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Three-Dimensional Arrays Using GlycoPEG Tags: Glycan Synthesis, Purification and Immobilisation**

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Abstract: Glycan arrays have become the premier tool for rapidly establishing the binding or substrate specificities of lectins and carbohydrate-processing enzymes. New approaches for accelerating carbohydrate synthesis to address the enormous complexity of natural glycan structures are necessary. Moreover, optimising glycan immobilisation is key for the development of selective, sensitive and reproducible array-based assays. We present a tag-based approach that accelerates the preparation of glycan arrays on all levels by improving the synthesis, the purification and immobilisation of oligosaccharides. Glycan primers were chemically attached to bifunctional polyethyleneglycol (PEG) tags, extended enzymatically with the help of recombinant glycosyltransferases and finally purified by ultrafiltration. When printed directly onto activated glass slides, these glycoPEG tags afforded arrays with exceptionally high sensitivity, low background and excellent spot morphology. Likewise, the conjugation of glycoPEG tags to latex nanoparticles yielded multivalent scaffolds for carbohydrate-binding assays with very low non-specific binding.

Keywords: carbohydrates · enzymes · glycan arrays · nanoparticles · oligosaccharides

Introduction

Ten years ago, several groups introduced the first glycan arrays as a new and promising tool for studying the binding and substrate specificities of carbohydrate-binding proteins and carbohydrate-processing enzymes with unprecedented throughput and minimal sample requirements.[1–6] Although much progress has been made since then, with some arrays that present over 600 glycan ligands[7,8] having been constructed, current formats still cover only a very small fraction of the estimated 10000–30000 biologically relevant glycan epitopes.[8,9] This is in large part due to the notorious difficulties in oligosaccharide synthesis or their isolation from natural sources. To prepare next-generation large glycan arrays that accommodate far higher numbers of ligands, new technologies are required that can accelerate carbohydrate synthesis or provide solutions for the high-throughput isolation, tagging and analysis of glycans from natural sources. (Automated) chemo-enzymatic solid or tagged solution-phase strategies are particularly promising for the rapid synthesis of oligosaccharides either directly on the chip or in solution with the help of solid and soluble supports.[10–13] Oligosaccharides assembled on a solid support usually have to be cleaved off the resin, purified and deprotected before they can be printed onto microarray slides.[14] This adds time-consuming steps to any automated solid-phase oligosaccharide synthesis. Perfluorinated linkers and protecting groups in combination with solid-phase extraction have been employed for some time in the tagged solution-phase synthesis of oligosaccharides and used as linkers for the noncovalent immobilisation of glycans on Teflon-coated microarray slides.[15] The low solubility of perfluorinated compounds (PFCs) and their conjugates in aqueous solutions,[16] however, has limited their synthetic use in organic-solvent-based chemical synthesis and their generally low biodegradability is now a major environmental concern, thereby threatening the future commercialisation of fluorous linkers.[17] In addition, the non-covalent attachment of glycoconjugates is limited to specially prepared perfluorinated surfaces.

We were interested in developing an environmentally friendly, tag-based method for the unified chemo-enzymatic synthesis, purification and immobilisation of glycans or other biomolecules on microarrays or other scaffolds. The chosen tag should be stable under harsh synthetic conditions, allow easy product purification and provide a covalent attachment point with uniform orientation for all ligands and low background in protein-binding assays.

Methylated polyethylene glycol (MPEG) is soluble both in water and many organic solvents and has been used as a

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[**] GlycoPEG = Glycan primers chemically attached to bifunctional polyethyleneglycol (PEG) tags.

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soluble support for solid-phase synthesis of oligosaccharides, -nucleotides and -peptides.[10,18,19] Reactions are carried out under homogeneous solution-phase conditions and intermediates are readily analysed in the presence of the polymer by NMR spectroscopy or mass spectrometry. The addition of ethanol or ether renders the PEG tag insoluble so reagents or other reaction impurities can be removed by simple filtration. Alternatively, membrane-based ultrafiltration can purify the tagged compounds in a single step by size exclusion, an especially important feature for microscale operations.[20] Furthermore, PEG tags are ideal spacers for covalently attaching biomolecules to surfaces at a tuned distance, which helps to improve receptor and enzyme accessibility, and their well-known protein-repelling properties result in arrays with exceptionally low background.[21]

Results and Discussion

These clear advantages of polyethylene glycol polymers prompted us to evaluate bifunctional PEG tags **1** and **2** (Figure 1) in the tag-supported chemo-enzymatic synthesis of oligosaccharides as well as a protein-repelling spacer for the direct attachment of glycans onto microarrays.

The use of glycosyltransferases with well-known substrate specificities avoids the use of protecting groups and compensates the lack of universal chemical methods for stereoselective formation of glycosidic bonds, the current limitations of chemical oligosaccharide synthesis. It also justifies the use of MALDI-TOF MS as the single method for the analysis of end products, an important feature for microscale chemistry. Not surprisingly, then, solid-supported enzymatic oligosaccharide synthesis has been investigated by a number of groups over the years with a strong focus on the type of solid support used for attaching either the enzyme or the growing sugar chain.[22–33] Generally, good to excellent coupling yields were achieved when soluble polymers[20,26,34] were used or longer oligoethylene-type linkers attached to the solid support.[22] Other more rigid polymers were less accessible for the enzymes and hence provided lower coupling yields.[24–26] More recent strategies that employed pH-[28] or thermoresponsive[35] polymers rendered the tagged reaction products insoluble after a specific stimulus. Enzymes and co-factors were then removed by simple filtration. In a related recent study, ionic liquid tags were applied to monitor the action of glycosyltransferases on several substrates by liquid chromatography (LC)-MS.[36] These techniques, however, are prone to difficulties when employed in microscale reactions.

Essential to our strategy, which is schematically depicted in Figure 1, was the efficient immobilisation of glycans tagged with a large PEG chain (approximately 5000 Da) by robotic spotting.

As a proof of concept, PEG-tagged mannose **3** and *N*-acetylglucosamine (GlcNAc) **4** were prepared (Scheme 1). The carbobenzyloxy (Cbz)-protected hetero-bifunctional polymer **1** was conjugated with trichloroacetimidates **24** and **25** to furnish conjugates **26** and **27**, respectively. Stepwise deprotection of glycoside **26** by aminolysis of the phthalimido group, acetylation and Birch reduction followed by rapid pu-

