, 49]. Incubation was performed for 24 h at 37°C with slight agitation. The progress of the reaction was followed by HPTLC as described above. By this method gangliosides G_{M1} (**1**), G_{M2} (**2**), G_{M3} (**3**), G_{A1} (**8**), G_{A2} (**9**), G_{D1a} (**10a**), G_{D1b} (**10b**), G_{D3} (**11**), G_{T1b} (**12**), and G_{Q1b} (**13**) were converted to lyso-gangliosides **("-Sph")**, respectively. Following aspiration of the decane layer, the reaction mixture was applied to a 1 cc Sep-Pak^R Vac C-18 SPE cartridge (Waters Corporation, Milford MA); following a water wash to desalt, a step gradient of aqueous methanol was applied to the cartridge (2 mL each of 20, 40, 60, 80, and 100% methanol). The eluted fractions were dried down and assayed by HPTLC as described above; fraction(s) containing lyso-ganglioside free of TDC were used for microarray printing. Production of compounds of the proper molecular weight was verified by MALDI-TOF-MS (DHB matrix). For a summary list of all arrayed compounds, see Table 1 and (Scheme 2).

NHS-PEO_2-maleimide-2-mercaptoethylamine derivatization of lyso-GSLs

Lyso-GM1 (100 μg) was dissolved in PBS-EDTA conjugation buffer (500 μL), and NHS-PEO_2-maleimide solution (4 μL) was added. Incubation was performed for 1 h at room temperature with slight agitation. The sample was desalted



Scheme 1 Enzymatic de-*N*-acylation of a typical glycosphingolipid, $G_{M1(a)}$, by SCDase. Subsequent derivatization of the resulting lyso-G_{M1} with NHS-PEO_2-maleimide (succinimidyl-[(*N*-maleimidopropionamido)-diethyleneglycol] ester) and 2-mercaptoethylamine (2-MEA) generates a primary amine-terminated linker reactive with either NHS- or epoxy-functionalized surfaces

on a 1 cc C-18 SPE cartridge; the sample was applied to the cartridge, washed with water, and eluted in 100% methanol. The NHS-PEO_2 maleimide derivatized sample was dried down under N_2, re-suspended in PBS-EDTA (100 μL). To generate a derivative with a free primary amine (Scheme 1), 10 uL of the 2-MEA solution in PBS-EDTA was added and the reaction incubated for 90 min at 37°C. The progress of the reaction was monitored using HPTLC, and the product desalted by C18-SPE as above. The molecular weight of the final product was verified by MALDI-TOF-MS (DHB matrix), *i.e.*, four major peaks observed at nominal, mono-isotopic *m/z* 1689, 1717, 1711, and 1739 are consistent with the target molecule containing d18:1 and d20:1 sphing-4-enines ($\Delta m/z$=28), represented as sodium adducts ([M(H)+Na]$^+$) and as di-sodium salt adducts ([M(Na)+Na]$^+$; $\Delta m/z$=22), respectively. The ratio of the final product to unreacted lyso-G_{M1} starting material was estimated by HPTLC to be >90%.

Printing of GSLs, microarray binding assay, and image acquisition

Lyso-GSLs and the lyso-G_{M1} NHS-PEO_2-maleimide-2-MEA derivative were dissolved in either 150 mM print

Table 1 List of compounds used in fabrication of microarrays. For detailed structures of ganglio-series gangliosides and their asialo derivatives, see Scheme 2

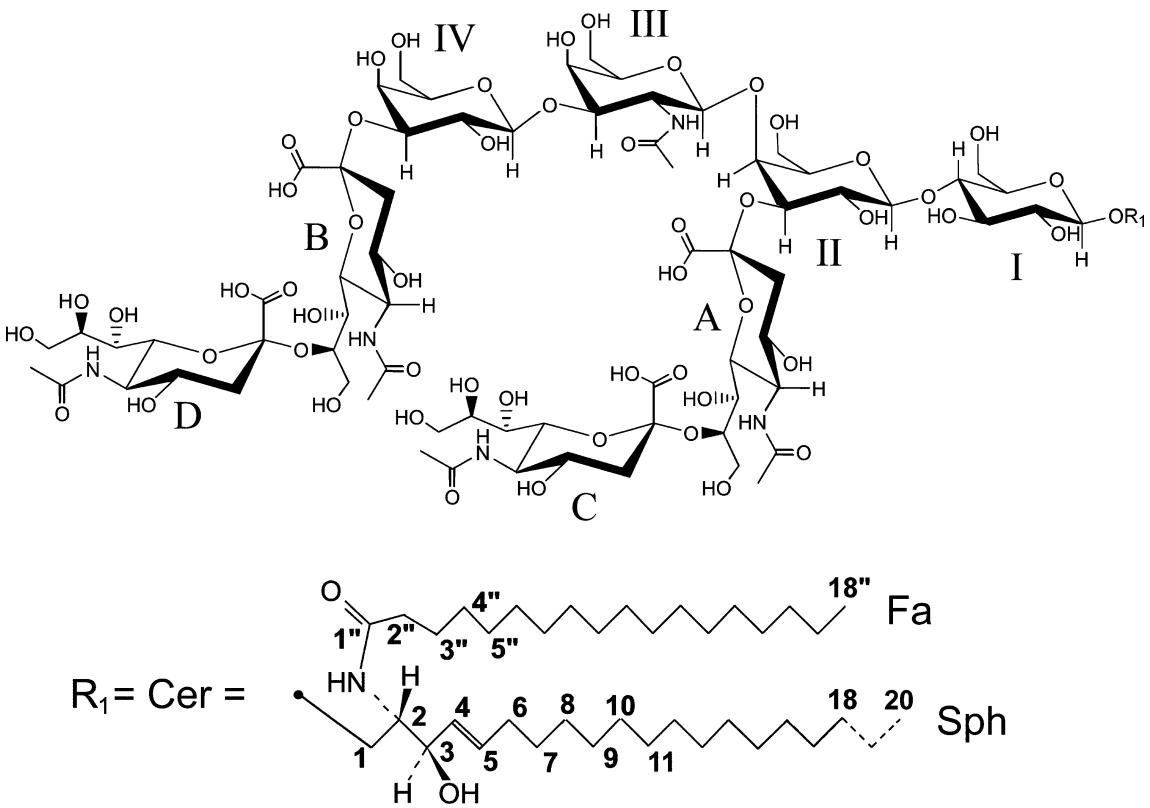
GSL as	Lyso	Glycan Structure
(**1**) $G_{M1(a)}$	**G_{M1}-Sph**[a]	Galβ3GalNAcβ4(Neu5Acα3)Galβ4Glcβ-
(**2**) G_{M2}	**G_{M2}-Sph**	GalNAcβ4(Neu5Acα3)Galβ4Glcβ-
(**3**) G_{M3}	**G_{M3}-Sph**[a]	Neu5Acα3Galβ4Glcβ-
(**4**) GalCer	**Gal-Sph**	Galβ-
(**5**) GlcCer	**Glc-Sph**	Glcβ-
(**6**) LacCer	**Lac-Sph**[a]	Galβ4Glcβ-
(**7**) Sulfatide	**S3Gal-Sph**	SO_3-3Galβ-
(**8**) G_{A1} (Gg_4)	**G_{A1}-Sph**	Galβ3GalNAcβ4Galβ4Glcβ-
(**9**) G_{A2} (Gg_3)	**G_{A2}-Sph**	GalNAcβ4Galβ4Glcβ-
(**10a**) G_{D1a}	**G_{D1a}-Sph**	Neu5Acα3Galβ3GalNAcβ4(Neu5Acα3)Galβ4Glcβ1-
(**10b**) G_{D1b}	**G_{D1b}-Sph**	Galβ3GalNAcβ4(Neu5Acα8Neu5Acα3)Galβ4Glcβ1-
(**11**) G_{D3}	**G_{D3}-Sph**[a]	Neu5Acα8Neu5Acα3Galβ4Glcβ-
(**12**) G_{T1b}	**G_{T1b}-Sph**	Neu5Acα3Galβ3GalNAcβ4(Neu5Acα8Neu5Acα3)Galβ4Glcβ-
(**13**) G_{Q1b}	**G_{Q1b}-Sph**	Neu5Acα8Neu5Acα3Galβ3GalNAcβ4(Neu5Acα8Neu5Acα3)Galβ4Glcβ-

