Surface chemistry and linker effects on lectin-carbohydrate recognition for glycan microarrays

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Introduction

Carbohydrates coat all living cells, forming the glycocalyx, and they play crucial roles in biological processes, including cell-cell and pathogen-host interactions, inflammation, neural development and fertilisation.[1-3] *In vivo*, the effects of carbohydrates are mediated by lectins, proteins of non-immune origin which recognise specific carbohydrate motifs.[3,4] Carbohydrates are structurally more complex than proteins and nucleic acids, varying in constituent residues, residue ring size, linkage position and anomericity, branching, and non-sugar substituents, such as sulfation and phosphorylation, which leads to an enormous number of possible structural isomers for an oligosaccharide.[1,5,6] Thus, a variety of specialised analytical techniques are required to elucidate the primary structures of biologically heterogeneous oligosaccharides.[7] Examining carbohydrate-lectin interactions has been similarly complex, involving specialised instrumentation and expertise and large quantities of often scarely available material, e.g. isothermal titration calorimetry (ITC) and surface plasom resonance (SPR).

For rapid and robust high throughput screening of carbohydrate-protein interactions, the glycan microarray has become recognised as an essential tool in recent years.[8] It greatly increases the number of experiments possible with limited sample amounts and facilitates a profiling or screening approach prior to subsequent focused investigation.[1] Hence, glycan microarrays have greatly helped to progress glycomics research and provided insights into the roles of carbohydrates in biological processes including assessment of antibody and lectin specificities, bacterial, viral and parasitic interactions and the individual effects of extracellular matrix components.[3] A variety of glycan-containing molecules and surface chemistries have been used to array carbohydrates on microarray slides. The molecules

arrayed have included neoglycolipids,[9] natural glycoproteins and neoglycoproteins,[8,10] carbohydrates with various linkers,[10,11] polysaccharides,[12,13] glycosaminoglycans,[14] and most recently, mucins.[15] The molecular scaffolding and density of the carbohydrate ligands presented varies depending on the molecule printed and consequently lectin recognition and binding will be affected,[16,17] as multivalent presentation of carbohydrate ligands generally enhances the avidity of lectins.[8]

Slide surfaces and chemistries are often dictated by the choice of molecule being printed and available functional group(s). A wide variety of coated and functionalised glass slide surfaces have been used for microarray printing of the probes mentioned above, including epoxide,[10,11] aldehyde,[10,14] polylysine,[14] hydrogel,[18] nitrocellulose[9] and maleimide.[19] Another recent strategy has been to chemo-enzymatically synthesise complex *N*-linked oligosaccharides *in situ* on the slide surface on a nanoscale.[20] Fluorescence-based detection of an appropriately labelled recognition molecule using a laser scanner is the most common method of observing binding events. Alternatively, labelling with nanoparticles coupled with resonance light scattering detection,[10] fluorescence labelling with evanescent-field fluorescence-assisted detection[8] and label-free SPR detection[11] have also been used.

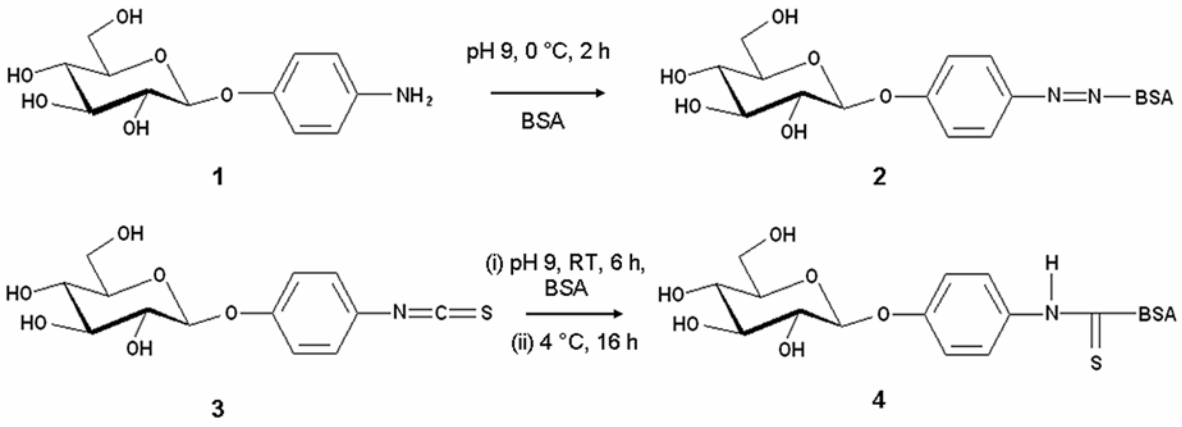


Fig. 1 Structures of the linkers before and after conjugation to the BSA backbone as exemplified by glucopyranosides, adapted from McBroom, *et al.*, 1972.[26] **1**, 4-Aminophenyl-β-D-glucopyranoside, **2**, Glc-β-4AP-BSA, **3**, β-D-glucopyranosyl phenylisothiocyanate, and **4**, Glc-β-PITC-BSA.