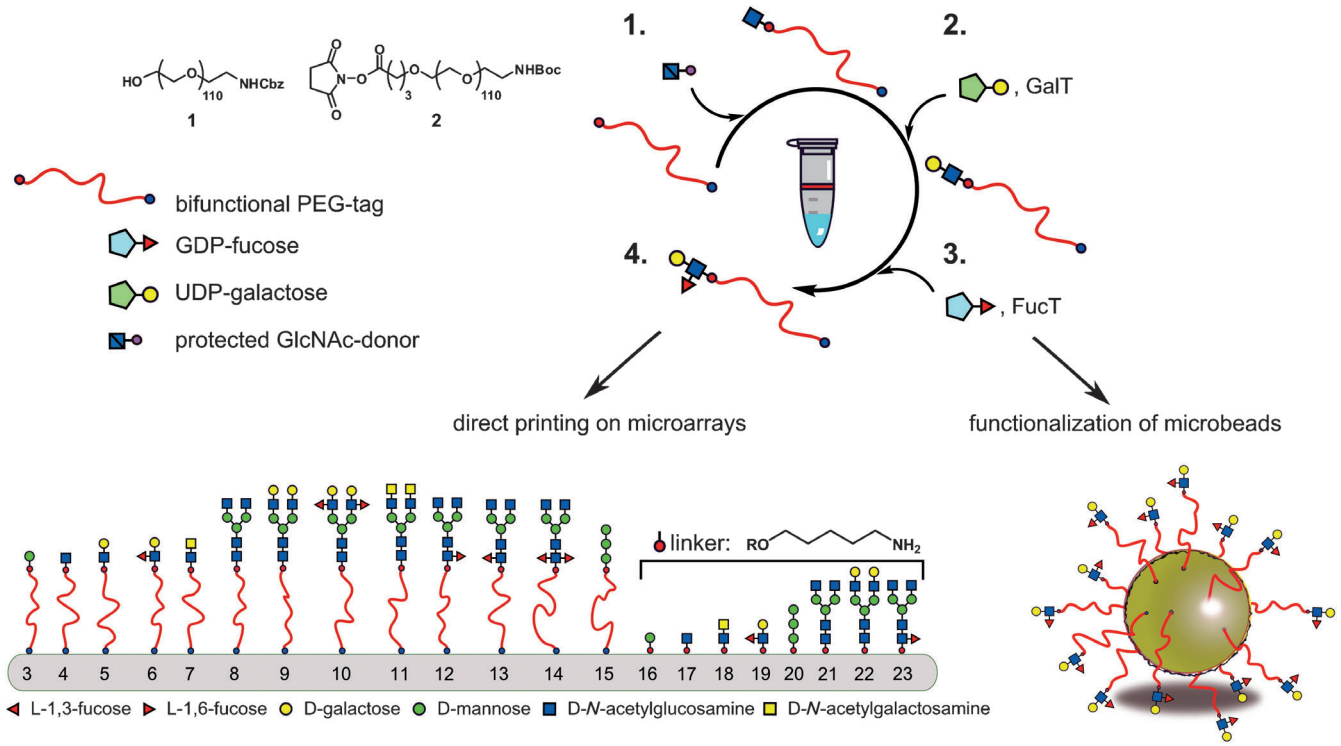
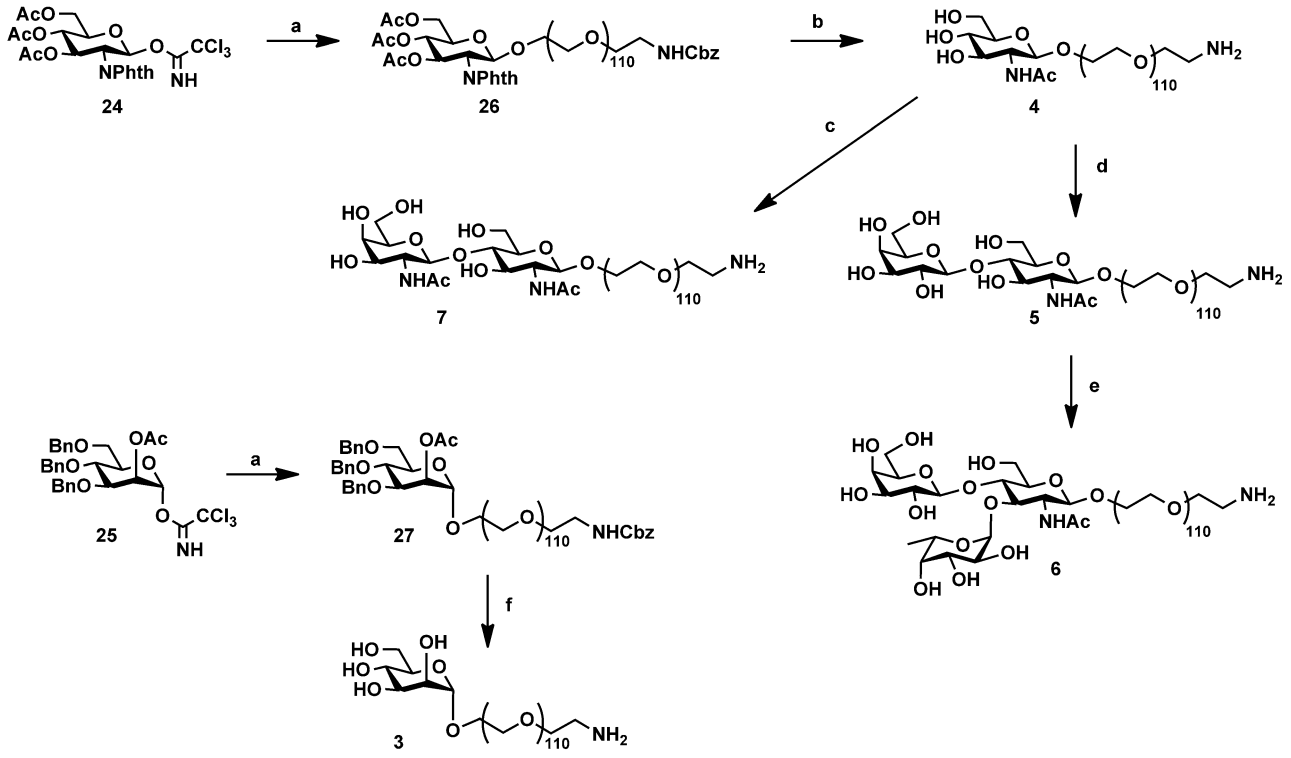


Figure 1. PEG-tag-supported synthesis, purification and immobilisation of glycans for 3D microarrays and functionalised microbeads.



Scheme 1. Examples of the chemo-enzymatic synthesis of glycoPEGtags: a) Compound **1**, TMSOTf, CH_2Cl_2; b) 1) NH_2EtNH_2, *n*BuOH, MW, 2) Ac_2O, pyridine; 3) Na, NH_3,THF, −78 °C; c) UDP-GalNAc, *C. elegans* β-1,4-GalNAcT, BSA, $MnCl_2$, alkaline phosphatase (EC 3.1.3.1) in cacodylate buffer 80 mM, pH 7.4, 25 °C, 18 h; d) β-1,4-GalT, UDP-Gal, $MnCl_2$, HEPES 50 mM, pH 7.4, 37 °C; e) *C. elegans* α-1,3-fucosyltransferase, GDP-Fuc, $MnCl_2$, MES 80 mM, pH 6.5, RT; f) Na, NH_3,THF, −78 °C.

rification through precipitation afforded PEG-tagged GlcNAc **4**. PEG-tagged mannose **3** was obtained after single-step Birch reduction of conjugate **27**. The deprotection and PEG-tag-based purification of both glycoconjugates proceeded with excellent conversion as confirmed by MALDI-MS analysis of the final products (**3** and **4**).

Both constructs were then robotically printed onto an *N*-hydroxysuccinimide (NHS)-activated glass slide at concentrations between 50 and 500 μM and non-printed areas passivated with a shorter hetero-bifunctional amino-PEG derivative (average mass 600 Da). Incubation of the slide with fluorescently tagged α-mannose binding lectin Concanavalin A (ConA) and a glucosamine binding lectin from *Bandeira simplicifolia* (BS II) clearly demonstrated the successful attachment of the large PEG glycoconjugates to the activated surface with excellent spot-to-spot reproducibility and homogeneity (see Figure S32 in the Supporting Information).

