Non-Covalent Microarrays from Synthetic Amino-Terminating Glycans: Implications in Expanding Glycan Microarray Diversity and Platform Comparison

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SUMMARY

Glycan microarrays have played important roles in detection and specificity assignment of glycan-recognition by proteins. However, the size and diversity of glycan libraries in current microarray systems are small compared to estimated glycomes, and these may lead to missed detection or incomplete assignment. For microarray construction, covalent and non-covalent immobilization are the two types of methods used, but a direct comparison of results from the two platforms is required. Here we develop a chemical strategy to prepare lipid-linked probes from both naturally-derived aldehyde-terminating and synthetic amino-terminating glycans that addresses the two aspects: expansion of sequence-defined glycan libraries and comparison of the two platforms. We demonstrate the specific recognition by plant and mammalian lectins, carbohydrate-binding modules and antibodies, and the overall similarities from the two platforms. Our results provide new knowledge on unique glycan-binding specificities for the immune-receptor Dectin-1 towards β-glucans and the interaction of rotavirus P[19] adhesive protein with mucin *O*-glycan cores.

glycan microarray, covalent immobilization, non-covalent immobilization, neoglycolipid, Dectin-1, rotavirus, mucin *O*-glycan core, β1,3-glucans,

INTRODUCTION

Recognition of glycans by proteins is crucial to understand molecular mechanisms in health and disease. Carbohydrate microarrays, including those of polysaccharides (Wang et al., 2002) and sequence-defined glycans (Fukui et al., 2002), have played a major role in dissecting glycan-protein interactions after their emergence as a natural follow-up to the development of the microarray technologies for nucleic acids (Schena and Shalon, 1995) and proteins (MacBeath and Schreiber, 2000). Since their inception in 2002, glycan microarrays have proven to be powerful tools in the detection and specificity assignment of glycan-protein interactions with implications in biology and medicine.

Natural glycans cannot be arrayed directly due mainly to their highly hydrophilic nature and the incompatibility of the functional groups of carbohydrate molecules with readily available microarray slides. Various approaches were developed to convert glycans into forms suitable for printing and immobilization on different surface-modified glass slides used for arrays of nucleic acids and proteins. As carbohydrate molecules cannot be cloned, their isolation from natural glycome sources (Li et al., 2018; Palma et al., 2015; Song et al., 2011) or synthesis by chemical (Cheng et al., 2018; Geissner et al., 2019) and enzymatic means (Gao et al., 2019; Prudden et al., 2017) are the main methods for building up libraries of glycans. Many microarray platforms using sequence-defined glycans have been developed using different chemistries and immobilisation strategies to address specific biological questions or to target specific glycomes (Ban and Mrksich, 2008; Blixt et al., 2004; Fukui et al., 2002; Geissner et al., 2019; Park et al., 2007; Pedersen et al., 2012; Sanchez-Ruiz et al., 2011; Šardzík et al., 2011; Shipp and Hsieh-Wilson, 2007; Wang et al., 2009; Xia and Gildersleeve, 2015). Among these, the neoglycolipid (NGL)-based microarray system of the Imperial College Glycosciences Laboratory (Fukui et al., 2002; Palma et al., 2014), the platform of US Consortium for Functional Glycomics (CFG) (Blixt et al., 2004) and the microbe-focused Max Planck Institute (MPI) platform (Geissner et al., 2019) have glycan libraries in a scale and diversity suitable for broad screening analyses and are major international resources serving the wide scientific community. The CFG and MPI arrays

comprise amino-terminating synthetic glycans that are covalently immobilized on NHS-functionalized slides, whereas the NGL arrays comprise mainly naturally-derived aldehyde (in the form of hemiacetal)-terminating glycans conjugated to a long chain amino phospholipid and non-covalently immobilized on nitrocellulose-coated slides. The three platforms are in some ways complementary with partial overlap in their glycan repertoires.

Although debatable, it has been estimated that there are 100,000-500,000 glycan structures in the mammalian glycome (Freeze, 2006; Rillahan and Paulson, 2011) present on glycoproteins, glycolipids and polysaccharides, and as secreted free sugars. The numbers of peripheral sequences (Drickamer and Taylor, 2002) or glycan determinants (Cummings, 2009) are in the range of 7,000. Therefore the glycome is considered larger than the genome and proteome, but the size of mammalian glycan array libraries up to now is small compared with those assembled for DNA and protein microarrays (Zhu et al., 2001). Currently in each of the two largest glycan microarrays there are around 1,000 probes; some of which have the same glycan structures but with different linkers or tags. There is an obvious need to expand the libraries of sequence-defined glycan probes to cover the major part of glycan structures within glycomes.

