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Sugar-coated microarrays: A novel slide surface for the high-throughput analysis of glycans

The development of DNA and protein microarrays represents a significant advance in transcriptomics and proteomics research. Such arrays allow the high-throughput, parallel analysis of protein occurrence and interactions and gene expression. However, this advance has not been matched by equivalent technology for analysis of glycomes. One reason for this is that compared to proteins, it is difficult to reliably immobilise populations of chemically and structurally diverse glycans. We describe the development of a new microarray slide surface to which diverse glycan structures can be directly immobilised without prior derivatisation of the slide surface or any modification of the arrayed samples. The slides can be used to produce comprehensive microarrays of carbohydrates, glycoproteins and proteoglycans using isolated samples or cell extracts. Using standard microarray equipment, a series of carbohydrate microarrays were generated and probed with a panel of monoclonal antibodies with specificities for glycan epitopes. The arrays were highly reproducible, stable, and could be stored dry for several months. Glycans play central roles in development, carcinogenesis, cell adhesion, and immunity and are increasingly the subject of therapeutic approaches. We anticipate that the development of carbohydrate microarrays will be important for the high-throughput analysis of glycans and their molecular interactions.

Keywords: Antibodies / Carbohydrate microarrays / Glycans / Novel slide surface PRO 0310

1 Introduction

The development of DNA microarrays has been one of the most significant advances in biology and medicine in recent decades and allows the high-throughput analysis of gene expression [1–5]. Powerful though DNA microarrays are, they do have some important limitations. One is that they can provide only an indirect inventory of the extent and functionality of protein populations. Changes in mRNA levels are not necessarily proportional to changes at the protein level because of differences in rates of translation and degradation [6]. Moreover, nucleotide-based arrays do not directly provide information on post-translational modifications of proteins that may be critical for function, such as acetylation, phosphorylation or hetero- and homo-dimerisation of subunits. For glycans, the link between gene expression levels and biosynthesis is even less direct since glycans are not encoded directly but are result of the co-operative activity of arrays of synthetic and modifying enzymes [7]. For these reasons there is a pressing need to develop high-throughput microarray approaches for the direct analysis of proteins and carbohydrates. However, while protein microarrays are now a well established technology [6, 8–10], the development of equivalent strategies for the direct analysis of carbohydrates has been hampered by technical limitations. All microarrays are underpinned by a non-porous surface onto which molecules can be stably immobilised, and the lack of suitable surfaces has been the limiting factor for the development of carbohydrate microarrays. In this report we describe the development of a new microarray slide surface that is capable of the direct, stable immobilisation of structurally and chemically diverse glycan structures without prior modification and therefore allows the development of comprehensive carbohydrate microarrays.

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Abbreviation: DE, degree of methyl-esterification

2 Materials and methods

2.1 Slide and microarray production

Slides were produced by injection moulding of black polystyrene and a surface modification was generated by oxidation. The resin used has high dimensional stability and good chemical resistance to the aqueous solutions used during arraying and probing. Slides were obtained from Nunc Roskilde, Denmark; http://www.nuncbrand.com/docs/doc_Products_OEM.asp). The microstructure of the slide surface was analysed using an atomic force microscope (DSM NSIII-AFM) used at a scan rate of 16.3 Hz. The scan size was 3.88 μm. Linear arrays were produced using a standard four pin, pin-and-ring™ type

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microarrayer (Affymetrix 417; Santa Clara, CA, USA) with a pin diameter of 150 μm. Spots were spaced 375 μm apart and applied at a rate of four spots/second. The spot volume was approximately 50 pL. Samples were arrayed from 30 μL aliquots contained in 96-well microtitre plates with conical wells (Corning, Cambridge, MA, USA). Up to 42 slides were arrayed from each microtitre plate and in all cases 15 replicates of each sample was arrayed. For each sample array, a corresponding register array was generated in which carmoisine dye was arrayed in the same configuration as the samples. This enabled the spatial relationships between the spots to be accurately determined during subsequent analysis of the probed arrays. The extent of subarrays was delineated by the inclusion of dextran conjugated to fluoroscein isothiocyanate (detran/FITC; Sigma-Aldrich, Poole, UK) into selected wells. Arrayed slides were stored dry at room temperature for up to three months prior to probing and analysis. The signal from probed arrays was collected using a laser array scanner (Affymetrix 418). Selected arrays were analysed using image analysis software (ImaGene™; BioDiscovery, Marina del Rey, CA, USA) which enabled the relative signal from each spot to be quantified. Data were not collected from arrays containing obvious defects such as incomplete removal of buffer salts, or complete sample drop outs caused by depletion of sample within spotting rings during arraying.