Once PEG-tagged sugars had proven to be excellent ligands for the robotic printing of glycan arrays, we next investigated their enzymatic extension by exploiting the large bifunctional PEG tag (5 kD) for rapid purification. Conjugated to the large soluble tag, glycans can be handled on the low microgram scale with great ease by employing standard biochemistry laboratory equipment. PEG-tagged GlcNAc **4** (300 μg, 15 μg of sugar) was incubated with a bovine milk β-1,4-galactosyltransferase and uridine 5′-diphosphogalactose disodium salt (UDP-galactose) for 20 h. The enzyme was precipitated, and the PEG-tagged *N*-acetyl lactosamine **5** was purified by simple ultrafiltration by taking advantage of the large 5 kD PEG tag for the separation from excess amounts of nucleotide donor and buffer salts[20] (Scheme 1). The tedious chromatographic purification steps required in the traditional solution-phase enzymatic oligosaccharide synthesis were thus avoided. MALDI-TOF MS analysis, which did not require more than 0.5 μg aliquot of sugar–PEG conjugate, showed complete reaction to the PEG-tagged *N*-acetyl lactosamine **5** by a 162 Da shift of the peak pattern[37] (see Figure S25 in the Supporting Information).

Subsequent incubation with a recombinant α-1,3-fucosyltransferase from *Caenorhabditis elegans* produced cleanly the Lewis X trisaccharide polymer conjugate **6**, which was purified by filtration from enzyme and excess amount of nucleotide donor. Treatment of the PEG-tagged GlcNAc primer **4** with GalNAc transferase from *C. elegans* and UDP-GalNAc as cofactor produced the PEG-tagged GalNAcβ1→4GlcNAc disaccharide **7** (Scheme 1).

To demonstrate the potential of PEG-supported microscale enzymatic oligosaccharide synthesis for the preparation of glycan arrays, we envisaged the formation of a small library of *N*-glycans (**8**–**14**) with variations in terminal sugars and core modification starting from a single *N*-glycan core structure (Figure 2). Towards this end we coupled biantennary C5-amino-linked *N*-glycan **21**[38] (1 mg; Figure 1) to the NHS-activated polymer **2** in nearly quantitative yield

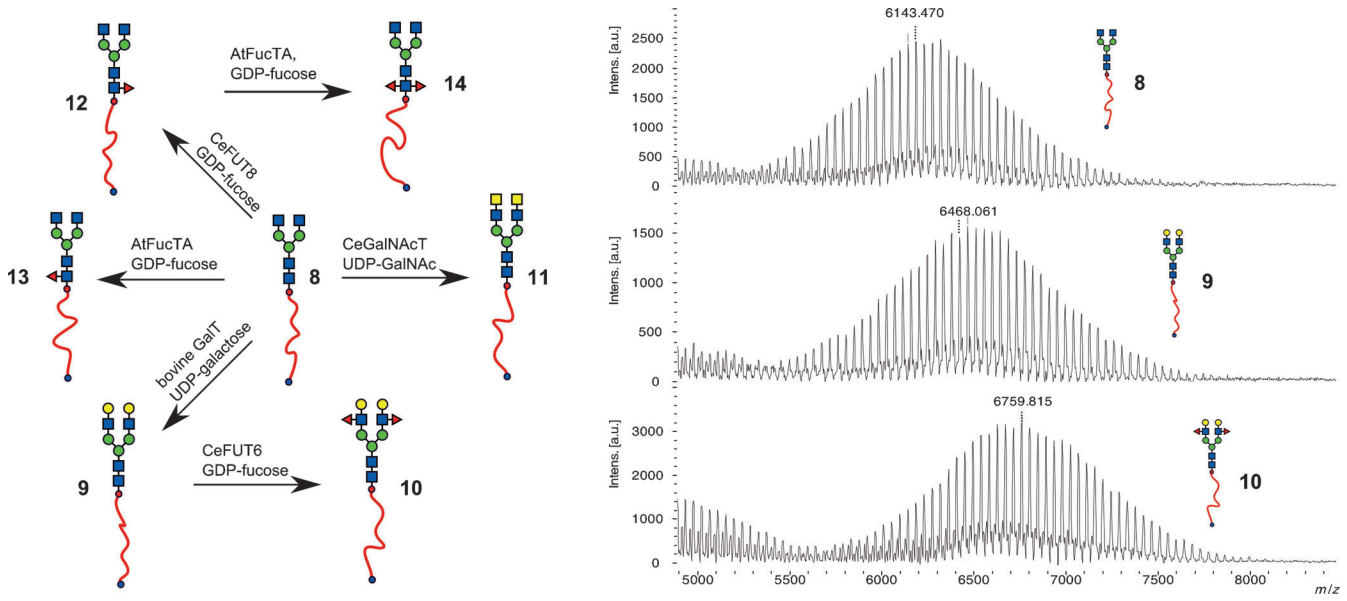


Figure 2. Enzymatic diversification of PEGylated bi-antennary glycan **8** and MALDI-TOF MS of selected compounds **8**, **9** and **10**.

and removed the *tert*-butyloxycarbonyl (Boc) protection to obtain PEG-tagged sugar **8** as the scaffold for the enzymatic library synthesis (see Figure S23 and Scheme S6 in the Supporting Information). Aliquots of sugar–polymer (200–300 μg) were dissolved in buffer and incubated with six recombinant glycosyltransferases to produce PEG-tagged glycans **8**–**14** by enzymatic soluble-polymer-supported synthesis in only a few days.

Incubation of **8** with GalT and UDP galactose gave rise to bis-galactosylated conjugate **9**, which was further elongated with a Lewis-type α-1,3-fucosyltransferase from *C. elegans* (CeFUT6) to produce conjugate **10**. Treatment of **8** with a GalNAc-transferase from *C. elegans* and UDP-GalNAc as the nucleotide donor furnished quantitatively **11** with two terminal GalNAc residues. The α-1,6- and α-1,3-core-fucosylated conjugates **12** and **13** were accessible by employing two recombinant fucosyltransferases from *Arabidopsis thaliana* (AtFucTA) and *C. elegans* (CeFUT8), respectively. Sequential incubation of **8** with both enzymes furnished the bis-fucosylated conjugate **14**[39] (Figure 2).

Thus, by means of PEG-tag-supported enzymatic synthesis, we were able to produce seven different *N*-glycan conjugates in a few days from a single compound. The soluble tag allowed a rapid purification of the glycoconjugate by filtration of the precipitated enzyme and then removal of low-molecular-weight impurities on a standard ultrafiltration device.[40] PEGylated trisaccharide **15** obtained by coupling the trimannoside **20** with linker **2** (Figure 1) followed by Boc deprotection was included as a larger mannoside for the lectin-binding studies into the ligand base.