Given the different ways of constructing glycan microarrays and the vast diversity of carbohydrate molecules with different structural and chemical/physical properties over nucleic acids and proteins, careful comparisons across different platforms are necessary for widening the scope and future use of microarrays in diverse applications and in deriving glycan binding specificities with confidence. In light of this, there have been studies comparing glycan binding profiles obtained with microarrays that use different chemistries for glycan derivatization, glycan linker types, glycan probe densities and modes of presentation (Grant et al., 2014; Padler-Karavani et al., 2012; Temme et al., 2019; Wang et al., 2014). However, a focused study on comparison of the two of the major platforms, covalent and non-covalent, has not been carried out.

The present work has aimed to address the two aspects: the need to expand libraries of sequence-defined glycans and for comparison of two of the major microarray platforms, by developing a new lipid reagent which is suitable for preparation of NGL probes from amino-terminating glycans. This opens the way for NGL microarrays to be sourced from both

naturally-derived aldehyde-terminating and synthetic amino-terminating glycans, and therefore expansion of the microarray coverage. It is now also possible to compare two platforms using the very same amino-terminating glycans as probes for both covalent and non-covalent microarrays after their conversion into NGLs.

RESULTS

Synthesis of novel phospholipid reagents active for amino-terminating glycans

Currently, the amino-phospholipid 1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine (DHPE) is used to conjugate aldehyde-terminating glycans by reductive-amination to form NGLs (Chai et al., 2003). Here we attempted two strategies to convert DHPE into lipid reagents with functionalities reactive with amino-terminating glycans.

A carboxyl-terminating lipid was designed for amide condensation with amino-terminating sugars. The amino group of DHPE reacted with succinic anhydride to form *N*-(4-oxobutanoic acid)-DHPE (DHPC) (**Figure 1A**) which contains a terminal carboxyl to be used for conjugation with amino-terminating sugars. The new lipid reagent DHPC was obtained in good yield (97%).

An aldehyde-terminating lipid was also designed by reaction of DHPE and hetero-bifunctional 4-carboxybenzaldehyde (**Figure 1B**). The carboxyl was used for conjugation to the amino group of DHPE by formation of an amide bond with the aid of activation by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxybenzotriazole (HOBT), leaving the free aldehyde for linking to amino-terminating glycans by reductive-amination. The aldehyde-terminating lipid product *N*-(4-formylbenzamide)-DHPE (DHPA) was obtained only in a moderate yield (63%) due mainly to the poor solubility of DHPE in the reaction solvent (dichloromethane or chloroform).

For assessing the use of the two lipid reagents in preparation of NGLs the aminoethyl glycoside of galactose, Galβ-O-CH_2CH_2-NH_2 (abbreviated as Gal-C2-NH_2), was used as the model sugar.

DHPC conjugation with Gal-C2-NH_2 via amide condensation was carried out in the presence of the activation reagents EDC and NHS (**Figure 1A**) (Sam et al., 2010). However,

as indicated by high-resolution (HP) TLC, only a limited amount NGL was generated (**Figure S1A**).

DHPA was conjugated to Gal-C2-NH_2 via reductive-amination (**Figure 1B**). The amino group of the amino-terminating sugar was linked to the aldehyde of DHPA in the presence of reducing agent cyanoborohydride. HP-TLC analysis showed that Gal-C2-NH_2 was almost completely converted into NGL (**Figure S1B** and **Figure S2A**). Due to the higher yield (85%, **Figure S1B** and **Figure S2A**) of NGL products and the potential use of the UV chromophore afforded by the benzene ring, DHPA was selected for preparation of a library of NGL probes.

Preparation of DA-NGLs for exploratory non-covalent microarray construction and analysis

Analyses by HP-TLC and MS indicated that multiple NGL products can be formed with DHPA. In the case of Glc-C2-NH_2, three DHPA-NGL products were found: NGL with single lipid (DA1), two lipids (DA2), and methylated single lipid (DA1+Me), as revealed by HP-TLC (**Figure 2A**) and MALDI-MS (**Figure S2B**). The double lipid conjugation by reductive-amination with the secondary amine was unpredicted. The formation of DA2-NGLs could not be minimized under different conjugation conditions (see **Methods** section for details). Aiming to minimize the formation of methylated products, EtOH, DMSO or DMF were used instead of MeOH, but methylated product was still formed (**Figure 2B**).

To obtain products with a single lipid, methylamino-terminating sugars were used. In this case, a clean single product was obtained (**Figure 2C-D**). An aminooxy-functionalized glycan, GalNAcα1-ONH_2 can also be conjugated to DHPA by generating an oxime without reduction to give a single lipid-linked DA-NGL (**Figure S3**).