2.2 Sample preparation

Arrayed samples were complex polysaccharides, proteoglycans and neo-glycoproteins. Lime pectic polysaccharide samples with known degrees (DE) and patterns of methyl-esterification were prepared as described previously [11] from a highly methyl-esterified sample (E81) by treatment with pectin methyl esterase from *Aspergillus niger* (F-series samples) or orange (P-series samples) or by base catalysis (B-series samples). Galactan from *Solanum tuberosum* (Galactan 2 (*St*)) and *Lupinus augustifolius* (Galactan 1 (*La*)) and arabinan from *Beta vulgaris* (Arabinan (*Sb*)) were supplied by Megazyme (Bray, Ireland). Xylogalacturonan from *Pisum sativum* was a generous gift from Professor Jean François Thibault (INRA, Nantes, France). Arabinogalactan-protein proteoglycans (AGP 1(*Dc*) and AGP 2(*Dc*)) were purified form *Daucus carota* cell cultures [12]. Mixtures of complex polysaccharides were extracted from suspension cultured *Nicotiana tabacum* (line BY2) and *D. carota* (line Ox6) cells and *N. tabacum* leaves by homogenisation in a buffer containing *trans*-1,2-diaminocyclo-hexane-*N*,*N*,*N*',*N*'-tetra-acetic acid (CDTA). The conditioned medium of BY2 and Ox6 cells was obtained prepared by filtration of cell cultures. The neo-glycoprotein (1→4)-β-D-galactan-BSA was prepared by conjugation of four residues of (1→4)-β-inked-ID-galactose to BSA *via* a 3 atom spacer as described previously [13]. All purified samples were applied at a level of 0.5 mg/mL in PBS (0.14 M NaCl, 2.7 mM KCl, 7.8 mM $Na_2HPO_4.12H_2O$, 1.5 mM KH_2PO_4, pH 7.2) and conditioned media and cell and tissue extracts were applied as three-fold dilutions in PBS. Selected samples were applied as five-fold dilution series in PBS with a starting concentration of 1 mg/mL.

2.3 Monoclonal antibody probes

Glycan microarrays were probed with a panel of mAbs with specificities for a range of glycan eptiopes. The antibodies JIM5 and JIM7 bind to homogalacturonan with low and high degrees of methyl-esterification respectively [14], while $PAM1_{scFv}$ recognises an epitope consisting of un-esterified homogalacturonan with a degree of polymerisation of at least 30 galacturonic acid residues [14]. LM5 and LM6 recognise (1→4)-β-D-galactan and (1→5)-α-L-arabinan respectively [14]. LM8 recognises xylogalacturonan [14], and LM2 recognises a glycan epitope of arabinogalactan-proteins proteoglycans. All antibodies were generated by conventional hybridoma technology, except $PAM1_{scFv}$ which was isolated from a phage display single chain synthetic antibody library [15].

2.4 Probing arrays

Arrayed slides were blocked with PBS containing BSA (3% v/w) (3% BSA/PBS) for at least 1 h. After washing for 2 min in PBS, slides were incubated in antibody solutions for at least 1 h. Hybridoma antibodies were used as 1/20 dilution of hybridoma supernatants in 3% BSA/PBS. $PAM1_{scFv}$ was used at a concentration of 10 μg/mL, also diluted in 3% BSA/PBS. Following antibody incubations, slides were washed twice for 10 min in PBS containing 0.1% v/v polyoxyethylene sorbitan monolaurate (Tween 20, PBST), followed by one 5 min wash in PBS. Slides were then incubated in solutions containing secondary antibodies conjugated to the fluorescent dye cyanine (Cy3). For hybridoma antibodies, an anti-rat/Cy3 conjugate was used (Amersham Biosciences, Little Chalfont, UK), while $PAM1_{scFv}$ binding was detected using anti-c-myc/Cy3 conjugate (Sigma). All secondary antibodies were used as 1/100 dilutions in 3% BSA/PBS and incubations were for at least 1 h. All primary and secondary antibody incubations were performed either under cover slips (using a volume of 80 μL) or in custom – made 28 mm × 80 mm plastic envelopes (using a volume of 3 mL). Incubations in envelopes tended to result in superior S/N ratios and all results shown were obtained using envelope incubations. Following incubation in secondary