All PEG-tagged glycans **3**–**15** and C5-amino-linked glycans **16**–**23** (see Figure 1) were then arrayed at 200, 100, 50 and 25 μM concentrations onto NHS-activated slides for a comparison of linker length and printing concentration on the sensitivity and spot homogeneity. Individual subarrays that contained all ligands were incubated with the fluorescently marked lectins ConA, *Ricinus communis* agglutinin (RCA), *Aleuria aurantia* lectin (AAL), *Wisteria floribunda* lectin (WFL), *Vicia villosa* lectin (VVL), wheat germ agglutinin (WGA), *Erythrina cristagalli* lectin (ECA), *Lens culinaris* agglutinin (LCA), *Galanthus nivalis* agglutinin (GNA), *Narcissus pseudonarcissus* lectin (NPL), BS II and an antibody against horseradish peroxidase (anti-HRP); the fluorescence intensity was quantified and used as a measure for the lectin–ligand binding strength (Figure 3).

Any differences in the immobilisation between ligands **3**–**15** were compensated by the large PEG tag and, in general, well-defined intense spots with excellent homogeneity were obtained for all printed samples at all concentrations. C5-amino-linked printed glycans **16**–**23** gave smaller and partially misaligned spots with a concentration- and ligand-dependent variation in size.

In general, all lectins bound with the expected selectivity but usually with stronger bonding to the PEGylated glycans (**3**–**15**) than to their respective C5-amino-linked homologues (**16**–**23**), which suggests a pronounced effect of the PEG polymers on the ligand presentation. Monosaccharides and some smaller glycan fragments were bound in their PEGylated form only, thus highlighting the need for a longer spacer to access the surface-bound glycans.

More precisely, PEGylated GlcNAc **4** bound to BS II, a terminal GlcNAc-recognising lectin, with considerable strength down to 50 μM spotting concentration, whereas the C5-linked analogue **17** was not bound at all. PEGylated GlcNAc-terminating *N*-glycans **8** and **12** bound nearly twice as strong to BS II than C5-linked congeners **21** and **23**, thus showing that the effect was preserved for more complex structures as well (Figure 3a). Similar results were obtained for the terminal galactose binding lectins ECA and RCA. PEGylated *N*-acetyl lactosamine **5** and GalNAcβ1→

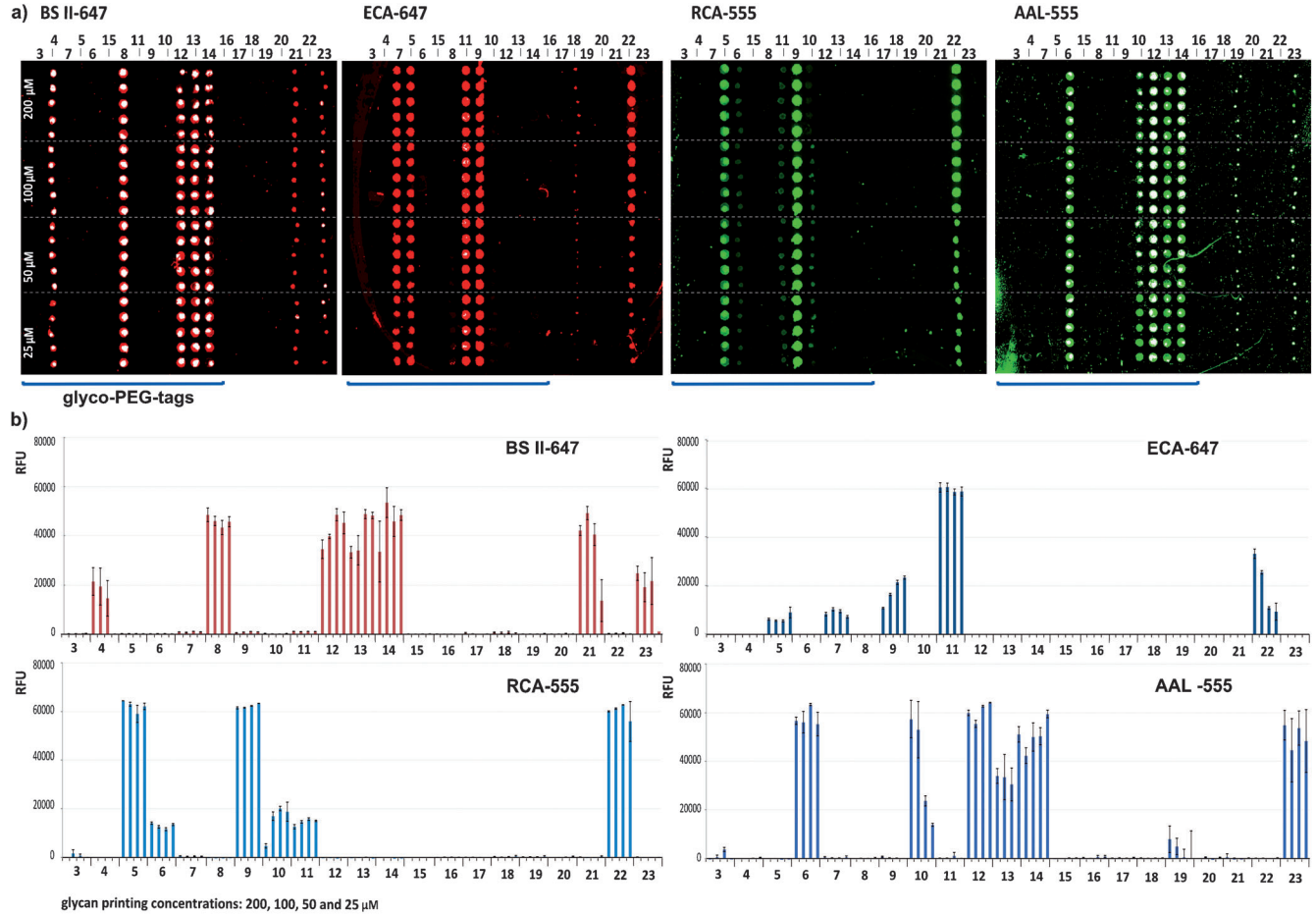


Figure 3. Comparison of immobilised glyco-PEG tags (**3**–**15**) and C5-linked glycans (**16**–**23**) in lectin binding assays. a) Fluorescence scans for arrays incubated with BS II, ECA, RCA and AAL. b) Histogram view of binding data at four different ligand printing concentrations.

4GlcNAc **7** bound to ECA with medium intensity at all spotting concentrations, whereas almost no interaction was detected for disaccharide **18** immobilised through the short C5 linker. RCA bound strongly to both PEGylated galactosylated bi-antennary glycan **9** and its C5-linked homologue **22**, independent of the linker used. Reduced but still significant binding was observed in the presence of α-1,3-fucose present in glycans **6** and **10**; the C5-linked homologue, however, showed no measurable interaction with the lectin (Figure 3).

As an example of the fucose binding probes tested, AAL showed the known broad specificity including core α-1,3- and core α-1,6-fucosylation and Lewis-type fucosylation present on the array. Significantly, AAL binding to the Le X epitope was only observed for its PEGylated form **6** with fluorescence values approaching saturation. Interestingly, AAL binding to the 1,6-core-fucosylated bi-antennary C5-linked glycan (**19**) remained strong, which suggests that other epitopes apart from the core fucose are recognised by the lectin.