HP-TLC analysis showed that the yield of these products (including NGLs with single and double lipid and methylated) was greater than 80% after 6–24 h for most of the oligosaccharides used in this work (**Table S1**). Conjugation was less efficient for aromatic amine-functionalized heparin-derived glycans, Hep-4-NS-PhNH_2 and Hep-4-NAc-PhNH_2. For these two oligosaccharides, incubation was prolonged to 48 h and at a higher temperature (80^{o}C). The conjugation efficiency was thereby increased to ~50% (data not shown).

As methylamino-terminating glycan analogues are not readily available, we evaluated the binding signals elicited by NGLs with single or double lipid chains. The isolated products were arrayed and the binding with ten carbohydrate sequence-specific proteins were analysed (**Table S2**). The binding patterns with the DA1- and DA2-NGL pairs immobilised non-covalently on nitrocellulose-coated slides were similar overall (**Figure S4**, selected shown in **Figure 3**) and were consistent with prior knowledge of glycan recognition by these proteins (**Table S2**). These included binding by the α-fucose-specific proteins, *Aleuria aurantia* lectin (AAL), *Ulex europeus* agglutinin (UEA-1) and the anti-blood group H type 1 and H type 2 antibodies; the β-galactoside specific *Ricinus communis* agglutinin I (RCA_{120}); the core 1 specific peanut agglutinin (PNA); the α-GalNAc-specific proteins, human macrophage galactose-type lectin (MGL), *Vicia villosa* lectin (VVL) and *Helix pomatia* agglutinin (HPA); and the *O*-β-GlcNAc-specific antibody CTD110.6 (**Figure 3**).

Construction and validation of DA-NGL microarray using sequence-specific carbohydrate binding proteins

Taking into account the above results, NGLs of the DA1 series were used to construct a microarray of 60 structurally diverse glycan sequences comprising both mucin-type *O*-glycan cores and *O*-GlcNAc linked to Ser/Thr, blood-group-, *N*-glycan-, glycosaminoglycan- and glycolipid-related sequences, and β1,3-gluco-oligosaccharides (linear or branched) with degree-of-polymerization (DP) of 12, 13 and 15 (position #1–#60 **Table S1**), referred to as DA-NGL microarray hereinafter; conventional NGLs and glycolipids (position #61–#82) were also included for reference .

The DA-NGL microarray was probed with lectins, antibodies and carbohydrate binding modules (CBMs) with known specificities (**Table S2**). The microarray analyses showed a good correlation of the binding profiles with the reported carbohydrate-binding for the proteins analysed and also provided new information on their specificities, which served to validate the DA-NGLs for binding studies (**Figure 4** and **Figure S5**).

Lectins and antibodies specific to mucin O-GalNAc cores or O-GlcNAc

The human MGL, which is specific to the tumour-associated *O*-glycan Tn antigen (GalNAcα-

Ser/Thr), showed binding to all the GalNAcα-terminating probes (#21–#23, **Figure 4A, Table S1**), including the GalNAcα-Ser/Thr substituted with an α2,6-linked Neu5Ac (#28, #29), which comprises the tumour associated α2,6-sialyl-Tn antigen, in accord with published interaction data (Mortezai et al., 2013). MGL could additionally bind to GalNAcβ-linked to Ser/Thr (#24 and #25). Binding, albeit weak, was also elicited with the synthetic probe presenting a terminal GalNAcβ1-4-linked to Gal in the extension of the core 3 *O*-glycan (#44). These results are in agreement with the reported plasticity of the MGL carbohydrate recognition domain to accommodate GalNAc in different structures present on pathogens, endogenous glycoproteins, and tumours (Diniz et al., 2019). The two other α-GalNAc-specific lectins VVL/VVA and HPA also showed their specific binding the GalNAcα-terminating probes (**Figure S5A-B**). The core 1 specific lectin PNA (**Figure 4B**) showed binding to Galβ1-3GalNAc-Ser/Thr (#30, #31) and to the probes containing this epitope at the non-reducing terminal (#19, #20 and #45). Noteworthy, PNA showed a strong binding to core 2 Galβ1-3(GlcNAcβ1-6)GalNAc- sequence linked to either an aminopropyl linker (#38) or to Thr (#39). The anti-sialyl-Tn specific mAb 3F1-IgG (**Figure 4D**) showed restricted binding to the two α2,6-sialyl-Tn probes (#28, #29) with a preference for α2,6-sialyl-Tn-Ser.

The mAb CTD110.6, which has been widely used to detect *O*-GlcNAc, showed strong binding to GlcNAcβ-Ser/Thr (#26, #27, **Figure 4E**) and exhibited cross-reactivity with chitobiose (GlcNAcβ1-4GlcNAc, #11) and GalNAcβ-Thr (#25), as reported previously (Reeves et al., 2014). The antibody also showed strong binding to core 2 (#39) and to GlcNAcβ6/β3-terminating core 4 (#46, #47) with a preference for Thr over the aminopropyl linker. The Ser analogues were not analysed.