[a] Compounds **1**, **3**, **6**, **11** also arrayed as spacer-terminated synthetic oligosaccharides, **G_{M1}-ose**, **G_{M3}-ose**, **Lac-ose**, **G_{D3}-ose**, respectively. Compound **1** also arrayed as a spacer-linked lyso-GSL, **G_{M1}-link**.

buffer (17 mM $NaH_2PO4.H_2O$, 133 mM $Na_2HPO4.2H_2O$, 0.005% CHAPS, 0.03% NaN_3, pH 8.5; 1% DMSO in 150 mM print buffer; or pure DMSO); serial dilutions were made in the same solvents to reach concentrations within the range between ~1000 μM and 1 μM. The GSLs were spotted on NHS-activated glass slides (Schott Nexterion[R] slide H or Schott Nexterion[R] MPX 16; Schott Nexterion, Mainz, Germany) using a Biorobotic Microgrid II arrayer (Genomic Solutions, Ann Arbor, MI) in quadruplicates at different concentrations and incubated for 1 h at room temperature and 80% humidity. Blocking of unreacted NHS groups was carried out by immersing slides in blocking solution (25 mM ethanolamine in 100 mM sodium borate, pH 8.5) for 1 h, and rinsing in PBS. Appropriate conjugates (AF555-CTX-B or AF488-PNA) or MAb (Sulph 1) were added to the wells and incubation performed as follows. Incubations with AF555-CTX-B (0.2 μg/mL) or AF488-PNA (20 μg/mL) were performed in the dark for 1 h; at the end of the incubation the slides were washed with PBS containing 0.05% Tween 20 (PBS-T) washing buffer, PBS, and finally deionized water before being dried down by centrifugation (200× *g*). Incubation with MAb was carried out for 1 h, followed by rinsing with PBS-T and incubation with Cy3 conjugated goat goat- anti- mouse IgG (H+L) secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), diluted 1:5000 in assay buffer. The rabbit pAb to G_{M2} was detected with biotinylated polyclonal swine anti-rabbit IgGs (DAKO, Denmark) and Cy3-conjugated streptavidin (Invitrogen). Final rinsing and drying was carried out as previously described. Fluorescent images of glass slides (microarrays) were obtained using a ProScanArray™ HT Microarray Scanner (Perkin-Elmer, Waltham MA, USA), and image analysis performed with ProScan Array Express 4.0 software (Perkin-Elmer) using the method of adaptive circles with local background subtraction.

Results and discussion

N-hydroxysuccinimide (NHS) modified glass slides are a well-established platform for construction of robust glycan microarrays from oligosaccharides immobilized via an amino-functionalized linker [14]. It was reasoned that all GSLs possess a cryptic amino functionality on the sphingoid moiety, which could be universally employed for immobilization in an analogous fashion, provided a convenient and widely applicable method for de-*N*-acylation were available. In fact, an already commercially available enzyme, sphingolipid ceramide de-*N*-acylase (SCDase), appeared to fulfill all the requirements, including applicability to GSLs with functional groups sensitive to the harsh alkaline conditions required for chemical de-*N*-acylation. In addition, although a number of chemistries are available for derivatization of the sphingoid moiety, these all require the presence of an activating Δ^4-unsaturation, which many classes of GSLs do not possess. The SCDase reaction is illustrated in Scheme 1 for G_{M1} ganglioside. It was further reasoned that the disadvantage of losing the chemical information in the fatty-*N*-acyl group could be in principle at least partially made up by incorporation of



Scheme 2 Composite structure for ganglio-series gangliosides and their asialo derivatives. Structures relevant to this work are (R_1=Cer=-Ceramide): (**1**) $G_{M1(a)}$ (I–IV, A); (**2**) G_{M2} (I, II, III, A); (**3**) G_{M3} (composed of residues I, II, A,); (**6**) lactosylceramide (I, II); (**8**) G_{A1} (I–IV); (**9**) G_{A2} (I–III); (**10a**) G_{D1a} (I–IV, A, B); (**10b**) G_{D1b} (I–IV, A, C); (**11**) G_{D3} (I, II, A, C); (**12**) G_{T1b} (I–IV, A, B, C); and (**13**) G_{Q1b} (I–IV, A, B, C, D). Ceramide (R_1) structures for bovine brain gangliosides are predominantly composed of d18:1 or d20:1 sphing-4-enine (Sph) with 18:0 fatty-*N*-acylation (Fa), as shown; lyso-GSLs are missing fatty-*N*-acylation

appropriately functionalized linkers interposed between the sphingoid amino group and the microarray surface. A prototype linker, incorporating a hydrophilic acyl chain analog and a terminal primary amine function, was assembled in two subsequent steps, as illustrated in Scheme 1. For convenience, initial validation of the GSL microarray concept was carried out using commercially available lyso-GSLs for most components. Epoxide functionalized microarray slides were also tested as an alternative chemistry for immobilization.