Pronounced differences in binding to PEGylated and non-PEGylated glycans were also observed for the other lectins tested (see Figures S34 and S35 in the Supporting Information). WGA showed strong binding at all printing concentrations for PEGylated glycans that present terminal GlcNAc and chitobiose, as in *N*-glycans **8**–**14** (see the Supporting Information). Fucosylation and specifically core fucosylation decreased binding to WGA significantly.[41] Single GlcNAc was only recognised in its PEGylated form **4** and fluorescence values for WGA binding to PEGylated GalNAcβ1→4GlcNAc (LacdiNAc; **7**) reached saturation levels, whereas binding to the C5-linked homologue **18** was moderate. Anti-HRP is a highly specific probe for core α-1,3-fucose, which was confirmed by binding exclusively to mono- and bis-fucosylated glycans **13** and **14** with very good signal intensity at all spotting concentrations. The higher specificity of WFL for GalNAc residues was reflected in a stronger binding to the two PEGylated ligands **7** and **11** (see the Supporting Information).

To determine the limit of detection for the immobilised glycoPEG tags, we printed a dilution series of the Le X–PEG conjugate **6** (Figure 4a) and LacdiNAc disaccharide **7** (Figure 4b). Both arrays were incubated with fucose binding lectin from *Aleuria aurantia* and GalNAc binding *Wisteria floribunda lectin* at a nanomolar concentration. The interaction of AAL with ligand **6** could be detected down to 25 attomole of printed glycan at a S/N > 5. (Figure 4a). This sen-

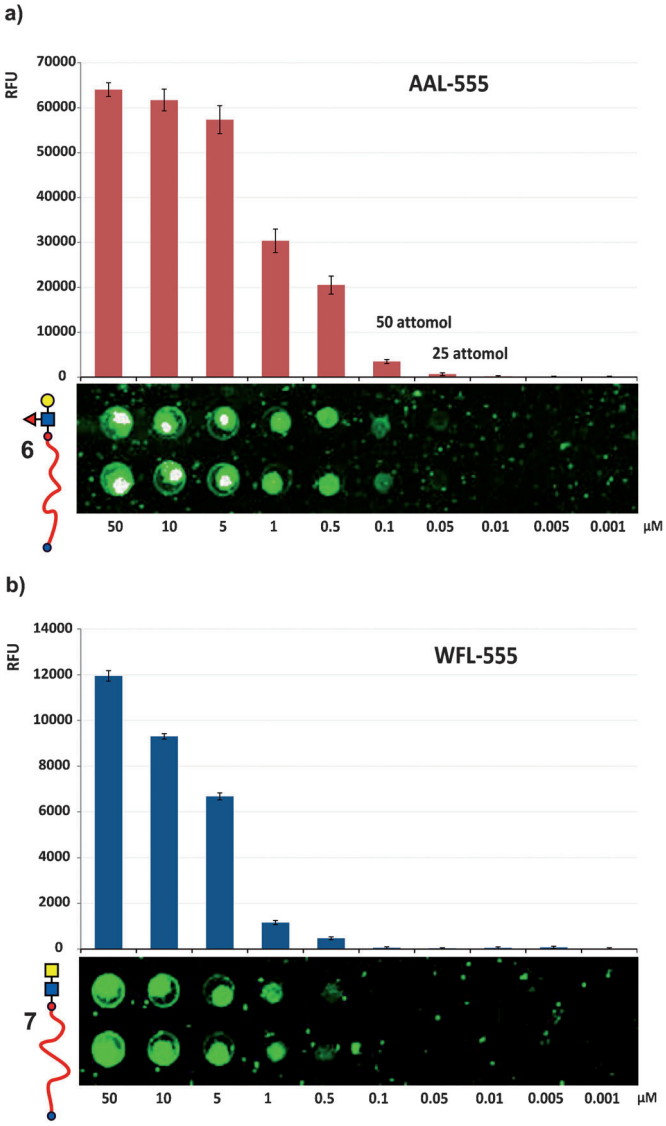


Figure 4. Assay sensitivity measured for binding of a) AAL to Le X-PEG tag and b) WFL to LacDiNAc-PEG tag at different spotting concentrations.

sitivity is comparable to reported values for the mannose/ConA ligand receptor pair studied on three-dimensional hydrogel slides, which are frequently used in glycan array research and are considered the gold standard.[42]

The above results clearly show that printed PEG-tagged glycans outperformed the smaller linkers in spot morphology and sensitivity for all lectins studied. The random coil conformation of large PEG chains upon immobilisation ("entangled mushroom-like PEG", 25% polymer extension)[43] most probably leads to a reduced ligand density for the PEGylated glycans relative to the C5-tagged glycans with a smaller footprint.[43] The lower sugar content per area, however, is more than compensated by the excellent accessibility of proteins to the binding epitope,[44] most clearly demonstrated by the exclusive binding of PEG-tagged small glycans **3**–**7**. Relative to other reported microarray formats in which ligands are attached by means of short linkers directly onto a flat Teflon or metal oxide surface,[17,45] the immobilisation of glycans through long PEG tags reaches a sensitivity otherwise only achieved by employing slide surfaces functionalised with a 3D hydrogel matrix.[42]

Although glycan arrays are valuable surface-based assays for the high-throughput screening of weak carbohydrate–protein interactions, other applications like suspension assays, cell labelling, flow cytometry, immuno-separation of biomolecules or in vivo imaging require spherical 3D scaffolds like functionalised nanoparticles or microbeads. We functionalised aliquots of fluorescent 300 nm-sized latex beads with equimolar amounts of either glycoPEG tags (**3**, **5**, **6, 7, 12** and **15)** or the corresponding C5-linked glycans (**16, 19**–**21**) to study the influence of the PEG spacer on the binding to a lectin array that comprised ConA, WGA, RCA, SNA, MAL-1, AAL, PSA, LCA, WFL, VVL and control buffer (Figure 5). Specific binding of beads to the lectin-functionalised wells could be visualised by scanning electron microscopy (SEM) after sputter-coating the slide with a thin layer of gold. At 200-fold higher resolution than scanometric fluorescence measurements, single beads can be visualised and the images obtained can be used for quantification of binding interactions.[46] Specific carbohydrate–carbohydrate interaction between Le X-functionalised beads could be the cause for the observed clustering and multilayer stacking of beads (Figure 5b).[47]

PEGylated Le X beads bound strongly only to RCA and fucose binding lectin AAL; beads functionalised with PEGylated mannose **3** selectively bound to ConA, whereas PEG-tagged trimannoside **15** beads also bound to mannose binding lectins PSA and LCA. Beads functionalised with C5-linked glycans, however, did not bind to the lectin array in a selective manner unless a tenfold-higher glycan concentration was used for their preparation (see the Supporting Information). Beads functionalised with C5-linked glycans **3** and **15** at tenfold-higher concentration bound to ConA, PSA and LCA with similar intensity but also showed non-specific binding to AAL, WGA and RCA.

Beads functionalised with PEGylated GalNAcβ1→4GlcNAc **7** bound with high affinity and excellent selectivity to RCA, WGA, ECA and WFL. PEGylated *N*-acetyl lactosamine beads **5** bound exclusively to RCA, whereas the α-1,6-core-fucosylated bi-antennary glycoPEG tag **12** showed a strong interaction with WGA, AAL, PSA, LCA and, to a lower degree, ConA lectins. These examples show that glycoPEG tags provide superior sensitivity and selectivity also in bead-based assays with interesting prospects for array-based studies of weak carbohydrate–carbohydrate interactions.