Lectins and antibodies specific to terminal NeuAc, Fuc, Gal, Man or GlcNAc sequences

Human Siglec-15 has been shown to have the α2,6-sialyl-Tn antigen as its ligand (Angata et al., 2007; MacAuley et al., 2014). Here, Siglec-15 was demonstrated to bind to sialylated glycans with other backbone-types (**Figure 4C**). The α2,3- and α2,6-sialyl lactose (#5, #6) were bound with similar intensities as α2,6-sialyl-Tn (#28, #29) and related O-glycan probes (#33 #36, #37), whereas stronger binding was observed to the extended α2,6-sialylated LSTb, α2,3/α2,6-disialylated DSLNT and DSMFLNH probes (#8–#10), which contain the Neu5Ac

α2,6-linked to an internal GlcNAc. Weak binding was detected to non-sialylated core 3-Thr (#41) and heparin-derived tetrasaccharides (#51, #52). The binding is unexplained, but may indicate that the carboxyl group in these probes behave similarly to the carboxyl functionality in sialic acid residues.

The terminal Manα1-, Galβ1- and GlcNAcβ1,4-probes also elicited specific binding signals with the plant lectins ConA, RCA_{120}, and WGA, respectively (**Figure S5C-F**). Additionally, ConA showed binding to the *O*-mannosylated peptide (#50) and WGA to probes with mucin *O*-glycan core sequences, albeit weakly: GlcNAcβ1-6-terminating core 2 (#38, #39), GlcNAc1-β6/β3- terminating core 4 (#47), and α2,6-sialyl-Tn-Ser/Thr (#28, #29). The fucose specific lectin AAL and the anti-blood group H type 1 and type 2 antibodies showed the specific binding to the terminal fucosylated DA-NGL probes (**Figure S5F-H**). AAL also showed binding to the core-fucosylated *N*-glycan probes with fucose α1,3- and/or α1,6-linked to GlcNAc (#12-#14 and #18).

CBMs specific to β-glucans

Two β-glucan-specific bacterial CBMs that exhibit different modes of interaction with glucan polysaccharides were analysed (**Table S2**). *Tm*CBM4-2 is an endo-type B CBM from a *Thermotogoa maritime* laminarinase, which binds to internal sequences of oligosaccharides and is highly specific for the β1,3-linkage (Palma et al., 2015). *Cm*CBM6-2, a CBM from a *Cellvibrio mixtus* endoglucanase 5A, has a broad specificity to β-glucans (Palma et al., 2015) and contains two binding sites – an endo-type B site and an exo-type C site – and binds to the non-reducing end of oligosaccharides. In accord with their specificities and topologies of the binding sites, *Cm*CBM6-2 bound with similar intensities to the linear β1,3-glucan probes with DP12 and DP15 (#53 and #54) and to the branched β1,3/1,6-glucan DP13 (#55) (**Figure 4F**), whereas *Tm*CBM4-2 showed stronger binding to the probe with the linear DP 12 (**Figure 4G**), compared to the branched probe with the same C5 linker. A new finding for *Cm*CBM6-2 was the consistent binding pattern observed to all GlcNAcβ1-terminating probes: chitobiose (#11), GlcNAcβ1-*O*-Ser (#26), and GlcNAcβ1-terminating core 2 (#38, #39), core 3 (#41) and core 4 (#46, #47), possibly through its exo-type C site.

Application of DA-NGL microarray to derive specificities of glycan-protein interactions

As the glycan probe repertoire in the NGL microarray system has now been increased to include synthetic amino-terminating glycans, it allowed a more comprehensive study and better understanding of glycan-protein interactions than in the previous investigations using conventional NGL arrays as exemplified by the mammalian immune receptor Dectin-1 (Brown and Gordon, 2005; Palma et al., 2006) and the VP8* domain of rotavirus [P19] (Li et al., 2018; Liu et al., 2016).

Dectin-1 binding to β-glucans

Dectin-1 interaction with glucan polysaccharides is highly specific for backbone sequences of β1,3-linked glucose with a minimum chain length of DP10 (Palma et al., 2006, 2015). There has been evidence suggestive of the additional involvement of β1,6-glucosyl branching in Dectin-1 specificity (Adams et al., 2008), but there has not yet been direct binding data to support this, due mainly to the unavailability of sequence-defined long chain β1,3/1,6-branched gluco-oligosaccharides. Isolation and purification of branched long chain gluco-oligosaccharides from β-glucan polysaccharides has been difficult (Palma et al., 2006, 2015). Here, chemically synthesized, amino-terminating linear β1,3-gluco-oligosaccharides with DP12 and DP15 (#53 and #54, respectively) and a branched DP13 (#55) were used after their conversion into DA-NGLs and probed for Dectin-1 binding (**Figure 5A**). The results clearly showed that at these oligosaccharide chain lengths the strongest binding of Dectin-1 was to the linear β1,3-gluco-oligosaccharides, whereas the binding to the branched probe was markedly reduced. This unequivocally shows the chain length dependency and a negative influence of a β1,6-monoglucosyl branch closer to the non-reducing end on Dectin-1 binding.