Typical results for the de-*N*-acylation reaction, as followed by HPTLC, are shown in Fig. 1; higher and lower Rf bands correspond to intact GSLs and their de-*N*-acylated products (lyso-GSLs), respectively. In our hands, the SCDase reaction with gangliosides in particular went essentially to completion, as observed previously [46–50]. Asialo-gangliosides G_{A1} and G_{A2} were prepared by mild acid de-sialylation of the parent gangliosides. In addition to generating a panel of lyso-GSLs, lyso-G_{M1} was successfully derivatized with the prototype linker.

Immobilization and detection of GSLs using appropriate binding proteins was carried out as follows. To determine a suitable working concentration, lyso-GSLs (**"-Sph"**) were printed in a range of starting concentrations between 1000 μM to 0.5 μM onto NHS-activated hydrogel or epoxy-modified glass slides (Fig. 2). As controls, GalNAcα-Thr (**Tn**), Galβ1-3GalNAcα-Thr (**T**), and GSL-derived oligosaccharides with amine-terminated linkers (**G_{M1}-ose**, **G_{D3}-ose**, **G_{M3}-ose**, and **Lac-ose**) were also incorporated into the print. In order to account for the range of polarities of the GSL derivatives, two different solvents were used to disperse the lyso-GSLs prior to printing, 1% DMSO in print buffer (labels superscripted "a") and 100% DMSO (labels superscripted "b"). The linker-terminated glycans and glycopeptides were dispersed in print buffer without DMSO. Initial experiments were performed with a Ctx-B conjugate (AF555-CTX-B), since the reactivity of Ctx-B has been well studied and documented in numerous publications—high binding affinity with G_{M1} ganglioside, much weaker affinity pattern with other gangliosides and their derivatives, occasionally differing in order depending on the method employed [51, 52] (see also, *e.g.*, [53, 54]. Although it has also been established that Ctx-B binding is not dependent on attachment of the oligosaccharide to ceramide, it should be an excellent probe for a properly functioning GSL microarray. As shown in

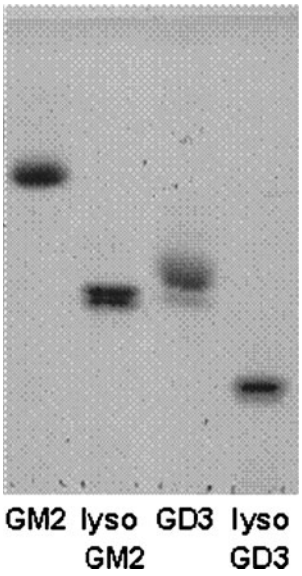


Fig. 1 HPTLC profile of gangliosides G_{M2} and G_{D3} with their SCDase de-*N*-acylated (lyso) derivatives. Silica gel 60 plate developed with $CHCl_3$-MeOH-water (50:47:14 v/v/v +0.03% w/v $CaCl_2$) mobile phase; detection with Bial's orcinol stain

Fig. 2, Panel A, reactivity with Ctx-B (**G_{M1}-Sph**) was achieved within the concentration range of ~1000 μM to 3.9 μM. Consistent with the known specificity of Ctx-B for the tetrasaccharide determinant (Galβ1-3GalNAcβ1-4 [Neu5Acα2-3]Galβ1) of G_{M1} ganglioside and related compounds [51, 52], binding was observed to lyso-G_{M1} printed with both solvents (**G_{M1}-Sph^a and G_{M1}-Sph^b**) and, to a lesser extent, to G_{M1} oligosaccharide [**G_{M1}-ose**]; none of the other compounds printed displayed Ctx-B reactivity. A parallel assay performed on the epoxy-modified slide, to assess the possible influence of differences in the substrate surface, gave similar results.

In a second set of experiments with this print, the GSL microarray was probed with a murine monoclonal antibody to sulfatide, Sulph 1, via a sandwich assay using anti-mouse IgG-Cy3 as secondary antibody (Fig. 2, Panel B). The Sulph 1 MAb reacted only with lyso-sulfatide (**S3Gal-Sph**), consistent with previous ELISA studies showing that 3′-O-Sulf-β-Gal acts as a binding epitope for the Sulph 1 MAb [27]. In our case, however, binding to lyso-sulfatide printed in DMSO on the NHS slide was virtually unobservable (Panel B, NHS, **S3Gal-Sph^b**). The reason