Conclusion

We have shown that bifunctional PEG tags provide a versatile handle that can streamline the preparation of glycan arrays and glycobeads from chemo-enzymatic synthesis and

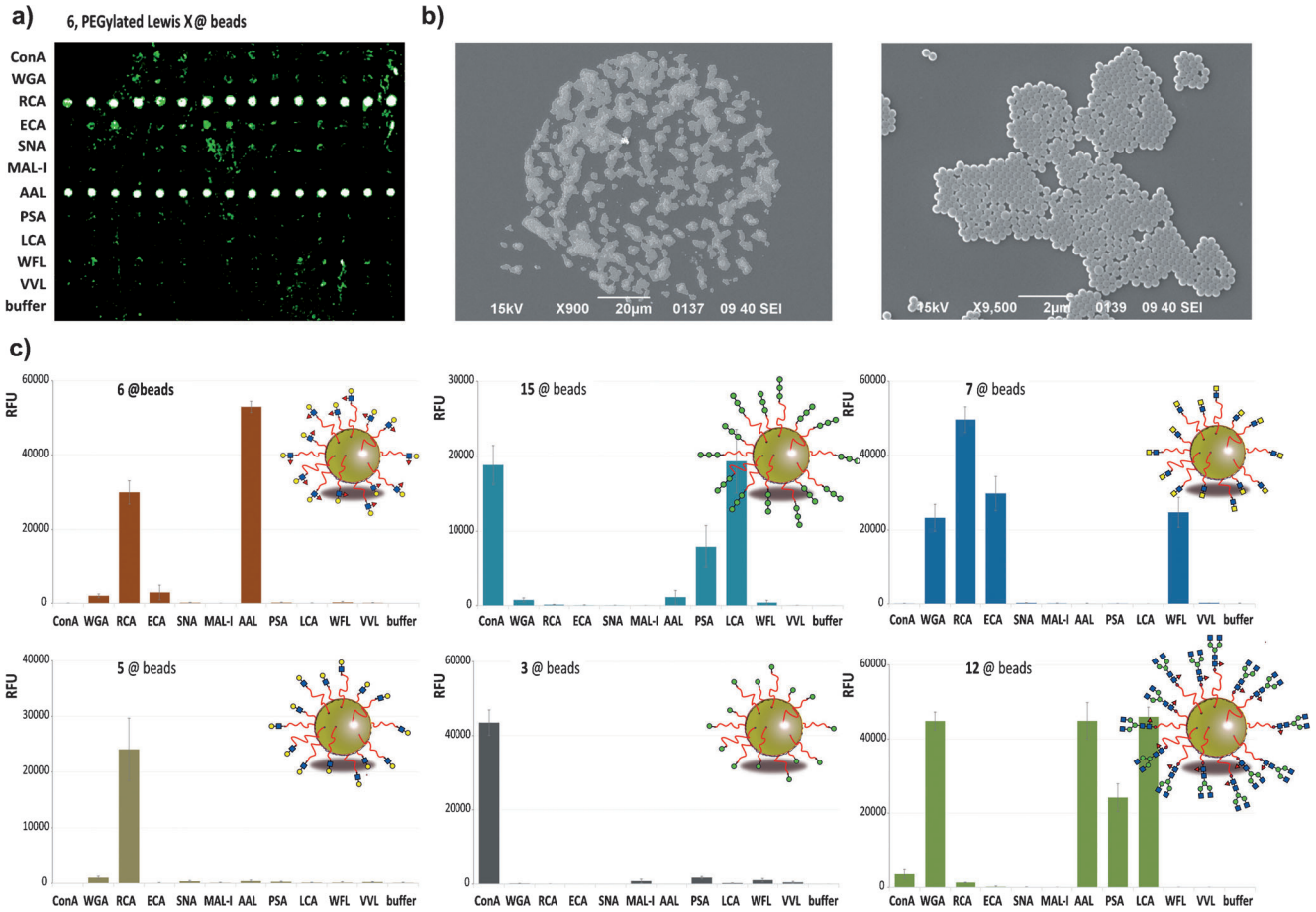


Figure 5. Binding of glyco-PEG-tag-functionalised fluorescent latex nanoparticles on lectin arrays. a) Fluorescence scan of Le X-PEG-tag-functionalised particles binding to lectin array. b) SEM images of beads binding to lectin-functionalised surface and close up. c) Quantification of binding to lectin arrays for beads functionalised with glycoPEG-tags **6**, **15**, **7**, **5**, **3** and **12**.

purification until the final immobilisation of complex glycans. Like no other tag-based system, PEG tags provide simple purification by size exclusion or filtration, compatibility with enzymatic and chemical synthesis in a homogeneous reaction medium and a superior linkage for biomolecules to surfaces or beads to result in assays with exceptionally low background, greatly improved sensitivity and excellent spot morphology. The latter is particularly relevant for quantitative diagnostic tests that require a high degree of reproducibility. To increase the synthetic throughput, the development of automated PEG-tag-based chemo-enzymatic synthesis that combines membrane filtration and robotic dispensing technology should be feasible and is currently being studied in our laboratory.

Experimental Section

The synthetic procedure and characterisation of key compounds are described below. Synthetic details and spectroscopic data for all other compounds can be found in the Supporting Information.

General methods: Chemicals were purchased from Sigma–Aldrich or Acros Organics and were used without further purification. THF was freshly distilled from Na/benzophenone and dichloromethane was freshly distilled from CaH_2. Thin-layer chromatography was carried out using Merck aluminium sheets silica gel 60 F_{254} and visualised by UV irradiation (254 nm) or by staining (vanillin (15 g) and H_2SO_4 (2.5 mL) concentrated in EtOH (250 mL)). Microwave irradiation was performed using a Biotage Initiator monomode oven, Biotage AB, Uppsala, Sweden. Purification of compounds was performed by flash chromatography using Merck 62 Å 230–400 mesh silica gel. Size-exclusion chromatography was performed using a Biorad P2 gel, Biorad, Hercules, USA. Pooled glycan-containing fractions were lyophilised using an ALPHA-2-4 LSC freeze-dryer from Christ, Osterode, Germany. All organic solvents were concentrated using rotary evaporation. ^1H and ^{13}C spectra were acquired using Bruker 500 MHz spectrometer and chemical shifts (δ) are given in ppm relative to the residual signal of the solvent used. Coupling constants (J) are reported in Hz. The mass spectrometric data were obtained using a Waters LCT Premier XE instrument, Waters, Manchester, UK with a standard ESI source by direct injection. The instrument was operated with a capillary voltage of 1.0 kV and a cone voltage of 200 V. Cone and desolvation gas flow were set to 50 and 600 L h^{-1}, respectively; source and desolvation temperatures were 100 °C. MALDI-TOF mass analyses were performed using an Ultraflextreme III time-of-flight mass spectrometer equipped with a pulsed N_2/Nd laser (355 nm) and controlled by FlexControl 3.3 software (Bruker Daltonics). The acquisitions were carried out in positive-ion reflectron mode at a laser frequency of 500 Hz. Microarrays were printed on glass slides employing a robotic non-contact spotter S11 from Scienion, Berlin, Germany. Aminosilane-coated glass slides, Nexterion Slide A+, and NHS-activated glass slides, Nexterion H, were purchased from Schott AG, Mainz, Germany. Lectins were purchased

from Vector Laboratories, Burlingame, USA and labelled with Hilyte Plus 647 and Hilyte Plus 555 protein-labelling kits from AnaSpec, Freemont, USA. Lectin incubations were performed using the Fast Frame incubation chambers from Whatman, Kent, UK. Fluorescence measurements were performed using an Agilent G265BA microarray scanner system, Agilent Technologies, Santa Clara, USA. Quantification was performed using ProScanArray Express software, Perkin–Elmer, Shelton, USA. SEM images were recorded using a JSM-6490LV, implemented with an ion beam sputtering unit.