antibodies, slides were again washed in PBST and PBS as described above then rinsed briefly in deionised water to remove PBS buffer salts. In order to remove surface water, slides were placed in 50 mL centrifuge tubes and centrifuged for 3 min at 1500 *g*. Any remaining water was removed by briefly holding slides in an air stream. Slides were stored at room temperature in an airtight box.

3 Results

Black polystyrene slides were produced with a physical surface modification (known as MaxiSorp™; Nunc, Roskilde, Denmark) (Fig. 1A). Slides were made to a standard size (25 mm × 75 mm) and were used in conventional microarray equipment. The microstructure of the slide surface, revealed by scanning atomic force microscopy (Fig. 1B), consists of a series of ridges that increase the surface area available for binding. However, the gross slide surface is extremely flat with a typical maximum height variation of less than 40 nm over 10 μm (Fig. 1C). Moreover, because the binding surface is created by physical modification to the polymer rather than by a coating, the surface is also highly consistent in nature, both within and between slides. An advantage of creating slides using the MaxiSorp™ surface is that the binding properties of this modification have been characterised previously [16, 17]. Binding is established by passive adsorption mediated by hydrogen bonding, ionic bonding and van de Waals hydrophobic interactions. Hydrogen and ionic interactions are primarily involved in capturing polar parts of molecules and securing a tight junction between the surface and the molecule. Immobilisation is further stabilised by the removal of water molecules between the relatively hydrophobic surface and the immobilised molecule. Water removal is driven by a lowering of the free energy in the system by exchanging the weak water bonds with hydrophobic bonds. This combination of binding mechanisms allows the stable immobilisation of a range of glycan structures with differing physical and chemical properties.

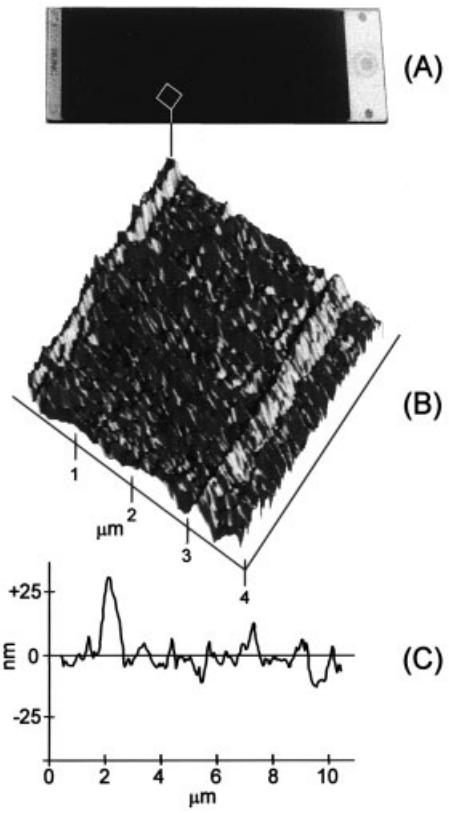

Figure 1. Development of novel slide surfaces for the direct immobilisation of carbohydrates. Slides were cast in black polystyrene (A) with a physical modification that created a surface with the capacity to immobilise structurally and chemically diverse glycans. The area available for binding is increased by the peaks and troughs in slide surface microstructure revealed by scanning atomic force microscopy (AFM) (B). However, the overall slide surface is extremely flat, as shown by AFM sectioning through a 10 μm portion of a slide surface (C).