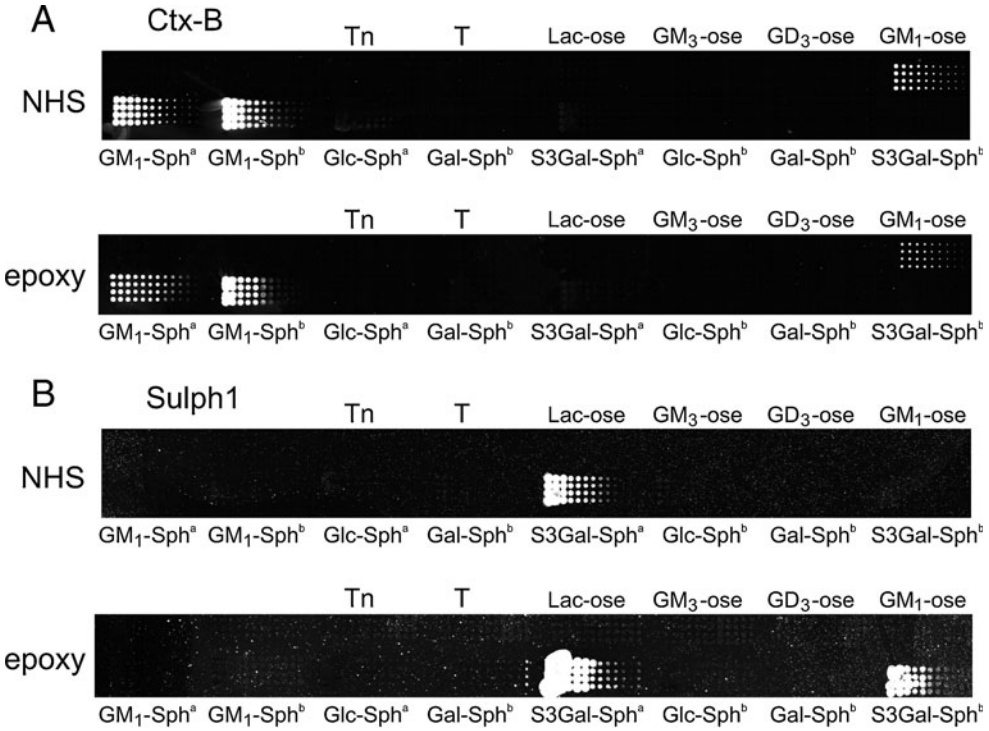


Fig. 2 Microarray analysis comparing binding of various immobilized lyso-GSLs to Ctx-B (Panel A) and to an anti-sulfatide monoclonal antibody (Panel B). Lyso-compounds **("-Sph")** were dispersed and spotted in two different solvents, 1% DMSO in print buffer (**a**) and pure DMSO (**b**), and assays were carried out in parallel on both NHS- and epoxy-modified glass slides to compare the potential effects of different immobilization and surface chemistries. Synthetic GSL-derived glycans (**"-ose"**) and simple glycopeptides (**Tn, T**), dispersed in print buffer without DMSO, were included as controls. Compounds were arrayed in columns of 4 spots of ~0.8 nL each, conc. ~1000, 500, 250, 125, 62.5, 31.3, 15.6, 7.8 and 3.9 μM. In Panel A, incubation was carried out with AF555-CTX-B conjugate (0.2 μg/mL); in Panel B, a sandwich assay consisted of anti-sulfatide MAb Sulph 1 followed by fluorophore-conjugated anti-mouse IgG-Cy3

for this may have to do with the loss of buffering capacity when using pure DMSO; thus, if **S3Gal-Sph** was mainly in the ammonium form when dissolved, it would remain poorly reactive with the NHS functionality, which requires a free amine to initiate conjugation. This would not have caused a problem with the epoxy-slide, which normally requires conjugation in a low pH buffer, since the reaction is initiated by a proton transfer to the epoxide function. This problem is under study.

Regarding previous claims that have implicated the proximal part of the ceramide as also essential for binding [27], this point deserves further investigation, since an independent probe of Sulph 1 binding utilizing a conventional glycan microarray showed some interaction with 3′-O-Sulf-β-Gal-ose and related oligosaccharides possessing terminal 3′-O-sulfated galactose (Arigi *et al*, unpublished results). It is also worth noting that the lower binding of lyso-sulfatide *versus* sulfatide observed in the original ELISA assays of Sulph1 specificity could be attributed at least in part to the significantly decreased adsorbance of the former to the microtiter wells, as demonstrated by washout of the radiolabeled lipids in control experiments [27].

In a third experiment that incorporated a broader panel of lyso-gangliosides printed within a concentration range of 100 μM to 12.5 μM in print buffer (Fig. 3), Ctx-B displayed the strongest binding to lyso-G_{M1}, as expected. Structures closely related to G_{M1}, lyso-G_{D1a} and lyso-G_{D1b}, displayed significantly weaker binding, with the weakest reaction to the latter. In the absence of absolute quantitation of immobilized ligands, it is best to avoid making strong conclusions from small differences in intensity; nevertheless this pattern of weaker binding to disialosyl gangliosides is not inconsistent with previously published results based on surface plasmon resonance (SPR) measurements. One such study showed low but measurable association of Ctx-B with G_{D1a}, and negligible interaction with G_{D1b} [53]; while a later study showed Ctx-B binding to G_{D1b}, with K_D an order of magnitude lower than for G_{M1}, but no significant binding to G_{D1a} [54]. On the other hand, the observed binding of asialo-G_{M1} (G_{A1}) is inconsistent with previously published results, none of which have shown detectable binding in the absence of the NeuAc linked α2-3 to the internal Gal residue. No reactivity to the rest of the test compounds was observed.

In another set of experiments, the GSL array was probed with (i) a polyclonal serum possessing anti-G_{A2} reactivity (Fig. 4, Panel A) and (ii) a lectin, PNA (Fig. 4, Panel B). With the polyclonal serum, the anticipated reactivity was detected with G_{A2} (Panel A, $\mathbf{G_{A2}}$), but a somewhat stronger cross reactivity was detected with G_{A1} (Panel A, $\mathbf{G_{A1}}$). Minor cross-reactivity was also observed with T and Tn glycopeptides (Panel A, **T**, **Tn**). This pattern is not unexpected from a commercial unpurified polyclonal