Chemo-enzymatic synthesis of PEG-tagged Lewis X derivative 6

*PEG-tagged monosaccharide **26***: Trichloroacetimidate **24** (50 mg, 0.01 mmol) and **1** (18 mg, 0.003 mmol) were dissolved in dry CH_2Cl_2 (2 mL) in the presence of 4 Å MS. The mixture was stirred for 1 h, cooled to −20 °C and then trimethylsilyl trifluoromethanesulfonate (TMSOTf; 1 μL, 0.007 mmol) was added. The mixture was stirred for 2 h from −20 to 0 °C, then quenched with a drop of Et_3N and diluted with CH_2Cl_2 (10 mL). The reaction mixture was evaporated and the product was precipitated by stirring the crude material in CH_2Cl_2/Et_2O (1:50) at 0 °C to afford compound **26** as a white solid (40 mg, 80 % yield). MALDI-TOF: *m*/*z*: 5354.94 [*M*+Na], most abundant peak.

*Amino-polyethylene glycolyl 2-acetamido-2-deoxy-β-*D*-glucopyranoside (**4**)*: A solution of **26** (0.046 g, 0.0086 mmol) and ethylenediamine (0.4 mL) in *n*BuOH (0.6 mL) was irradiated three times with microwaves at 120 °C for 30 min. Solvent was evaporated and the reaction crude was subjected to acetylation with Ac_2O (0.4 mL) in pyridine (0.8 mL) at 0 °C for 18 h. The solvent was evaporated and the product was precipitated by stirring the crude material in CH_2Cl_2/Et_2O (1:50) at 0 °C to afford the compound as yellowish solid (0.039 g). This material was dissolved in THF (1 mL) and added to a suspension of Na in liquid NH_3 at −78 °C. The mixture was stirred for 15 min at this temperature and then the reaction was quenched by the addition of NH_4Cl. The solvent was then evaporated. Purification by size-exclusion chromatography (Biogel-P2, NH_4HCO_3 as eluent) afforded product **4** as a colourless oil (23 mg, 60 % yield). MALDI-TOF MS: *m*/*z*: 4962.38 [*M*+Na], most abundant peak.

β-1,4-Galactosylation: A solution (200 μL) of **4** (2 mg, 0.39 μmol), UDP-Gal (uridine 5′-diphosphogalactose disodium salt; 1.6 mg), bovine serum albumin (BSA; 1 mg), bovine milk β-1,4-galactosyltransferase (50 mU; EC 2.4.1.22), alkaline phosphatase (2.3 U; EC 3.1.3.1) and $MnCl_2$ (10 mM) in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (50 mM, pH 7.4), was incubated at 37 °C for 20 h. The resulting mixture was heated at 95 °C for 5 min. The yellowish precipitate was removed and the solution was purified and buffer exchanged to water using an Amicon spin filter of 10 kDa. The isolated polymer was freeze-dried to obtain compound **5** as a yellowish powder (1.7 mg, 85 % yield). MALDI-TOF MS: *m*/*z*: 5119.143 [*M*+Na].

α-1,3-Fucosylation: A solution (250 μL) of **5** (0.4 mg, 75 nmol), GDP-Fuc (0.5 μmol), *C. elegans* Lewis X type α-1,3-fucosyltransferase (CeFuT6; 69 μg) and $MnCl_2$ (20 mM) in MES buffer (80 mM, pH 6.5) was incubated at 25 °C for 40 h. The resulting mixture was heated at 95 °C for 5 min. The precipitate was removed and the solution was purified and the buffer exchanged to water using an Amicon spin filter of 3 kDa. The isolated polymer was freeze-dried to obtain compound **6** as a white powder (0.3 mg, 75 % yield). MALDI-TOF MS: *m*/*z*: 5266.52 [*M*+Na].

Chemo-enzymatic synthesis of core-type fucosylated PEG-tagged *N*-glycan derivatives 12, 13 and 14

Bi-antennary N*-glycan coupling to the PEG tag*: A mixture of glycan **21** (0.23 mg, 0.16 μmol), the *N*-Boc-protected NHS-activated PEG **2** (1.00 mg, 0.20 μmol) and *N,N*-diisopropylethylamine (DIPEA; 5 μL, 28.70 μmol) in dry DMF (0.2 mL) was stirred for 2 h at RT and then the excess amount of activated polymer was removed by addition of Tentagel-NH_2 resin (20 mg, 8.6 μmol) overnight at RT. The resin was filtered off and the solvent removed under vacuum. The crude was re-dissolved in dH_2O (200 μL) and filtered using Amicon spin filters (10 kDa). The polymeric conjugate was treated with aqueous 10 % trifluoroacetic acid (TFA) solution (0.5 mL) for 2 h at 37 °C. The solution was then filtered using Amicon spin filters (10 kDa) to remove the excess amount of TFA. The polymer aqueous solution was lyophilised to obtain compound **8** as a white solid (0.78 mg, 76 % yield). The coupling conversion was calculated (97 %) by ultra performance liquid chromatography (UPLC) from the reaction crude by derivatisation with a 9-fluorenylmethoxycarbonyl (Fmoc) reporter group. The isolated compound was analysed by MALDI-TOF MS using pre-spotted MALDI targets with α-cyano-4-hydroxy-cinnamic acid (HCCA) matrix (Anchor Chip, Bruker Daltonics): *m*/*z*: 6232.04 [*M*+Na], most abundant peak.

Core-type α-1,6-fucosylation: A solution (200 μL) of compound **8** (0.3 mg, 46 nmol), GDP-fucose (200 nmol), and *C. elegans* core-type α-1,6-fucosyltransferase (55 μg) in MES buffer (80 mM, pH 6.5), $MnCl_2$ (20 mM) was incubated at 25 °C for 24 h. The resulting mixture was heated at 95 °C for 5 min. The precipitate was removed, the solution was purified and the buffer exchanged to water using an Amicon spin filter of 10 kDa. The isolated polymer was freeze-dried to obtain **12** as a white powder (0.3 mg, 95 % yield). MALDI-TOF MS: *m*/*z*: 6377.16 [*M*+Na].

Core-type α-1,3-fucosylation: A solution (200 μL) of compound **8** (0.3 mg, 46 nmol), GDP-fucose (100 nmol), and *A. thaliana* core-type α-1,3-fucosyltransferase (70 μg) in MES buffer (80 mM, pH 6.5), $MnCl_2$ (20 mM) was incubated at 25 °C for 40 h. The resulting mixture was heated at 95 °C for 5 min. The precipitate was removed, the solution was purified and the buffer exchanged to water using an Amicon spin filter of 10 kDa. The isolated polymer was freeze-dried to obtain **13** as a white powder (0.3 mg, 95 % yield). MALDI-TOF MS: *m*/*z*: 6377.28 [*M*+Na].