Rotavirus P[19] VP8 binding to mucin O-glycan cores and blood group H type 1*

The rotavirus P[19] VP8* specificity towards glycans has been investigated earlier in independent studies and it has been shown toward both mucin *O*-glycan cores and the blood group H type 1 sequence (Liu et al., 2016, 2017; Sun et al., 2018). Although conventional NGL microarray screening analysis has given useful information on the specificity of this

VP8*, it was not possible to directly compare the binding of the P[19] VP8* to these different types of glycans as the Ser/Thr-terminating mucin cores could not be prepared as NGL probes. In the present DA-NGL microarrays (**Figure 5B**) containing both types of glycan probes, the P[19] VP8* bound predominantly to the mucin core 2 (#38, #39) and core 4 (#46, #47), which share the core structure (GlcNAcβ1-6GalNAcα-), but not to core 1 or core 3 providing evidence for the role of the β1,3-linked Gal and β1,6-GlcNAc for the interaction (Liu et al., 2016). In comparison, the LNFP-I pentasaccharide probe (#7), which presents the blood-group H type 1 sequence Fucα1-2Galβ1-3GlcNAcβ1-3Gal- bound by P[19] VP8* (Liu et al., 2016), elicited only a weak binding signal. The earlier observation of unpredicted binding of P[19] VP8* to β1,3-glucan sequences using conventional NGLs (Li et al., 2018) is also supported by the strong binding to probes #53–#55 in the DA-NGL microarray (**Figure 5B**).

Comparison of binding signals with the non-covalent NGL and covalent microarrays

A panel of 46 amino-terminating sugars used to prepare DA-NGLs, including the neutral, fucosylated and sialylated glycans, high-mannose and bi-antennary *N*-glycans, glucan oligosaccharides, and Ser/Thr-terminating mucin *O*-glycan cores, were selected for covalent printing onto NHS slides. These were assessed for comparison of the binding signals using the glycan-binding proteins analysed in the DA-NGL microarrays (**Table S1**, covalent/NGL chart positions #1-#46). As shown in **Figure 6** and **Figure S6A-N**, similar binding profiles were observed using the two types of microarrays. Among the eighteen proteins analysed, three showed identical binding profiles: P[19]VP8* (**Figure 6A**) anti-STn 3F1 (**Figure S6D**) and anti-H type 2 (**Figure S6I**), twelve gave similar binding patterns with some minor differences in terms of the probes bound or the binding intensity: mDectin-1 (**Figure 6B**), PNA, (**Figure 6C**), MGL (**Figure S6A**), VVL/VVA (**Figure S6B**), HPA (**Figure S6C**), mAb CTD110.6 (**Figure S6D**), UEA-1(**Figure S6H**), anti-H type 1 (**Figure S6J**), RCA-120 (**Figure S6K**), ConA (**Figure S6L**), *Cm*CBM6-2 (**Figure S6M**) and *Tm*CBM4-2 (**Figure S6N**). There were three that showed some major differences with respect to the glycan probes bound and signal intensity human Siglec-15 (**Figure 6D**), WGA (**Figure S6F**) and AAL (**Figure S6G**).

These findings are highlighted in the four selected typical examples shown in **Figure 6**. In all four examples, the binding specificities and binding signals detected are the same. Rotavirus [P19] VP8* showed very similar binding in both platforms in terms of both binding patterns and intensity values (**Figure 6A**). However, in some cases NGLs showed more intense signals, e.g. Dectin-1 binding to linear β1,3-gluco-oligosaccharides with DP12 and DP13 (#42 and #43, **Figure 6B**) and branched DP13 (#44), whereas in other cases covalent arrays exhibited binding to weaker binders that was only marginally detected in the DA-NGL arrays, e.g. PNA binding to 2,6-sialylated core 1 (#29, #30, **Figure 6C**). Human Siglec-15 is among the very few examples analysed showing a difference between the two platforms: in the covalent array, Siglec-15 showed a restricted binding profile to the sialylated milk sugars LSTb and DSLNT (#8, #9, **Figure 6D**) with weak binding to the branched DSMFLNH probe (#10), whereas in the NGL arrays exhibited similar binding to these three probes, in addition to the binding detected to α2,6-sialyl-Tn-Ser (#23) and α2,3-sialyl core 1-Thr (#28) (in this comparison Siglec-15 was tested as a non-pre-complex and in **Figure 4C** as a pre-complex with the detection antibody, which enhances the binding signal).