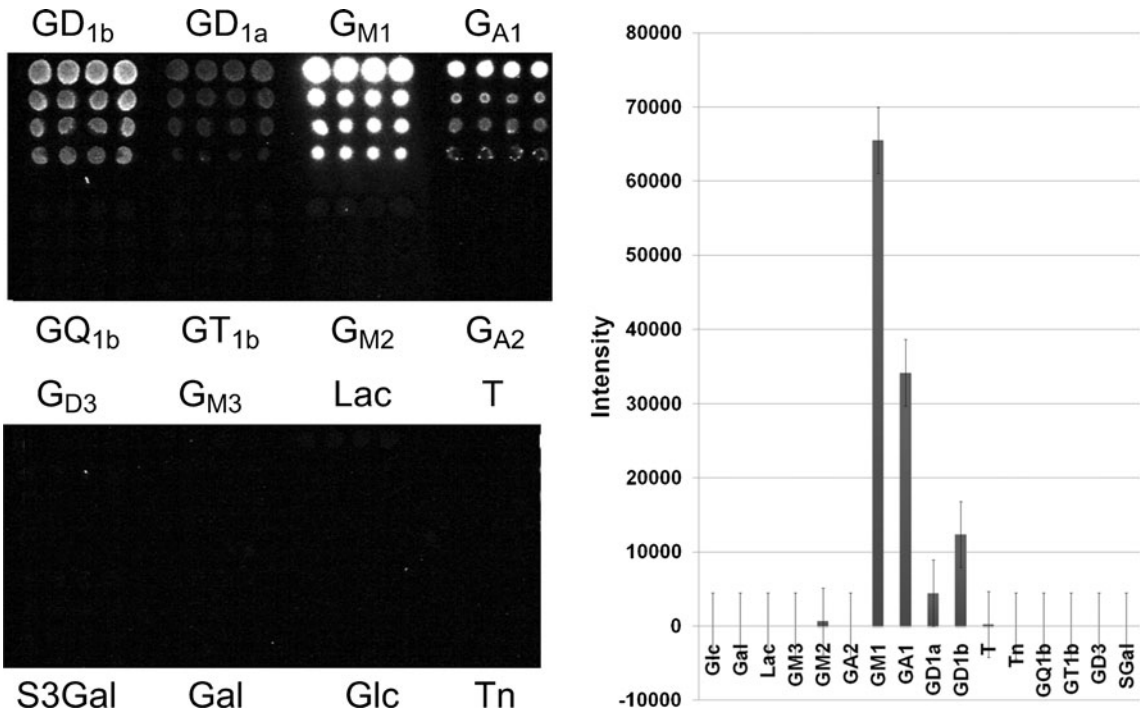


Fig. 3 Analysis of interaction of AF555-CTX-B conjugate (0.2 μg/mL) with an expanded panel of lyso-GSLs ("-Sph" omitted) arrayed on an NHS-modified glass slide. Synthetic glycopeptides (**Tn** and **T**) were spotted as controls. Compounds were arrayed in columns of 4 spots, conc. ~200, 100, 50 and 25 μM, ~0.8 nL each

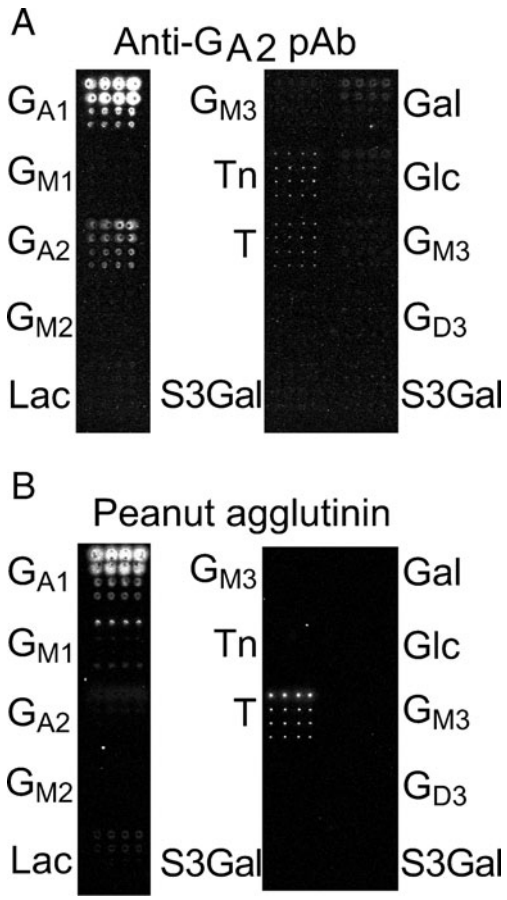


Fig. 4 Reactivity of immobilized lyso-GSLs ("-Sph" omitted) with a polyclonal antibody to G_{A2} (Panel A) and with AF488-PNA conjugate (Panel B). Compounds were arrayed in columns of four spots, 200 μM to 25 μM

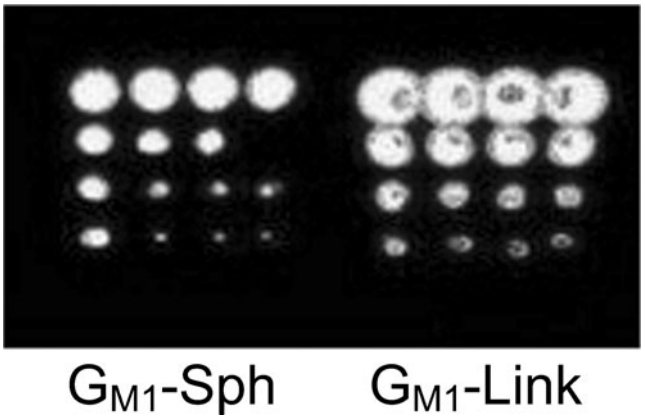


Fig. 5 Comparison of reactivity of fluorescent-labeled cholera toxin B chain (AF555-CTX-B) with underivatized lyso-G_{M1} ("G_{M1}-Sph") and lyso-G_{M1} derivatized with NHS-PEO_2-maleimide (succinimidyl-[(*N*-maleimidopropionamido)-diethyleneglycol] ester) and 2-mercaptoethylamine ("G_{M1}-link"). Compounds were arrayed in columns of four spots, 200 μM to 25 μM

antibody preparation. PNA showed reactivity to G_{A1} and T glycopeptide (Panel B, $\mathbf{G_{A1}}$ and **T**), as expected; in addition, some reactivity to G_{M1} was also detected (Panel B, $\mathbf{G_{M1}}$). It is worth noting that some early studies of PNA interactions with gangliosides and asialogangliosides yielded conflicting results. Thus, while Momoi *et al* [55] reported strong interaction of PNA with G_{A1}, but no measurable binding with G_{M1} in ELISA and TLC overlay assays, Månsson and Olofsson [56] reported similar behavior of both G_{A1}- and G_{M1}-containing liposomes towards an affinity column of immobilized PNA. Results available at the Center for Functional Glycomics public glycan array database (primary glycan screen data; http://www.functionalglycomics.org/glycomics/publicdata/selec

Table 2 List of carbohydrate-binding proteins and their expected binding specificities

Protein	Optimal Glycan Binding Determinant	GSL Carrier	Notes
Cholera toxin B chain	Galβ3GalNAcβ4(Neu5Acα3)Galβ4Glcβ-	(**1**) $G_{M1(a)}$	1
Peanut agglutinin	Galβ3GalNAcα/β-	(**8**) G_{A1} (Gg_4Cer)	2
		(**1**) $G_{M1(a)}$	2
Anti-G_{A2} polyclonal	GalNAcβ4Galβ4Glcβ-	(**9**) G_{A2} (Gg_3Cer)	3
Sulph1 MAb	SO_3-3Galβ-(lipid)	(**7**) SO_3-3GalβCer	4

(1) Ceramide aglycone not essential; strongest binding with $G_{M1(a)}$ [51, 52], but weak cross-reactivities with related gangliosides have been noted, *e.g.*, [53, 54], as discussed in text.