Core-type α-1,6-fucosylation and α-1,3-fucosylation: A solution (200 μL) of compound **12** (0.15 mg, 23 nmol), GDP-fucose (100 nmol) and *A. thaliana* core-type α-1,3-fucosyltransferase (70 μg) in MES buffer (80 mM, pH 6.5) was incubated at 25 °C for 40 h. The resulting mixture was heated at 95 °C for 5 min. The precipitate was removed, the solution was purified and the buffer exchanged to water using an Amicon spin filter of 10 kDa. The isolated polymer was freeze-dried to obtain **14** as a white powder (0.1 mg, 67 % yield). MALDI-TOF MS: *m*/*z*: 6523.20 [*M*+Na].

3D microarray preparation

Functionalisation of aminosiloxane surfaces: Amino slides (Nexterion slide-A star) were blocked with succinic anhydride solution (5.0 g) in *N*-methyl-2-pyrrolidone (NMP) (250 mL) and borate buffer (30 mL, 0.2 M), pH 9) for 45 min at RT. The slides were washed with phosphate-buffered saline (PBS) and water and dried with Ar. Activation of the carboxylic acid slides as succinimide esters was performed by treatment with 0.2 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 0.05 M NHS in anhydrous DMF for 2 h at RT. The slides were rinsed with anhydrous DMF and dried with Ar. After the washing step with dry DMF, the surfaces were ready for the printing of the glycan arrays.

Microarray printing: Glycoconjugates **3**–**15** and the aminopentyl-linked glycans **16**–**23** were printed at different concentrations 200, 100, 50 and 25 μM in phosphate buffer (100 mM) that contained 0.002 % of Tween-20 at pH 8.4, on NHS-functionalised glass slides with a non-contact spotter. A number (3) of 270 pL drops of the buffered solutions were printed with a distance of 300 μm. Five replicates were printed of each sample as 21×20 matrixes. After printing, the slides were kept in a 75 % humidity chamber (saturated NaCl solution) at 25 °C for 18 h. Unreacted NHS groups were quenched by placing the slides in a 50 mM solution of PEG-600-NH_2 in sodium borate buffer 50 mM, pH 9.0, for 2 h. The standard washing of the slides was performed with PBST (PBS solution that contained 0.5 % Tween 20), PBS and water. The slides were dried in a slide spinner.

*Labelled lectin incubation of the 3*D *microarray*: Lectin stock solutions (4.0–5.0 mg mL^{-1}) were diluted 1:2 with phosphate buffer 300 mM, pH 8.5 and labelled with 1 μL of Hilyte Plus 647 and 555 (cy5/cy3 analogues) protein-labelling kit from AnaSpec, Freemont, USA, for each 100 μL of sample for 2 h at RT. Excess amounts of dye were removed by buffer exchange to PBS by employing 3 kDa Amicon filters, Millipore, Carrigtwohill, Co. Cork, Ireland. The labelled lectins were stored as 1–2 mg mL^{-1} solutions in PBS at 4 °C (D/P ratio: 1–2). ConA, RCA, AAL, WFL, VVL lectins and anti-HRP were labelled with the cyanine-3 analogue and WGA, ECA, LCA, GNA, NPL and BS II were labelled with the cyanine-5 analogue.

The microarrays were compartmentalised with a 16-well gasket. Lectin solutions were prepared in PBST (PBS with 0.05 % Tween-20) with

$CaCl_2$ (1 mM) and $MgCl_2$ (1 mM). Solutions (100 μL) of the corresponding labelled lectins were incubated in the dark over each microarray for 1 h at room temperature. Concentrations of 10 μg lectin per mL were used for all lectins except for BS II, which was incubated at 50 $\mu g\,mL^{-1}$. The slides were washed under standard conditions, dried with a slide spinner and fluorescence emission was analysed with a microarray scanner.

Histograms that show the carbohydrate–lectin interactions were prepared using Microsoft Excel software. Fluorescence measurements were performed using an Agilent G265BA microarray scanner, Agilent Technologies, Santa Clara, USA. Image quantification was performed with ProScanArray Express software from Perkin–Elmer, Shelton, USA and interaction profiles with the lectin array are displayed as histograms. The adaptive circle method with a diameter range of 65–70 μm was employed for spot quantification. The median value was used for the fluorescence of each spot and for every carbohydrate an average of five replicate spots was used to construct histograms that show the lectin-binding profile. Error bars are included that show the standard deviation for each carbohydrate–lectin interaction.

Lectin array analysis of PEG-tagged glycan-coated fluorescent beads

Glycan immobilisation on fluorescent beads: Fluorescent (cy3) latex beads 300 nm in diameter (50 μL, 5×10^9 beads) functionalised with carboxylic acid groups (1.08×10^6 groups per bead) were activated as NHS ester by treatment with solutions of EDC (2 mM, 200 μL) and NHS (1 mM) in H_2O overnight at RT. After washing by centrifugation, the activated beads were incubated with the corresponding solution of aminopentyl glycan (three concentrations: 100 nmol per 200 μL, 10 nmol per 200 μL or 5 nmol per 200 μL) or PEGylated glycan (two concentrations: 10 nmol per 200 μL or 5 nmol per 200 μL) in phosphate buffer (30 mM, pH 8.4) that contained 0.005% of Tween-20 overnight at RT. The beads were quenched by the addition of a solution (50 mM, 500 μL) of ethanolamine in borate buffer (50 mM, pH 9.0). After centrifugation and washing with phosphate buffer, the coated beads were stored in a final volume of 50 μL at 4 °C.

Lectin microarray preparation: Lectin solutions in print buffer (0.4 $mg\,mL^{-1}$; PBS that contained 1% glycerol and 0.001% of Tween-20) were freshly prepared for every print run to avoid activity loss due to protein denaturation in a source plate (384-well plate) loaded with aliquots (20 μL) of every lectin, which could be stored overnight at 4 °C without loss of function. These dilutions were spotted onto NHS-activated hydrogel glass slides (Nexterion H, Schott AG, Mainz, Germany) by employing a dispensing volume of 0.8 nL and at 300 μm pitch between spot centres and a robotic non-contact piezoelectric spotter (S11, Scienion, Berlin).

Lectins ConA, WGA, RCA, ECA, SNA, MAL-I, AAL, PSA, LCA, WFL and VVL were printed in 15 replicates as 15×12 matrixes in well format. Printing buffer was also spotted as a negative control. Humidity for the slide printing was maintained around 50% before and during printing. The printed slides were incubated at 75% humidity in a custom-made humidity chamber filled with saturated NaCl solution at 18 °C overnight and stored at −20 °C without quenching unless the slides were used immediately.

Before use of the slides for lectin-array blotting, unreacted NHS-activated carboxylate groups were quenched by placing the slides in a 50 mM ethanolamine solution in 50 mM borate buffer for 1 h and washed with PBS for 5 min. Slides were dried by centrifugation without any additional washing step.

Interaction of the glyco-conjugated beads with the lectin array: The bead solutions were diluted 1:100 in PBS buffer that contained 0.005% Tween-20 and these solutions (100 μL) were loaded into the microarray wells and incubated at RT for 1 h. The slides were washed under standard conditions, dried with a slide spinner. Fluorescence emission was analysed with a microarray scanner.

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