It is also interesting to note that in most cases NGL array showed 3-6 times more intense binding signals than the covalent arrays while the background of covalent arrays is generally lower than that of NGL array, although there are seven cases in which comparable binding intensities were observed in the two platforms.

DISCUSSION

With the new lipid reagent, we have demonstrated that the widely used synthetic amino-terminating glycans are well suited to the NGL-based microarrays and that the NGL probe library of one of the major international microarray resources can be much expanded in repertoire. Although limited in number, the selected glycan sequences analysed in this initial proof-of-concept study cover different glycan structural types, *e.g.* mucin-type *O*- glycan cores and *O*-GlcNAc linked to Ser or Thr, blood-group antigens and ganglioside-, *N*-glycan- and glucan-related sequences. The utility of the DA-NGL microarray was demonstrated by

the specific binding patterns obtained with plant and mammalian lectins, monoclonal antibodies and CBMs.

With the probe types extended to the short chain *O*-glycans with intact core sugars and the linked amino acid residues, we were able to broaden the knowledge on the specificities of glycan-binding proteins previously not available. We showed that PNA lectin, widely used in the detection of core 1 T-antigen, exhibits strong binding to the core 2 antigen and that the *O*-GlcNAc specific antibody CTD110.6 and *C. mixtus Cm*CBM6-2 are able to strongly interact with core 2 and core 4 antigens, through recognition of the terminal β-GlcNAc residues. In addition, here, and in a recent published study (Murugesan et al., 2020), we demonstrated that human Siglec-15 can bind to α2,3 and α2,6 sialylated glycan structures other than the tumour associated α2,6-sialyl-Tn. The additional glycan binding properties of Siglec-15 reported here were shown to involve the conserved essential Arg residue critical for interaction with sialic acid (Murugesan et al., 2020). The published study also showed that recognition of sialylated glycans on tumour cells by Siglec-15 can occur independently of sialyl-Tn antigen expression (Murugesan et al., 2020). Human Siglec-15 has recently gained research interest as its function is important for promoting a tumour immunosuppressive phenotype and tumour progression (Wang et al., 2019) and for osteoclast biology (MacAuley et al., 2014). Our results open the way to studies of the implication of recognition of sialyl glycans other than α2,6-sialyl-Tn antigen in the function of this Siglec.

The specific binding observed with the 3F1 mAb against the α2,6-sialyl-Tn *O*-glycan corroborates recent studies on the L2A5 antibody that is being developed for anti-cancer immunotherapy (Loureiro et al., 2018). Noteworthy, in our analysis of 3F1 and L2A5 antibodies, we observed a clear preference for the α2,6-sialyl-Tn glycan in Ser over Thr. The preference for Ser or Thr *O*-glycans has been reported for proteins targeting the Tn *O*-glycan antigen: while some anti-Tn antibodies (Coelho et al., 2015) and HPA lectin (Madariaga et al., 2014) prefer Tn-Ser, others such as anti-MUC1 antibodies (Martínez-Sáez et al., 2015) and VVL (Madariaga et al., 2014) have a higher affinity for Tn-Thr. Indeed our microarray data also show a preference of VVL for Tn-Thr. Published structural data showed that the Tn-Ser and Tn-Thr structures adopt different conformations in solution and in the protein-bound state, allowing to establish specific glycan and water-mediated interactions with the protein-

binding site (Bermejo et al., 2018). Our results further support the hypothesis that the preference of binding to the Ser or Thr structures may add to the specificity of a given glycan-binding protein and have biological significance in the molecular recognition of natural *O*-glycans.

The ability to prepare NGLs from the amino-terminating glycans made it possible for the very same glycan molecules to be used in the two major platforms. This is important for the comparison of the two platforms as this eliminates variables that may influence the binding results (Wang et al., 2014), *e.g.* the origin, the quality and quantity of glycan molecules used in different arrays. The data presented here showed, for the first time, that two of the major glycan microarray platforms and employed by the international resources give similar binding profiles with the different glycan binding proteins analysed.

Dectin-1 is the major receptor for β-glucans on macrophages. The recognition of β-glucans promotes oligomerization of the receptor at the cell surface and mediates cell signalling in the immune cell response to several fungal species (Plato et al., 2013). The interaction of Dectin-1 with β-glucans and the consequent cellular effects are thought to be dependent on the linkage, size and branching (Adams et al., 2008; Marakalala et al., 2011). Although the specificity for the β1,3-linkage and the chain length requirement for Dectin-1 binding have been well accepted using glucan-derived oligosaccharides, the influence of β1,6-branching of the glucan chain has needed corroboration (Adams et al., 2008; Palma et al., 2015). The microarray analysis reported here have enabled direct comparison of glucan-derived linear and chemically synthesized β1,6-branched gluco-oligosaccharides with identical backbone; our results show that a β1,6-linked glucose positioned at the non-reducing penultimate glucose has a damping effect on Dectin-1 binding of. The mono-glucosyl branching at this position likely interferes with the presentation of the hypothesized helical conformational epitope formed by the β1,3-linked glucose chain recognised by Dectin-1. It will be important to investigate the influence of other β1,6-branches on short and long β1,3-linked glucose backbone chains. This will lead to a better understanding of the molecular basis of the recognition of fungi by Dectin-1.