With the great variety of both platforms and ligands in use, the difference in ligand density and presentation across microarray platforms and the effect of these factors on lectin recognition have recently been identified as a potential issue.[3] However, the effect of the slide

surface itself on ligand presentation and recognition must also be considered. As has been shown for antibody microarrays, slide surface chemistry can have significant impact on the presentation, three-dimensional (3D) structure, capacity, background noise and spot morphology of the arrayed molecules, which in turn impacts upon their interactions and reproducibility.[21,22] Another variable in carbohydrate ligand presentation may be the linker used to attach the carbohydrate to the scaffold, as the linker selected has been shown to influence carbohydrate-lectin interactions in ITC, enzyme-linked immunosorbent assay (ELISA) and SPR studies.[23,24]

In this study, three different microarray surfaces were arrayed with the same mono- and di-saccharide neoglycoconjugates (NGCs), using bovine serum albumin (BSA) as a multivalent molecular scaffold, and glycoproteins for presentation of naturally occuring oligosaccharides. The glycan microarray slides were incubated with well-characterised, fluorescently-labelled plant lectins to evaluate the influence of slide chemistry and surface on background noise, spot size and morphology, lectin-ligand recognition and reproducibility. In addition, three monosaccharide neoglycoconjugate analogues with two different common linkers (4-aminophenyl (4AP) and phenylisothiocyanate (PITC) (Fig. 1)) were included in the test group to assess the influence of these linkers on lectin discrimination across platforms.

Experimental

Materials

Aldehyde ES microarray slides were from Pierce Biotechnology (Rockford, IL). Nexterion® Slide H microarray slides were purchased from Schott AG (Germany). Poly-L-lysine slides, BSA (cat. no. A7638, ≥99%), glycopyranosyl PITC and 4AP derivatives and goat anti-rabbit IgG labelled with Atto 633 were purchased from Sigma-Aldrich Co. (Dublin, Ireland). The BSA was periodate-treated[25] and used for neoglycoconjugate synthesis and microarray slide blocking. The bicinchoninic acid (BCA) Protein Assay Kit and sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) were from Pierce Biotechnology (Thermo Fisher Scientific Inc., Dublin, Ireland) and rabbit anti-cow albumin polyclonal antibody was from Dako (Glostrup, Denmark). Pure tetramethylrhodamine-(TRITC-) labelled lectins were from EY Laboratories, Inc. (San Mateo, CA). All other reagents were from Sigma-Aldrich Co. unless otherwise noted and were of the highest grade available.

NGC synthesis

A range of mono- and di-saccharide NGCs were synthesised from their commercially available PITC or 4AP derivatives (Fig. 1).[26] In brief, glycopyranosyl-PITC was added to BSA at pH 9.0 in 0.15 M NaCl in a 150:1 molar ratio (carbohydrate-linker to protein), incubated for 6 h at room temperature (approximately 20 °C) while maintaining basic pH and then kept at 4 °C for 16 h.

The pH was adjusted to 7.2, the reaction mixture dialysed exhaustively against water and the NGC purified on BioGel P-2 (Bio-Rad Laboratories, Hertfordshire, UK). The collected fractions were monitored by absorbance at 280 nm and Bradford assay.[27] Fractions containing NGC were pooled and lyophilised.

The 4AP-glycosides were dissolved in ice-cold 0.15 M HCl and 0.05 M $NaNO_2$ was added sufficient to make the solution oxidising (approximately 1:1.5 (v/v) ratio). The 4AP-glycoside solution was added to an ice-cold solution of BSA in 0.15 M NaCl, pH 9, in a molar ratio of 50:1, and the reaction was allowed to proceed at 0 °C for 2 h while pH was maintained at 9 by the addition of 0.5 M NaOH. The reaction mixture was then neutralised, dialysed exhaustively against distilled water and lyophilised. NGC protein and carbohydrate content were determined using the BCA assay,[28] with BSA as the standard, and Monsigny assay,[29] using the appropriate carbohydrate as the standard, i.e. Gal for Gal-α-PITC-BSA, Gal-β-PITC-BSA, Gal-β-4AP-BSA, lactose for Lac-β-4AP-BSA, etc., and the molar substitution ratio was determined (Table 1).