(2) Fairly promiscuous with respect to aglycone (or lack thereof); weaker binding to Galα/β−. Reaction with G_{A1} but not G_{M1} reported [55]; reaction with both G_{A1} and G_{M1} reported [56] and Center for Functional Glycomics primary glycan array screen http://www.functionalglycomics.org/glycomics/publicdata/selectedScreens.jsp.

(3) Unpurified rabbit serum, isotype IgG, IgM (Matreya LLC).

(4) Also recognizes sulfolactosylceramide (SO_3-3Galβ4GlcβCer) and seminolipid (SO_3-3Galβ1diacylglycerol); significant cross-reactivities with lyso-sulfatide and lyso-seminolipid also noted [27], as discussed in text. In our hands, a variety of sulfated β-Gal-containing oligosaccharides, including the monosaccharide SO_3-3Galβ−, also reacted on a glycan microarray format (Arigi *et al*, unpublished).

tedScreens.jsp) also show binding of PNA to both G_{A1}-ose and G_{M1}-ose. A list of carbohydrate binding proteins and their expected binding specificities, based on previously published studies, is presented in Table 2.

Finally, the potential effect of introducing a linker into the lyso-GSL array design is illustrated in Fig. 5, which shows a direct comparison of the binding of AF555-CTX-B to similar amounts of non-derivatized and linker-derivatized lyso-G_{M1} (**G_{M1}-Sph** and **G_{M1}-link**, respectively) bound to the NHS slide. In several experiments, the linker-derivatized compound appeared to bind Ctx-B somewhat better at higher concentrations, but the effect leveled off at lower concentrations spotted. Thus, at least in this limited context, there was no obvious advantage to incorporating a linker into the design. Most likely this is because the NHS groups on the Nexterion slide H are *already* presented at the end of a flexible stretch of polyethylene glycol or other linker capable of simulating an amide linked alkyl chain after coupling. However, it is possible to envision the rational design of a second generation linker incorporating additional features of the ceramide fatty-*N*-acyl moiety, such as the 2-hydroxy function, to test their effects on protein binding.

Conclusions

We have designed and tested a prototype microarray platform for displaying covalently attached GSLs, using a strategy that is complementary rather than identical to that employed by Song *et al* [45]. A number of features have been incorporated into the design that could make this a useful alternative to non-covalent GSL arrays [34], including the flexibility to use derivatized glass microarray surfaces not compatible with display of native GSLs. A significant feature of native GSLs, the fatty-*N*-acyl group, has been sacrificed, but this may not be critical for many applications; in any case, incorporating a fatty-*N*-acyl analog into an appendable linker is a viable solution. This idea has already been incorporated into the GSL-array by Liang *et al* [36], although the objective of that study and repertoire of compounds synthesized for it were highly limited. Such an appendage could incorporate additional ceramide features, including the fatty-*N*-acyl 2-hydroxy group found on GSLs from many sources; it could also incorporate other technical features, such as photolabile linkers or, importantly, fluorescent labels, as introduced by Song *et al* [45], to facilitate quantitation of printed compounds, without which comparisons of binding interactions lack quantitative precision.

A significant question is whether retaining all or part of the ceramide is essential for studying protein-GSL interactions. Although a number of studies cited suggest that it may not be, generation and array of lyso-GSLs still presents a number of advantages over display of only the oligosaccharide moiety, which must be generated either by chemical synthesis or by cleavage of the ceramide using an endo-glycoceramidase [57] or ceramide glycanase [58]. Both GSL-glycan methodologies require more steps to reach the final, immobilizable target compound. In addition, members of a large, structurally diverse family of GSLs, the GIPCs of fungi, plants, and certain parasites [44], are not accessible via a simple ceramide cleavage yielding a molecular form suitable for microarray display. However, as we have previously established, GIPCs can be de-*N*-acylated with SCDase [50]; thus, although we have not explored application to fungal and plant GIPC arrays in the current work, the path is open in principle for a more universal methodology that could include them. Furthermore, it seems unlikely that immobilized monosaccharides would be adequate surrogates for monoglycosylceramides such as glucosyl- and galactosylceramides, where proximal ceramide features are doubtless essential for proper recognition in some contexts. It is likely that retention of at least the proximal part of the sphingoid, as well as linking it through a properly configured fatty-*N*-acyl analog, will be required to study such interactions in an array format. These considerations will be an important part of designing a second generation covalent GSL array.

Additional issues not addressed in this study concern the presentation of the lyso-GSLs on the slide surface—for example whether during printing they become randomly distributed or organized as clusters due to lipophilic intermolecular interactions; their precise orientations are also unclear. These issues could perhaps be addressed by surface sampling techniques such as atomic force microscopy or three-dimensional fluorescence microscopy [59, 60], but are beyond the scope of this study.

Acknowledgements This work was supported by The Copenhagen Center for Glycomics at the University of Copenhagen, the Carlsberg Foundation, The Benzon Foundation, The Velux Foundation, The Danish Research Councils, NIH/NCI 5U01 CA128437 and 5U01 CA111294, EU FP7-HEALTH-2007-A 201381, and the University of Copenhagen Programme of Excellence. Early phases of the work were supported by an NIH grant (R21 RR20355) to S.B.L.