We tested the suitability of this surface for generating carbohydrate microarrays by analysing the immobilisation of a range of glycan structures derived from, or occurring in, plant cell walls which contain some of the most complex glycans found in nature. Samples analysed were polysaccharides, proteoglycans and neo-glycoproteins as well as plant cell extracts. A series of identical microarrays was created and immobilisation was assessed by the binding of a panel of previously characterised mAbs with specificity to carbohydrate epitopes [14, 15] (Fig. 2). For all the samples tested the antibody binding profiles indicated that antigens had been effectively immobilised and that epitope conformations were preserved. The epitopes recognised by the antibodies JIM5, JIM7 and $PAM1_{scFv}$ all consist of homogalacturonan (HG, a homopolymer of (1→4)-α-linked-D-galacturonic acid) but differ in degree and pattern of methyl-esterification (DE). The differential binding of these antibodies to a series of microarrayed pectic polysaccharides which differed only in DE indicated that subtle postsynthetic modifications to complex polysaccharides can be determined using these arrays (Fig. 2C–E).

In order to be able to eventually create comprehensive microarrays of glycomes it is necessary that the immobilisation surface used should be capable of binding not just pure carbohydrate structures but also glycoproteins and proteoglycans. This was tested by arraying the neoglycoprotein (1→4)-β-D-galactan-BSA and arabinogalactan-protein proteoglycans. The binding of LM5 and LM2