Glycan microarray printing

Poly-L-lysine slides were functionalised with sulfhydryl-reactive maleimide groups by incubation of the slide surface with 10 mM sulfo-SMCC prepared in phosphate buffered saline (PBS), pH 7.4 (137 mM NaCl, 2.7 mM KCl, 2 mM KH_2PO_4 and appropriate mixture of 10 mM Na_2HPO_4 and NaH_2PO_4 for correct pH) for 1 h at room temperature in a humidity chamber. The functionalised polylysine slides were washed twice in deionised water (approximately 1 min per wash), centrifuged dry (500 x g, 5 min) and stored at 4 °C with dessicant until use.

NGCs were prepared at a concentration of 1 mg/mL in PBS, pH 7.4, based on their protein content measured by the BCA assay, and glycoproteins at 1 mg/mL based on weight to ensure saturation of the feature area on the slide surfaces. Probes (NGCs and glycoproteins) were printed at approximately 1 nL per feature on Nexterion® Slide H, Aldehyde ES or functionalised poly-L-lysine slides in a humid environment (62% +/-2%) using a

piezoelectric microarray printer equipped with an uncoated glass nozzle with a 90 μm orifice (SciFlexArrayer S3, Scienion, Germany). Six replicate subarrays were printed per slide, with each probe spotted in replicates of twelve per subarray (see Fig. 2 for layout and Table 1 for print order). Slides were incubated in a humidity chamber overnight after printing to ensure complete conjugation. The functional groups on the Nexterion® slide H slides were then deactivated or capped by incubation with 100 mM ethanolamine in 50 mM sodium borate, pH 8, the aldehyde slides with 50 mM ethanolamine[10] in PBS, pH 7.4 and the functionalised polylysine slides with 1.4 mM β-mercaptoethanol in PBS pH 7.4 for 1 h at room temperature (see Table 2). The slides were washed with PBS, pH 7.4 with 0.05% Tween-20 (PBS-T) three times and once with PBS. Slides were then centrifuged dry (1,500 rpm, 5 min) and stored dry at 4 °C with desiccant until use.

Slide incubation and scanning

Just before use, the Aldehyde ES slides were blocked with 2% BSA in PBS, pH 7.4, supplemented with 50 mM ethanolamine, and functionalised poly-L-lysine slides were blocked with 2% BSA in PBS, pH 7.4 supplemented with 1.4 mM β-mercaptoethanol for 2 h at room temperature in a humidity chamber. The slides were washed three times in PBS-T, once in PBS (2 min each wash), centrifuged dry and incubated immediately. Nexterion® Slide H did not require protein blocking prior to use.[15,18]

All microarray slides were incubated using an 8-well gasket slide and incubation cassette system (Agilent Technologies Ireland, Ltd., Cork, Ireland) and were protected from light throughout the procedure. Briefly, 70 μL of dilute TRITC-labelled lectin (see Table 3 for appropriate dilution of each lectin) in Tris-buffered saline supplemented with Ca^{2+} and Mg^{2+} ions (TBS; 20 mM Tris-HCl, 100 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.2) and with 0.05% Tween-20 (TBS-T) was applied to each well of the gasket slide. In the cases of Con A and WFA, lectins were also diluted in 100 mM of appropriate haptenic sugar in TBS-T (Table 3) and compared to an uninhibited lectin incubated subarray on the same slide to verify carbohydrate-based lectin binding.[30] The microarray slide was then sandwiched with the gasket, the cassette assembled and placed in a rotating incubation oven (23 °C, approximately 4 rpm) for 1 h. Slides were disassembled under TBS-T, washed 3 times in TBS-T for 2 min each with gentle agitation in a Coplin jar, with a final 3 min wash in TBS. The microarrays were dried by centrifugation and scanned immediately with a 543 nm laser (90% laser power, 70% PMT, TRITC emission filter, 5 μm resolution) in a Perkin-Elmer

Scanarray Express HT.

For antibody incubations, the primary antibody was incubated as above at a 1 in 2500 dilution in TBS-T on the microarray slide for 1 h, washed and dried by centrifugation and then immediately incubated with the fluorescently-labelled secondary antibody at a 1 in 1000 dilution in TBS-T. Microarray slides were washed and dried as above and scanned immediately.

Table 1 Neoglycoconjugates printed, codes and average molar substitution ratios.