References

1. Zhang, X., Kiechle, F.L.: Review: glycosphingolipids in health and disease. Ann. Clin. Lab. Sci. **34**, 3–13 (2004)
2. Ariga, T., Miyatake, T., Yu, R.K.: Recent studies on the roles of antiglycosphingolipids in the pathogenesis of neurological disorders. J. Neurosci. Res. **65**, 363–370 (2001)
3. Fredman, P., Vedeler, C.A., Nyland, H., Aarli, J.A., Svennerholm, L.: Antibodies in sera from patients with inflammatory demyelinating polyradiculoneuropathy react with ganglioside LM1 and sulphatide of peripheral nerve myelin. J. Neurol. **238**, 75–79 (1991)
4. Hakomori, S.: Tumor-associated carbohydrate antigens defining tumor malignancy: basis for development of anti-cancer vaccines. Adv. Exp. Med. Biol. **491**, 369–402 (2001)

5. Buschard, K., Josefsen, K., Horn, T., Larsen, S., Fredman, P.: Sulphatide antigen in islets of Langerhans and in diabetic glomeruli, and anti-sulphatide antibodies in type 1 diabetes mellitus. APMIS **101**, 963–970 (1993)
6. Buschard, K., Josefsen, K., Horn, T., Fredman, P.: Sulphatide and sulphatide antibodies in insulin-dependent diabetes mellitus. Lancet **342**, 840 (1993)
7. Buschard, K., *et al*.: Sulphatide in islets of Langerhans and in organs affected in diabetic late complications: a study in human and animal tissue. Diabetologia **37**, 1000–1006 (1994)
8. Blomqvist, M., *et al*.: Sulfatide is associated with insulin granules and located to microdomains of a cultured beta cell line. Glycoconj. J. **19**, 403–413 (2002)
9. Andersson, K., *et al*.: Patients with insulin-dependent diabetes but not those with non-insulin-dependent diabetes have anti-sulfatide antibodies as determined with a new ELISA assay. Autoimmunity **35**, 463–468 (2002)
10. Fukui, S., Feizi, T., Galustian, C., Lawson, A.M., Chai, W.: Oligosaccharide microarrays for high-throughput detection and specificity assignments of carbohydrate-protein interactions. Nat. Biotechnol. **20**, 1011–1017 (2002)
11. Willats, W.G., Rasmussen, S.E., Kristensen, T., Mikkelsen, J.D., Knox, J.P.: Sugar-coated microarrays: a novel slide surface for the high-throughput analysis of glycans. Proteomics **2**, 1666–1671 (2002)
12. Wang, D., Liu, S., Trummer, B.J., Deng, C., Wang, A.: Carbohydrate microarrays for the recognition of cross-reactive molecular markers of microbes and host cells. Nat. Biotechnol. **20**, 275–281 (2002)
13. Houseman, B.T., Mrksich, M.: Carbohydrate arrays for the evaluation of protein binding and enzymatic modification. Chem. Biol. **9**, 443–454 (2002)
14. Blixt, O., *et al*.: Printed covalent glycan array for ligand profiling of diverse glycan binding proteins. Proc. Natl. Acad. Sci. U. S. A. **101**, 17033–17038 (2004)
15. Mamidyala, S.K., Ko, K.-S., Jaipuri, F.A., Park, G., Pohl, N.L.: Noncovalent fluorous interactions for the synthesis of carbohydrate microarrays. J. Fluorine Chem. **127**, 571–579 (2006)
16. Shin, I., Park, S., Lee, M.R.: Carbohydrate microarrays: an advanced technology for functional studies of glycans. Chemistry **11, 2894–2901 (2005)**
17. Larsen, K., Thygesen, M.B., Guillaumie, F., Willats, W.G., Jensen, K.J.: Solid-phase chemical tools for glycobiology. Carbohydr. Res. **341**, 1209–1234 (2006)
18. Horlacher, T., Seeberger, P.H.: Carbohydrate arrays as tools for research and diagnostics. Chem. Soc. Rev. **37**, 1414–1422 (2008)
19. Liu, Y., Palma, A.S., Feizi, T.: Carbohydrate microarrays: key developments in glycobiology. Biol. Chem. **390**, 647–656 (2009)
20. Lonardi, E., Balog, C.I., Deelder, A.M., Wuhrer, M.: Natural glycan microarrays. Exp. Rev. Proteomics. **7**, 761–774 (2010)
21. von Gunten, S., *et al*.: Intravenous immunoglobulin contains a broad repertoire of anticarbohydrate antibodies that is not restricted to the IgG2 subclass. J. Allergy Clin. Immunol. **123**, 1268–1276 (2009)
22. Oyelaran, O., McShane, L.M., Dodd, L., Gildersleeve, J.C.: Profiling human serum antibodies with a carbohydrate antigen microarray. J. Proteome Res. **8**, 4301–4310 (2009)
23. Springer, G.F.: Immunoreactive T and Tn epitopes in cancer diagnosis, prognosis, and immunotherapy. J. Mol. Med. **75**, 594–602 (1997)
24. Reis, C.A., *et al*.: Development and characterization of an antibody directed to an alpha-N-acetyl-D-galactosamine glycosylated MUC2 peptide. Glycoconj. J. **15**, 51–62 (1998)
25. Price, M.R., *et al*.: Summary report on the ISOBM TD-4 Workshop: analysis of 56 monoclonal antibodies against the MUC1 mucin. San Diego, Calif., November 17–23, 1996. Tumour. Biol. **19**(Suppl 1), 1–20 (1998)
26. Ryuko, K., *et al*.: Characterization of a new MUC1 monoclonal antibody (VU-2-G7) directed to the glycosylated PDTR sequence of MUC1. Tumour Biol. **21**, 197–210 (2000)
27. Fredman, P., *et al*.: Characterization of the binding epitope of a monoclonal antibody to sulphatide. Biochem. J. **251**, 17–22 (1988)
28. Magnani, J.L., Smith, D.F., Ginsburg, V.: Detection of gangliosides that bind cholera toxin: direct binding of 125 I-labeled toxin to thin-layer chromatography. Anal. Biochem. **109**, 399–402 (1980)
29. Magnani, J.L., Brockhaus, M., Smith, D.F., Ginsburg, V.: Detection of glycolipid ligands by direct binding of carbohydrate-binding proteins to thin-layer chromatograms. Methods Enzymol. **83**, 235–241 (1982)
30. Holmgren, J., Svennerholm, A.M.: Enzyme-linked immunosorbent assays for cholera serology. Infect. Immun. **7**, 759–763 (1973)
31. Holmgren, J., Elwing, H., Fredman, P., Svennerholm, L.: Immunoassays based on plastic-adsorbed gangliosides. Adv. Exp. Med. Biol. **125**, 339–348 (1980)
32. Feizi, T., Chai, W.: Oligosaccharide microarrays to decipher the glyco code. Nat. Rev. Mol. Cell Biol. **5**, 582–588 (2004)
33. Wallner, F.K., Norberg, H.A., Johansson, A.I., Mogemark, M., Elofsson, M.: Solid-phase synthesis of serine-based glycosphingolipid analogues for preparation of glycoconjugate arrays. Org. Biomol. Chem. **3**, 309–315 (2005)
34. Kanter, J.L., *et al*.: Lipid microarrays identify key mediators of autoimmune brain inflammation. Nat. Med. **12**, 138–143 (2006)
35. Imamura, A., *et al*.: Design and synthesis of versatile ganglioside probes for carbohydrate microarrays. Glycoconj. J. **25**, 269–278 (2008)
36. Liang, P.H., *et al*.: Quantitative microarray analysis of intact glycolipid-CD1d interaction and correlation with cell-based cytokine production. J. Am. Chem. Soc. **130**, 12348–12354 (2008)
37. Yamazaki, V., *et al*.: Cell membrane array fabrication and assay technology. BMC. Biotechnol. **5**, 18 (2005)
38. Isobe, T., Naiki, M., Handa, S., Taki, T.: A simple assay method for bacterial binding to glycosphingolipids on a polyvinylidene difluoride membrane after thin-layer chromatography blotting and in situ mass spectrometric analysis of the ligands. Anal. Biochem. **236**, 35–40 (1996)
39. Guittard, J., Hronowski, X.L., Costello, C.E.: Direct matrix-assisted laser desorption/ionization mass spectrometric analysis of glycosphingolipids on thin layer chromatographic plates and transfer membranes. Rapid Commun. Mass Spectrom. **13**, 1838–1849 (1999)
40. Distler, U., *et al*.: Matching IR-MALDI-o-TOF mass spectrometry with the TLC overlay binding assay and its clinical application for tracing tumor-associated glycosphingolipids in hepatocellular and pancreatic cancer. Anal. Chem. **80**, 1835–1846 (2008)
41. Xia, B., *et al*.: Versatile fluorescent derivatization of glycans for glycomic analysis. Nat. Methods **2**, 845–850 (2005)
42. Blixt, O., *et al*.: Chemoenzymatic synthesis of 2-azidoethyl-ganglio-oligosaccharides GD3, GT3, GM2, GD2, GT2, GM1, and GD1a. Carbohydr. Res. **340**, 1963–1972 (2005)
43. Bohorov, O., Andersson-Sand, H., Hoffmann, J., Blixt, O.: Arraying glycomics: a novel bi-functional spacer for one-step microscale derivatization of free reducing glycans. Glycobiology. **16**, 21C–27C (2006)
44. Lester, R.L., Dickson, R.C.: Sphingolipids with inositolphosphate-containing head groups. Adv. Lipid Res. **26**, 253–274 (1993)
45. Song, X., *et al*.: Shotgun glycomics: a microarray strategy for functional glycomics. Nat. Methods **8**, 85–90 (2011)
46. Ito, M., Kurita, T., Kita, K.: A novel enzyme that cleaves the N-acyl linkage of ceramides in various glycosphingolipids as well as