Rotaviruses comprise a genotypically variable family of viruses that cause severe gastroenteritis in human and animals and use glycans as receptors for infection. The

recognition of glycans by rotaviruses in a genotype-dependent manner is via the distal VP8* head of the spike protein VP4. For P[19] genotype, the glycan specificity of VP8* has been assigned to mucin *O*-glycan cores (particularly core 2) and H type 1 histo-blood group antigen (HBGA) precursors using different types of microarrays (Li et al., 2018; Liu et al., 2016). Here, we were able to compare directly the binding of P[19] VP8* to these types of antigens prepared as probes using the same lipid reagent for presentation on the microarray. The predominant binding was observed to mucin cores 2 and 4, with only weak binding to LNFP I presenting the H type 1 HBGA trisaccharide epitope (Fucα1-2Galβ1-3GlcNAcβ1-) required for binding. The results are in accord with solution NMR data (Liu et al., 2016) and also evidenced from earlier glycan microarray data (Liu et al., 2016; Sun et al., 2018). This evidence poses important questions on the functional significance of the preferential binding to the mucin core *O*-glycans for viral pathogenesis compared with H HBGAs. P[19] rotavirus commonly infect animals (porcine) and only sporadically humans. It is postulated that the P[19] genotype may represent an early evolutionary stage that started adapting to human receptors but retaining the binding specificities to the short chain mucin cores 2 and 4, and also H type 1 HBGAs. A more recent study using microarrays of *O*-glycans isolated from a porcine mucin by the beam search strategy (Li et al., 2018) showed a more potent P[19] VP8* binding to the extended H type 1 chain (Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-?Gal-). The reported crystal structures of the P[19] VP8* in complex with LNFP-I and core 2-Thr showed that both ligands are bound in a similar way but that the binding site is able to accommodate an extended glycan chain (Liu et al., 2017). Thus, further studies with structurally diverse elongated mucin *O*-glycans with intact cores and HBGA sequences are required to clearly answer VP8* specificity for P[19] infection and rotavirus evolution. The observation that P[19] VP8* also exhibits binding to non-mammalian β1,3-glucan sequences is consistent with previous microarray data (Li et al., 2018). These sequences are typical and highly abundant in fungal cell walls and act as pathogen-associated molecular recognition patterns (PAMPs) (Brown and Gordon, 2005). The glycan microarray data may be the first indication of interactions of enteric viruses with fungi within the intestinal microbiome, which deserves further investigation.

In summary, this study opens the way to extensively broaden the repertoire of glycan libraries. With the overall similarity of the glycan binding patterns in the two major platforms used by international resources, glycan microarrays will continue to play important roles in detection of glycan-protein interactions and provide new insights into glycan recognition systems.

SIGNIFICANCE

The specific interaction of proteins with glycans governs essential biological and physiological processes occurring within an organism. Glycan microarrays have become essential methods to study glycan recognition by proteins and have found wide applications in biology and biomedicine. However, there is an urgent need in microarray development to expand the libraries of sequence-defined glycan probes to cover the major part of glycan structures within glycomes in order to better define glycan-protein interactions. Among many microarray systems, covalent and non-covalent glycan immobilization are the two different principles, represented by the major international microarray resources, using mainly amino-terminating synthetic glycans covalently immobilized on NHS-functionalized slides or naturally-derived aldehyde-terminating glycans, after their conversion into neoglycolipid probes, non-covalently immobilized on nitrocellulose-coated slides. With the NGL array as the example, we demonstrate the benefit of combined chemical strategies taking both advantages of glycans of aldehyde-terminating from natural glycomes and amino-terminating by synthetic means to increase size and diversity of glycan probe libraries. We were able to use the very same glycans in both covalent and non-covalent platforms enabling a comparison of their performance. The analyses in the two types of microarrays demonstrate similar binding profiles with different classes of glycan-binding proteins and provide knowledge on the unique specificity of the immune-receptor Dectin-1 towards β-glucans and core O-glycan recognition by the adhesive protein VP8* of the rotavirus P[19]. The method can now be applied to expand the glycan library coverage in non-covalent microarrays by incorporating probes conventionally listed in the covalent arrays. This will provide better opportunities to decipher glycan recognition systems with implications in understanding

cellular mechanisms in health and disease.