Row	Neoglycoconjugate	Code	Average substitution
1	Lac-β-4AP-BSA	Lacb4	34
2	LacNAc-α-4AP-BSA	LNa4	27.6
3	LacNAc-β-4AP-BSA	LNb4	18.25
4	Gal-α-PITC-BSA	GalaP	19.4
5	Gal-β-PITC-BSA	GalbP	32.6
6	Gal-β-4AP-BSA	Galb4	15
7	Empty	Empty	-
8	Man-α-4AP-BSA	Mana4	11.8
9	Man-α-PITC-BSA	ManaP1	27.6
10	Xyl-β-4AP-BSA	Xylb4	5.6
11	Xyl-α-4AP-BSA	Xyla4	15
12	4AP-BSA	4AP	na
13	PITC-BSA	PITC	na
14	Fuc-α-4AP-BSA	Fuca4	11.4
15	Fuc-β-4AP-BSA	Fucb4	17.3
16	Glc-β-4AP-BSA	Glcb4	20.4
17	Glc-β-PITC-BSA	GlcbP	28.1
18	Fetuin	Fetuin	na
19	Asialofetuin	ASF	na
20	Man-α-PITC-BSA	ManaP2	22
21	Ribonuclease B	RB	na

na, not applicable

Data extraction and analysis

Raw intensity values were extracted from the image files using GenePix Pro v6.1.0.4 (Molecular Devices, Berkshire, U.K.) and a proprietary *.gal file using adaptive diameter (70-130%) circular alignment based on 230 μm features and exported as text to Excel (version 2007, Microsoft) where all data calculations were performed. Local background was subtracted and background-corrected median feature intensity (F543median-B543) was used for each feature intensity value. The median of twelve replicate spots per subarray was

handled as a single data point for graphical and statistical analysis (n = 3). Data intensities across three replicate microarray slides were normalised to the per-subarray total intensity mean and binding data was presented in histogram form of average intensity with standard deviation of three experimental replicates. The significance of inhibition data was evaluated using a standard Student's t-test (paired, two-tailed) ($p \leq 0.05$, see Table S-1[†]).

Table 2 Summary of the slide types used for NGC microarray printing. Functionalisation refers to any linker added, reactive chemistry to chemistry for conjugation of arrayed NGCs and glycoproteins, capping to the chemistry used to deactivate the remaining active functional groups on the slide surface after printing and blocking refers to the blocking conditions.

Slide type	Manufacturer	Functionalisation	Reactive chemistry	Capping	Blocking
Aldehyde ES	Pierce Biotechnology	n.a.	Reactive at pH 7-9 with primary amines.	50 mM ethanolamine in PBS, pH 7.4, 1 h	2% BSA, 50 mM ethanolamine, 1 h
Poly-L-lysine	Sigma Co.	Sulfo-SMCC	Maleimide group reacts with sulfhydryl group at pH 6.5-7.5 to form stable thioether bonds.	1.4 mM β-mercaptoethanol in PBS, pH 7.4, 1 h	2% BSA, 1.4 mM β-mercaptoethanol, 1 h
Nexterion slide H®	Schott AB	n.a.	NHS ester reacts with primary amines at pH 7.2-8.5.	100 mM ethanolamine in 50 mM sodium borate, pH 8.0, 1 h	n.a.

n.a. not applicable

Results

Three slide surface chemistries commonly used for microarray fabrication were selected for evaluation of carbohydrate ligand recognition by lectins, the three-dimensional (3D) hydrogel Nexterion® Slide H, Aldehyde ES (enhanced surface) and a functionalised poly-L-lysine. The poly-L-lysine slide surface was derivatised with sulfo-SMCC, a heterobifunctional linker with an amine-reactive *N*-hydroxylsuccinimide (NHS) ester and a sulfhydryl-reactive maleimide group which was available for the subsequent immobilisation of the NGCs. After printing the NGCs and glycoproteins, deactivation of the functionalised surfaces was done by capping or blocking the active functional groups on all surfaces. In the case of Slide H, no

additional blocking step was required after capping but both the Aldehyde ES and functionalised poly-L-lysine slides required further blocking with BSA to occupy any remaining active sites and block non-specific binding to the slide surface (Table 2).

Spot morphology and size

Spot morphology was poor for the functionalised poly-L-lysine slide surface while both the Aldehyde ES and Slide H surfaces had regular and easily extractable circular features (Fig. 2). The Aldehyde ES microarray slides have a micro-etched surface claimed to aid in the production of uniform, small features, but despite this, these slides had the largest average spot diameter of the three slide surfaces evaluated (250, 211 and 202 μm for the Aldehyde ES, functionalised poly-L-lysine and Slide H, respectively). The smaller spot diameter for Slide H was probably due to a greater density of functional groups, and therefore larger capacity per unit area, of the 3D surface compared to the two-dimensional (2D) surfaces of the aldehyde and poly-L-lysine slides. The average spot size formed by ribonuclease B (RB) was smaller than for the rest of the arrayed NGCs and glycoproteins, approximately 220, 180 and 170 μm for the Aldehyde ES, functionalised poly-L-lysine and slide H surfaces, respectively.