sphingomyelin to produce their lyso forms. J. Biol. Chem. **270**, 24370–24374 (1995)
47. Ito, M., Kita, K., Kurita, T., Sueyoshi, N., Izu, H.: Enzymatic N-deacylation of sphingolipids. Methods Enzymol. **311**, 297–303 (2000)
48. Kita, K., Kurita, T., Ito, M.: Characterization of the reversible nature of the reaction catalyzed by sphingolipid ceramide N-deacylase. A novel form of reverse hydrolysis reaction. Eur. J. Biochem. **268**, 592–602 (2001)
49. Kurita, T., Izu, H., Sano, M., Ito, M., Kato, I.: Enhancement of hydrolytic activity of sphingolipid ceramide N-deacylase in the aqueous-organic biphasic system. J. Lipid Res. **41**, 846–851 (2000)
50. Li, Y., Arigi, E., Eichert, H., Levery, S.B.: Mass spectrometry of fluorocarbon-labeled glycosphingolipids. J. Mass Spectrom. **45**, 504–519 (2010)
51. Cuatrecasas, P.: Gangliosides and membrane receptors for cholera toxin. Biochemistry **12**, 3558–3566 (1973)
52. Cuatrecasas, P.: Interaction of Vibrio cholerae enterotoxin with cell membranes. Biochemistry **12**, 3547–3558 (1973)
53. Kuziemko, G.M., Stroh, M., Stevens, R.C.: Cholera toxin binding affinity and specificity for gangliosides determined by surface plasmon resonance. Biochemistry **35**, 6375–6384 (1996)
54. MacKenzie, C.R., Hirama, T., Lee, K.K., Altman, E., Young, N. M.: Quantitative analysis of bacterial toxin affinity and specificity for glycolipid receptors by surface plasmon resonance. J. Biol. Chem. **272**, 5533–5538 (1997)
55. Momoi, T., Tokunaga, T., Nagai, Y.: Specific interaction of peanut agglutinin with the glycolipid asialo GM1. FEBS Lett. **141**, 6–10 (1982)
56. Månsson, J.E., Olofsson, S.: Binding specificities of the lectins from *Helix pomatia*, soybean and peanut against different glycosphingolipids in liposome membranes. FEBS Lett. **156**, 249–252 (1983)
57. Ito, M., Yamagata, T.: A novel glycosphingolipid-degrading enzyme cleaves the linkage between the oligosaccharide and ceramide of neutral and acidic glycosphingolipids. J. Biol. Chem. **261**, 14278–14282 (1986)
58. Zhou, B., Li, S.C., Laine, R.A., Huang, R.T., Li, Y.T.: Isolation and characterization of ceramide glycanase from the leech, Macrobdella decora. J. Biol. Chem. **264**, 12272–12277 (1989)
59. Linman, M.J., Yu, H., Chen, X., Cheng, Q.: Fabrication and characterization of a sialoside-based carbohydrate microarray biointerface for protein binding analysis with surface plasmon resonance imaging. ACS Appl. Mater. Interfaces **1**, 1755–1762 (2009)
60. Ke, B.B., Wan, L.S., Xu, Z.K.: Controllable construction of carbohydrate microarrays by site-directed grafting on self-organized porous films. Langmuir **26**, 8946–8952 (2010)