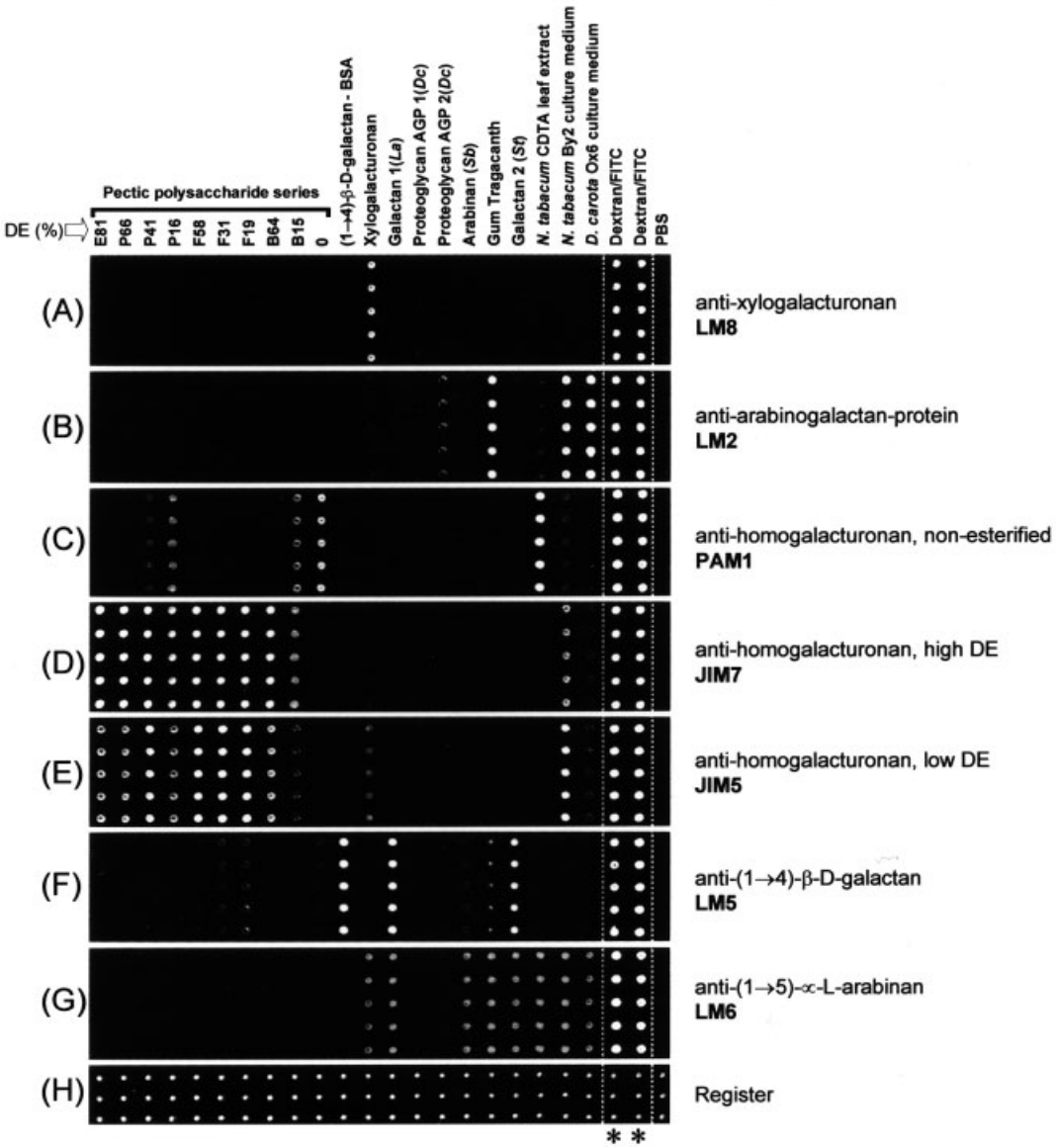

Figure 2. Carbohydrate detecting microarrays. A series of identical carbohydrate microarrays (A–G) were created by immobilising a series of polysaccharides, proteoglycans, neo-glycoproteins and plant cell extracts (listed in top line, for details see Section 2.2). Fifteen replicates of all samples were applied – five replicates are shown. The arrays included a series of pectic polysaccharides differing only in their DE. The immobilisation of arrayed glycans was assessed by probing with a panel of mAbs (listed right) with specificity to carbohydrate epitopes. Antibody binding was detected by probing with Cy3 conjugated secondary antibodies. A register array (H) of directly applied carmoisine dye was included in order to track the position of all samples and directly immobilised fluorescently labelled dextran (*) was used to establish array orientation. PBS was used as a negative control.

respectively to these antigens indicated that proteoglycans and glycoproteins can be effectively immobilised using these slides (Fig. 2. B, F). The reproducible and detection limits of antibody binding to immobilised glycans were also tested (Fig. 3). The detection limits of three antibodies were tested by probing arrayed dilution series of selected antigens and quantifying the signals obtained. Galactan polymer and pectic polysaccharide were detected to a level of 1.6 μg/mL by antibodies LM5 and JIM5 (Fig. 3. A, B), while xylogalacturonan was detected to a level of 40 μg/mL by antibody LM8 (Fig. 3. C). Based on a spot volume of 50 pL, a detection limit 1.6 μg/mL corresponds to a minimum detectable amount of 80 fg. The ability to detect very low levels of material arrayed at

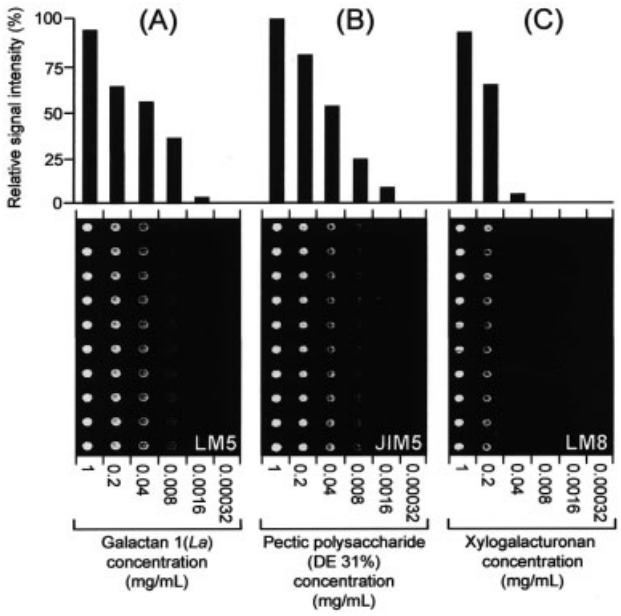

Figure 3. The detection limits of three antibodies was assessed by probing arrayed dilutions series (1 mg/mL–0.32 μg/mL) of galactan polymer (Galactan 1(*La*)), pectic polysaccharide and xylogalacturonan with antibodies LM5 (A), JIM5 (B) and LM8 (C) respectively. The signal from each spot was quantified and the relative means of signals of 10 replicates is indicated above each sample. All SEMs were less than 3.8% of the maximum relative signal.