Background

The microarray slides were incubated with fluorescently-labelled lectins reported to recognise the carbohydrates present (Table 3). Con A exhibited the lowest background noise of all the lectins with all surfaces, which facilitated feature extraction. However, it was clear even from visual inspection of the subarrays across the three different slide chemistries that the functionalised poly-L-lysine and Aldehyde ES microarray slides exhibited higher background upon incubation with the lectins WFA, SBA and UEA-I than Slide H, which had very low background noise (Fig. 2). Both the functionalised poly-L-lysine and Aldehyde ES slides were BSA-blocked while the 3D surface of slide H was unblocked. When the BSA blocking step was omitted on the Aldehyde ES and functionalised poly-L-lysine surfaces, the background was much higher again (data not shown) and did not permit differentiation between signal and noise. High background noise reduces the sensitivity of the binding by increasing the signal to noise ratio,[21] and thus can effectively swamp or mask any binding events which actually occur on the slide surface. The functionalised poly-L-lysine slide had

the highest average background noise for all lectins tested (1823.3-11982.3 RFU, Table 4) and, for the majority of lectin interactions, the background was more intense than the signal, rendering most of the background-subtracted signal intensities negative (Fig. 3B). On the other hand, Slide H displayed low background with all lectins tested (267-433.3 RFU, Table 4) which facilitated observation and extraction of the binding signal intensity.

Table 3 Lectins used to probe the glycan microarrays. The binding specifities[31] and concentrations used for the lectins are detailed, as well as the inhibitory carbohydrates used with respective concentrations.

Lectin	Abbreviation	Binding specificity	Conc (μg/mL)	Inhibitory carbohydrate
Ulex europaeus agglutinin I	UEA-I	Fuc-α-(1→2), H type 2 antigen	10	100 mM Fuc
Concanavalin A (jack bean lectin)	Con A	α-Man>α-Glc>α-GlcNAc, complex biantennary	10	100 mM Man
Wisteria floribunda agglutinin	WFA	GalNAc, GalNAc-α-(1→6)-Gal>GalNAc-α-(1→3)-GalNAc (Forsmann antigen) >GalNAc>>Lac>Gal, chondroitin sulfate	7	100 mM Gal
Soybean agglutinin	SBA	GalNAc>Gal	10	100 mM Gal

Lectin recognition across slide surfaces

In agreement with its known binding specificity, Con A recognised α-linked Man and the high mannose type *N*-linked glycosylation on RB on all three slide surfaces[31] (Table 3). It had low binding intensity with the glycoproteins fetuin and asialofetuin (ASF) on the functionalised poly-L-lysine and Slide H surface, with better recognition on the Aldehyde ES slide (Fig. 3). Con A is reported to recognise complex biantennary *N*-linked oligosaccharides,[32] and 17% of the *N*-linked oligosaccharide structures on fetuin (and asialofetuin) are biantennary.[33] Interestingly, Con A also bound with good intensity to β-linked Glc across all three slide surfaces, but this well characterised lectin is only known to typically interact with α-linked Glc[31] (Table 3). The binding was verified as carbohydrate-mediated (see below). The unusual interaction of Con A with a β-Glc NGC has been previously reported where the β-Glc was linked *via* a phenylazo linker to the BSA backbone,[34] as is the case here.

UEA-I bound to both β- and α-linked Fuc on the Slide H and Aldehyde ES microarrays, and also recognised the NGCs Gal-β-PITC-BSA and Man-α-PITC-BSA (with average

substitution ratio of 27.6, but not the 22). No carbohydrate ligands were recognised with this lectin on the functionalised poly-L-lysine surface and, in general, this slide gave the lowest binding signals with all lectins after background subtraction (Table 3 and Fig. 3).

The lectins SBA and WFA are both known to have greatest binding affinity for *N*-acetylgalactosamine (GalNAc), with a slight preference for the α-linkage, but also recognise Gal[31] (Table 3). Gal-β-PITC-BSA was well recognised across all slides by both lectins, and ASF, with its Gal-terminating oligosaccharides, was recognised by WFA, although to a lower extent. The printed NGC Gal-α-PITC-BSA was well recognised by SBA and WFA only on the Aldehyde ES microarray, although interestingly the β-linked version was preferred by both lectins on this platform. In addition, the lactose (Lac) and *N*-acetyllactosamine (LacNAc) NGCs were bound by WFA on the Aldehyde ES microarray, with LacNAc-β-4AP-BSA slightly favoured. The latter three NGCs were only very slightly recognised by WFA on the Slide H platform and not at all on the functionalised poly-L-lysine slide (Fig. 3).