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AUTHOR CONTRIBUTIONS

W.C. conceived the project. C.L. designed the chemical strategies, and A.S.P. designed and performed microarray binding experiments and carried out data analysis. P.Z. carried out the synthesis of the novel lipid reagents and related glycan standards. C.G., Z.L. and F.T. performed microarray binding experiments and contributed to data analysis. W.C. and Y.Z. carried out analysis of glycan probes and L.M.S. performed microarray printing. M.W., P.H.S., L.M.L., V.P., J.Y., U.W. contributed to the key glycan probes. W.C., A.S.P., and C.L. wrote the paper and P.Z., J.Y. and U.W. wrote the synthetic part. All co-authors edited and approved the manuscript.

FIGURE LEGENDS

Figure 1. Reaction schemes of novel lipid reagents for NGL preparation from amino-terminating sugars.

Figure 2. HPTLC analysis of DA-NGL products. (A) Multiple products formed from Glc-C2-NH2. (B) Reaction products from Lac-C2-NH2 using MeOH, EtOH, DMSO and DMF as the solvent. (C) Reaction products from methylamino-terminating Glc-C2-NHMe and Lac-C2-NHMe using MeOH and EtOH as the solvent. DA1, single lipid conjugation product; DA2, double lipid conjugation product; DA1+Me methylated single lipid conjugation product.

Figure 3. Comparison of the binding signal intensities of NGL products with single (DA1) or double (DA2) lipid chains immobilised non-covalently on nitrocellulose-coated slides. The probes are arranged according to their backbone-sequence type: lactose and *N*-acetyl lactosamine (Lac/LN), lacto-*N*-tetraose and lacto-*N*-neo-tetraose (LNT/LNnT), glycolipid and *O*-glycan core. The glycan sequence of probes eliciting binding signals are annotated (a more comprehensive comparison is shown as a heatmap, **Figure S4**). The representation of glycans follows the guidelines of Symbol Nomenclature for Glycans(Varki et al., 2015). The binding signals are means of fluorescence intensities of duplicate spots at 5 fmol of probe arrayed (with error bars) and are representative of at least two independent experiments. Binding signals are in red for the DA1 and blue for the DA2 conjugation products, respectively. The chart position assigned to each probe is referenced in **Table S1** (NGL Chart Pos.).

Figure 4. Glycan DA-NGL microarray validation using sequence-specific proteins. The microarray was probed with (A-C) lectins, (D-E) monoclonal antibodies, and (F-G) carbohydrate binding modules (CBMs) of bacterial glycoside hydrolases (See also **Figure S5** for additional analysis). The probes are arranged according to their backbone-sequence type as indicated in the colored panels: lactose and *N*-acetyl lactosamine (Lac/LN), lacto-*N*-

tetraose and lacto-*N*-neo-tetraose (LNT/LNnT), poly *N*-acetyllactosamine (PolyLN), *N*-glycans, glycolipid, *O*-glycan core, glycosaminoglycans (GAGs), glucose and *N*-acetylglucosamine homo-oligomers (Glc_n/$GlcNAc_n$), and monosaccharides (Mono). The representation of glycans follows the guidelines of Symbol Nomenclature for Glycans(Varki et al., 2015). The binding signals are means of fluorescence intensities of duplicate spots at 5 fmol of probe arrayed (with error bars) and are representative of at least two independent experiments. The chart position assigned to each probe is referenced in **Table S1** (NGL Chart Pos).

Figure 5. Application of DA-NGL microarray to derive specificities of glycan-protein interactions. (A) Murine Dectin-1; (B) Rotavirus [P19] VP8*. The probes are arranged according to their backbone-sequence type as in **Figure 4**. The glycan sequence of probes eliciting binding signals are annotated. The representation of glycans follows the guidelines of Symbol Nomenclature for Glycans (Varki et al., 2015). The binding signals are means of fluorescence intensities of duplicate spots at 5 fmol of probe arrayed (with error bars) and are representative of at least two independent experiments. The chart position assigned to each probe is referenced in **Table S1** (NGL Chart Pos).

Figure 6. Comparison of non-covalent NGL and covalent microarrays. The comparison is illustrated by four typical examples to highlight the binding by (A) rotavirus [P19]VP8*; (B) murine Dectin-1; (C) PNA; and (D) human Siglec-15 (See also **Figure S6A-N** for a more comprehensive comparison).The probes are arranged according to their backbone-sequence type as in **Figure 4 and 5**. The glycan sequence of probes eliciting binding signals are annotated. The representation of glycans follows the guidelines of Symbol Nomenclature for Glycans(Varki et al., 2015). The chart position assigned to each probe is referenced in **Table S1** (Covalent/NGL Chart Pos).

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