Table 1. The detection limit and spatial density of carbohydrate microarrays compared to ELISAs and immunodot assays. Values pertain to the binding of mAb JIM5 to partially methy-esterified lime pectin [22].

	Carbohydrate microarray	ELISA	Immunodot assay
Detection limit (concentration)	~ 1.6 μg/mL	~ 0.1 ng/mL	~ 10 μg/mL
Detection limit (amount)	~ 80 fg[a)]	~ 5 pg[b)]	~ 10 ng[c)]
Typical density of arrayed samples	Up to 10 000 *per* slide	96 *per* plate	~ 500 *per* membrane

a) For a spot volume of 50 pL
b) For a plate coating volume of 50 μL/well
c) For a spot volume of 1 μL

very high density is a significant advantage of microarrays compared to existing methods of carbohydrate analysis, such as ELISAs and immunodot assays (Table 1). Both intra- and inter-array consistency was high. Intra-array consistency was assessed by collecting signals from five separate sets (on different slides) of 15 replicates of spots of an F-series pectic polysaccharide sample with a DE of 31% (F31) as detected by probing with antibody JIM5. Inter-array consistency was assessed by quantifying the difference in signals from spot-pairs of two different pectic polysaccharides, F31 and a B-series pectic polysaccharide with a DE of 15%. Again, five sets of 15 replicates were arrayed over five separate slides and probed with JIM5. The SEM intra-array variation was 3.2% of the maximum relative signal, while the SEM of the inter-array variation was 5.1% of the maximum relative signal. The S/N ratio obtained was generally high and this was in part due to the fact that slides were produced using black polymer resin. Transparent slides with identical surface properties were also tested but gave significantly inferior S/N ratios (results not shown). Arrayed slides were stored dry for up to three months before probing and analysis. No significant qualitative differences in signals were obtained for stored slides compared to freshly arrayed slides and the immobilisation of glycans onto the slides therefore appeared to be stable during prolonged storage.

4 Discussion

The fundamental importance of carbohydrate structures in biology and pathology is becoming ever more apparent. Glycans are known to play central roles in development, carcinogenesis, cell adhesion, and immunity and are increasingly the subject of therapeutic approaches [18]. However, the indirect link between gene expression and glycan end products, as well as the often extreme complexity of glycans creates an urgent need for the development of high-throughput, direct physical methods of analysis. We have described a novel microarray slide surface that can be used for the immobilisation of diverse glycan structures. The arrays are highly reproducible, stable and are made using standard microarray equipment and simple probing procedures. Importantly, carbohydrates are arrayed directly without the need for modification to generate reactive groups. The utility of these microarrays is primarily for the rapid, sensitive and very high-throughput analysis of the occurrence of glycans, rather than as a tool for quantification. We believe that these slides make a significant contribution to the range of surfaces available for microarray production [19–21] and bring into reach the possibility of the global analysis of glycomes. However, as is the case for protein microarrays, some major challenges remain.

The nomenclature adopted for protein arrays provides a useful framework for developing strategies to develop this rapidly evolving area of technology [9]. Two types of arrays are defined: function arrays, and detecting arrays. In function arrays the arrayed targets are probed with a

fluorescently labelled bait or mixtures of baits. In contrast, the construction of detecting arrays involves the immobilisation of ligands (such as antibodies) which are used to capture their binding partners from complex mixtures. Binding is detected either by the bulk labelling of target mixtures prior to exposure to the array, or by probing for captured targets with antibodies or other ligands. We have reported here the development of carbohydrate function arrays and we envisage that such arrays will have several important applications. These include the identification of the glycan moieties recognised by putative carbohydrate binding proteins and, as we have demonstrated, the high-throughput characterisation of antibody specificities. We also anticipate that these arrays may be used to identify carbohydrate binding peptides by screening phage display peptide libraries. Such peptides may have use as therapeutic agents, and provide sequence data for use in convergent evolution studies to identify endogenous carbohydrate binding proteins. An important aim for the future is to array the output from separations of cell extracts in order to compare glycomes during development or disease. Recent advances in very small-scale extraction and fractionation technologies with nanoscale fraction outputs that can be directly arrayed, makes this a realistic goal for the near future.

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