There was no binding to the negative control 4AP-BSA by any lectin on any slide surface, but most lectins interacted with PITC-BSA on most surfaces, except for Con A on all surfaces and WFA on the Aldehyde ES slide (see Figs. 2 and 3). However, use of the unmodified linker conjugated to the protein backbone may not be a good indicator of the spacer contribution to overall lectin binding to an NGC (i.e. a negative control). The presentation of the 'bare' linker portion on the linker-BSA conjugate is not the same as when it is further conjugated to a carbohydrate. This is evident when the expected low signal intensities of UEA-I and SBA with Glc-β-PITC-BSA are compared with the higher signal from PITC-BSA with the same lectins on the Aldehyde ES slide, and also when the low signal intensities from Gal-α-PITC-BSA binding with WFA, SBA and UEA-I on Slide H are compared to the higher signal intensity with PITC-BSA for the same lectins (Fig. 3).

Con A and WFA lectins were also co-incubated with 100 mM of Man and Gal, respectively, to verify carbohydrate-mediated lectin binding.[30] The binding of Con A and WFA to printed NGCs and glycoproteins was inhibited from 83.9-99.4% and 60.5-98.2%, respectively, on Slide H and from 71.7-99.4% and 98.3-100%, respectively on Aldehyde ES slides ($p \leq 0.05$, Table S-1 in supplementary data). The high background for WFA binding to the functionalised poly-L-lysine slides meant that the majority of extractable signals were negative intensity values after background correction and thus no statistically significant data extraction was possible. However, Con A gave low background on the functionalised poly-L-lysine surface and binding signals were completely inhibited (99.7-100%) with Man (Table

S-1).

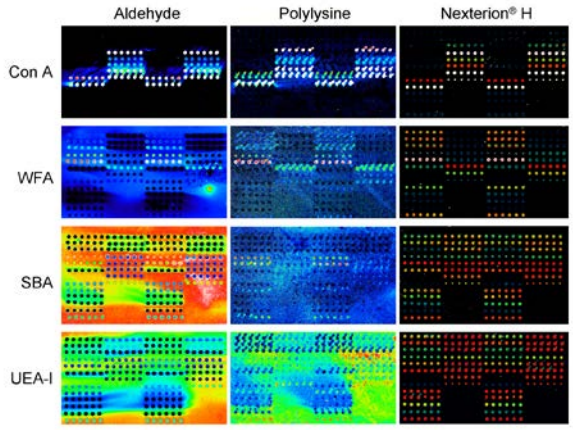
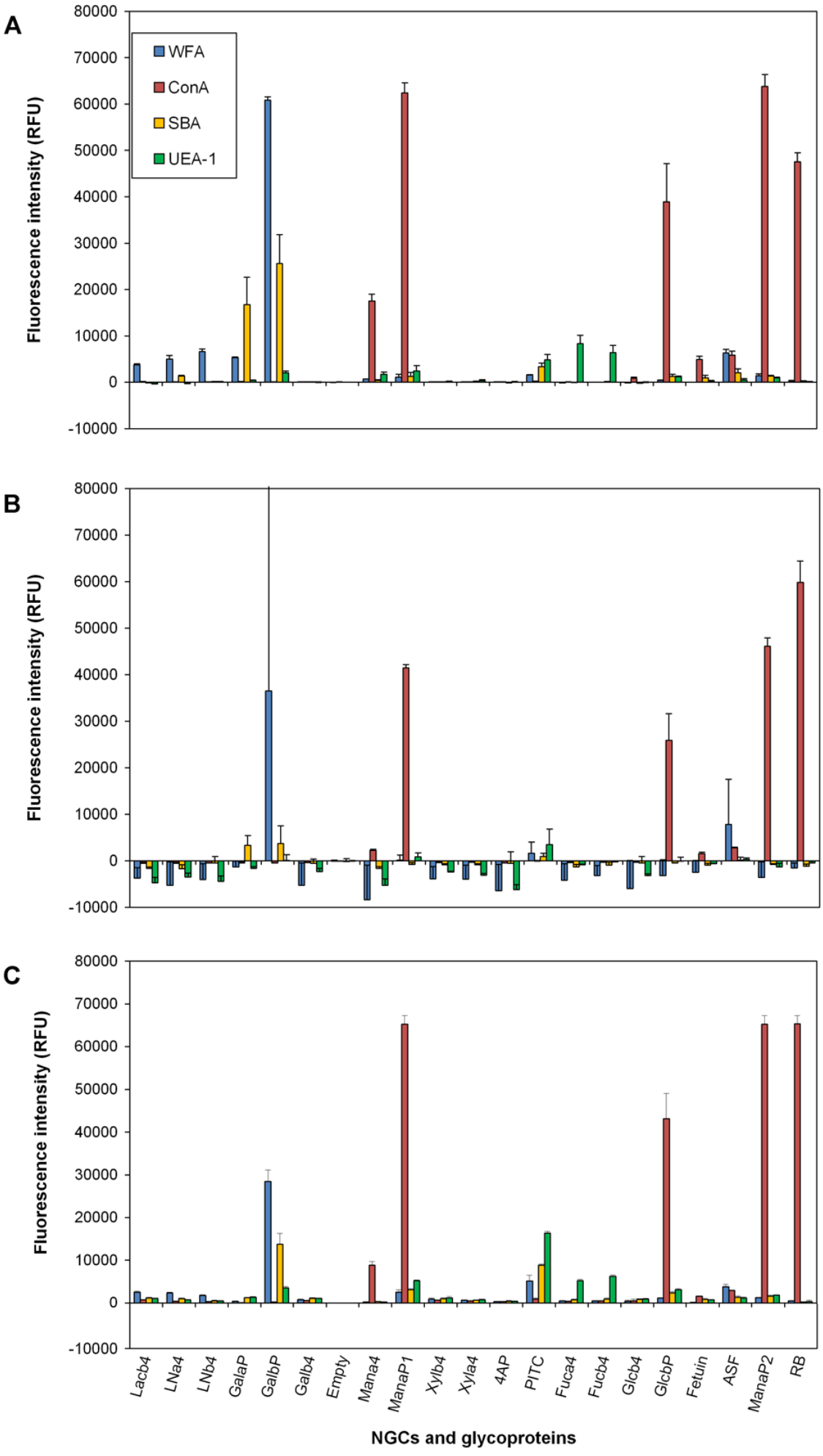


Fig. 2 Representative image from the three slide surface chemistries evaluated showing subarrays incubated with each lectin as indicated. Print order is as per Table 1, with the list printed twice to produce 12 spots per probe i.e. columns 3 and 4 of each subarray are replicates of columns 1 and 2.

Table 4 Average of %CV (based on absolute %CV) and median background intensity (RFU) from three replicate experiments for each lectin on the different slide surface chemistries (slide-to-slide variability).

Slide type	Lectin/ carbohydrate	Average background	Average signal %CV
Aldehyde ES	Con A	394.1	25.1
	Con A/Man	338.8	37.9
	WFA	942.9	45.5
	WFA/Gal	834	93.3
	SBA	1392.5	72.2
	UEA-I	1578.8	81.0
Functionalised poly-L-lysine	Con A	1866.0	48.0
	Con A/Man	1823.3	149.3
	WFA	8336.0	125.2
	WFA/Gal	5450.0	32.1
	SBA	6013.5	100.5
	UEA-I	11982.3	267.7
Nexterion slide H®	Con A	267.7	16.6
	Con A/Man	267	21.2
	WFA	313	20.3
	WFA/Gal	291	14.2
	SBA	294	17.8
	UEA-I	433.3	16.2

Fig. 3 Histograms representing the differences in recognition of neoglycoconjugates and glycoproteins by fluorescently-labelled lectins on the three slide surface chemistries evaluated. (A) Aldehyde ES, (B) functionalised poly-L-lysine, and (C) Nexterion® slide H. All slides were printed, incubated and processed at the same time. Histograms represent the average of three replicate experiments and error bars are 1 standard deviation. Each experiment is the median of 12 data points (12 features).



Reproducibility

The expected ligands were recognised by appropriate lectins on both the Slide H and Aldehyde ES platforms (see above). In addition, Slide H gave the most reproducible signals with an average % coefficient of variation (CV) ranging from 14.2 to 21.2% across the different lectins (Table 4), which is on the lower end for protein-based microarrays.[22,35] The reproducibility of lectin binding signals on the functionalised poly-L-lysine surface was poorest of the surfaces tested (32.1-267.7%CV, Table 4), which argues against their selection as a robust glycan microarray platform. Although performing better overall than the functionalised poly-L-lysine surface, the high, uneven background with the majority of lectins tested and overall poor reproducibility (25.1-93.3%CV) (Fig. 2 and Table 4) of Aldehyde ES slides also discourages the use as a general glycan microarray platform in spite of expected ligand recognition.

Effect of different linkers on lectin recognition

Three monosaccharide NGC analogues (α-Man, β-Gal and β-Glc) were printed with two different linkers, 4AP and PITC (Fig. 1 and Table 1), and lectin recognition of these monosaccharides was assessed across the different surfaces. Con A bound to both analogues of α-linked Man across all platforms, but displayed lower binding intensity to the 4AP analogue compared to the PITC-linked NGC. The 4AP α-Man NGC on the Aldehyde ES slide gave greatest binding intensity with Con A relative to the other surfaces (Fig. 3). Although a different average substitution ratio of carbohydrates on the BSA backbone can undoubtedly have an effect on the degree of lectin binding to the NGC,[17,36] Con A binding to Man-α-PITC-BSA with two different substitution values (ManaP1 and ManaP2, 27.6 and 22 moles carbohydrate per mole of protein, respectively, Table 1) had similar signal intensity to one another within the same platform (Fig. 3). This was probably due to similar ligand density of the two NGC features on the microarray surface.[3] Glc-β-PITC-BSA was well-recognised by Con A across all slide surfaces, but Glc--β-4AP-BSA was not recognised at all. Similarly, WFA and SBA bound to Gal-β-PITC-BSA on all three surface chemistries but Gal-β-4AP-BSA was not recognised by either lectin (Fig. 3). Conjugation of all NGCs to the microarray surface was confirmed by incubation with an anti-albumin antibody (not shown). The difference in lectin recognition between the monosaccharide NGC analogues is most likely due to the differences in the ligand presentation or accessibility because of the linker flexibility.[24] The N=N bond of the 4AP linker does not allow rotation about that bond in

contrast to the PITC linker with the more flexible N-C bond (Fig. 1).

Discussion

Overall, Con A performed well on all slide surfaces evaluated with low, non-interfering background noise and expected ligand recognition of α-linked Man and the glycoproteins RB, fetuin and asialofetuin (Figs. 2 and 3 and Table 4). The atypical carbohydrate-mediated recognition of the Glc-β-PITC-BSA NGC has been previously reported,[34] and the optimised ligand presentation for Con A recognition here may be due to the influence of the linker.[34,37]

The other three lectins tested showed varying degrees of background and specific recognition depending on the slide surface chemistry, with UEA-I being the least discriminatory lectin on all surfaces. In general, the functionalised poly-L-lysine slide showed the lowest intensity binding with all lectins after background subtraction and did not facilitate ligand recognition with UEA-I. The Aldehyde ES slide allowed good recognition of the expected ligands by the lectins but signals had poor reproducibility and the slides had high background with the lectins SBA, WFA and UEA-I. However, the 3D hydrogel Slide H surface had consistently lower background with expected recognition for all lectins binding to NGCs and glycoproteins. In contrast to these observations, a similar study assessing different surfaces for antigen binding to antibody microarrays found that 2D slide surfaces exhibited lower background than 3D slide surfaces.[21]

Thus, based on lowest background noise, expected ligand recognition, good spot morphology and high reproducibility, the 3D hydrogel surface was most suitable for lectin interrogation of the glycan microarray platform in this study. The results presented also highlight the need for assessment of glycan microarray platforms with multiple lectins, as the use of Con A alone would not have revealed the high background issues with the Aldehyde ES and functionalised poly-L-lysine surfaces, which were apparent only upon incubation with the three other lectins, SBA, WFA and UEA-I.

A pair-wise comparison of lectin interaction with analogues of three monosaccharide NGCs with two different linkers, 4AP and PITC, demonstrated differential lectin recognition of the carbohydrate depending on the linker identity. In agreement with previous literature,[24] the more flexible PITC linker afforded greater recognition of the monosaccharides by the relevant lectins on all platforms, with poor or no recognition of the 4AP conjugates.

The observed variations in lectin recognition between different surface chemistries and carbohydrates conjugated with different linkers may help explain differences in binding

reported for the same recognition elements using different platforms and ligand presentation. For example, the hemagglutinin (HA) of the H1N1 2009 influenza virus, A/California/4/2009, bound to both α-(2,3)- and α-(2,6)-linked sialic acid motifs on a neoglycolipid microarray,[38] while the same HA recognised only α-(2,6)-linked sialic acids on the Consortium for Functional Glycomics glycan microarray platform,[39,40] which utilises the Slide H surface.[18]

Conclusions

In this study, three microarray slide surface chemistries were evaluated for their effects on lectin-carbohydrate recognition using a panel of arrayed neoglycoconjugates and glycoproteins and four lectins for interaction. The 2D surfaces tested, Aldehyde ES and functionalised poly-L-lysine, displayed high background with all lectins except Con A even with blocking, which interfered in both feature detection and intensity extraction. In addition, the functionalised poly-L-lysine slides did not facilitate carbohydrate recognititon with the lectin UEA-I. The 3D hydrogel surface, Slide H, had greatest reproducibility, smallest feature diameter, good spot morphology, expected ligand interation and importantly, negligable background. The comparison of two different linkers for ligand presentation during this study demonstrated that use of a flexible linker allowed best recognition by the lectins selected. This paper highlights the importance of the effect of the selection of both slide surface chemistry and linkers for optimal carbohydrate recognition.

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Notes

†Electronic Supplementary Information (ESI) available: **Table S-1** Table of statistically evaluated inhibition data. See DOI: 10.1039/b000000x/

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