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Development of a multifunctional aminoxy-based fluorescent linker for glycan immobilization and analysis

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Abstract

Glycan arrays have become a technique of choice to screen glycan-protein interactions in a high-throughput manner with high sensitivity and low sample consumption. Here, the synthesis of a new multifunctional fluorescent linker for glycan labelling via aminoxy ligation and immobilization is described; the linker features a fluorescent naphthalene group suitable for highly sensitive HPLC-based purification and an azido- or amino-modified pentanoyl moiety for the immobilization onto solid supports. Several glycoconjugates displaying small sugar epitopes via chemical or chemoenzymatic synthesis were covalently attached onto a microarray support and tested with lectins of known carbohydrate binding specificity. The glycan library was extended using glycosyltransferases (e.g. galactosyl-, sialyl- and fucosyltransferases); the resulting neoglycoconjugates, which are easily detected by mass spectrometry, mimic antennal elements of N- and O-glycans, including ABH blood group epitopes and sialylated structures. Furthermore, an example natural plant N-glycan containing core α 1,3-fucose and β 1,2-xylose was also successfully conjugated to the fluorescent linker, immobilised and probed with lectins as well as anti-horseradish peroxidase. These experiments validate our linker as being a potentially valuable tool to study glycozyme and lectin specificities, sensitive enough to allow purification of natural glycans.

Keywords

Chemo-enzymatic synthesis; fluorescent labelling; glycan arrays; natural glycans; N-glycoside

Introduction

All biological processes involve recognition either of ligands or substrates, regardless of whether the 'partners' are nucleic acids, proteins, lipids, glycans or smaller molecules. High throughput analyses have become established as a means to identify recognition partners, but, while nucleic acid and protein/polypeptide arrays have become routine for mass production, glycan array methodologies are still in their infancy; this is partially due to the inherent difficulties of oligosaccharide chemistry and the branched, non-template-driven nature of oligosaccharide structures and biosynthesis. Nevertheless, over the years a number

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Conflicts of Interest

The authors have no conflicts of interest.

of platforms, which use a variety of approaches to immobilize glycans, have been reported (Park, et al. 2013, Rillahan and Paulson 2011, Song, et al. 2014). Especially the covalently printed arrays of the Consortium for Functional Glycomics have been highly valuable in identifying potential glycan partners for a range of lectins and antibodies (Blixt, et al. 2004, Smith, et al. 2010); in the most current version of this glycan array, 609 mammalian and 153 pathogen glycan determinants are displayed. These glycan arrays present well-defined glycan structures that have been prepared by means of chemical or chemo-enzymatic approaches; insights into recognition of glycans by, e.g., plant lectins, mammalian galectins, influenza haemagglutinins and C-type lectins of the immune system (Smith, et al. 2010, Taylor and Drickamer 2009, Walther, et al. 2013) have resulted from using glycan arrays. Nevertheless, there are still many biologically-relevant glycans found in nature that are not represented in these glycan libraries; for instance, although blood group glycans on the Consortium array were found to be ligands for an oyster galectin (Feng, et al. 2013), the sulphated forms of these epitopes (as present in the oyster itself) were absent and so the positive or negative effect of the anionic modification on binding could not be studied. Therefore, as part of strategies to extend the number of glycans available on arrays, there is increasing interest in developing fluorescent tags that can be employed for purification, analysis and immobilization of oligosaccharides derived from natural sources or from enzymatic reactions.

For the elucidation of the glycome of a living organism, glycans are first released from glycoproteins or glycolipids using enzymatic or chemical methods; a common option is to then derivatise the glycans with a fluorescent tag and, purify the products by HPLC and characterize these by mass spectrometry (Ruhaak, et al. 2010). 2-aminobenzamide (2-AB) and 2-aminopyridine (2-AP) are still the most commonly used fluorescent tags for glycan labelling. This fluorescent derivatisation step proceeds in high yield via reductive amination and has been used to immobilize glycans on microarray supports (de Boer, et al. 2007, Song, et al. 2008). More recently, a novel 2-amino-*N*-(2-aminoethyl)-benzamide-based fluorescent tag (AEAB) was developed with higher immobilization efficiency and has been used for 'shotgun glycomics' in which the glycan partners for lectins are identified and then only 'hit' glycans are structurally characterized (Byrd-Leotis, et al. 2014). In all these cases (2-AB, 2-AP and AEAB), there is a reductive step, which results in loss of the biological "closed-ring" form at the reducing end of the glycan. Although this is not crucial for glycan analysis by HPLC or MS, the existence of an open-ring reduced form of a glycan can reduce or abolish its biological function (i.e., its capacity to bind), which is particularly relevant for short-chain glycans (Jiménez-Castells, et al. 2008, Liu, et al. 2007, Thygesen, et al. 2009, Wilson, et al. 1998). One approach to at least partially retain the cyclic nature of the reducing unit in glycan arrays is to prepare neoglycolipids via oxime ligation (Liu, et al. 2007). Although a portion of such conjugates is in the closed-ring form, the yield is not quantitative and remaining open-ring forms will complicate interpretation of binding results.

To overcome the limitations of many conjugation methods, Peri proposed the use of alkyl *N,O*-substituted hydroxylamines instead of regular hydroxylamine, which force the retention of the closed-ring form (Goff and Thorson 2014, Peri, et al. 1998). To date, a few glycan array platforms have been prepared using this *N*-substituted hydroxylamine (Bohorov, et al. 2006, Chen and Pohl 2008, Cló, et al. 2010) and a selection of the glycoconjugates on the

Consortium array (those with spacer 21) were prepared with this method. Amongst these, only the linker developed by Cló with an UV-active aromatic chromophore allows purification and quantification of glycoconjugates by HPLC (Cló, et al. 2010). Another strategy to prepare glycan microarrays with a closed-ring structure at the reducing end was developed by Song *et al.* based on the AEAB linker. However, this strategy requires four reactions (i.e.: glycosylamine formation, reaction with acryloyl chloride, ozonolysis and reductive amination) to obtain the final fluorescent glycoconjugate (Song, et al. 2009b).

With the ultimate goal of producing fluorescent arrays with natural glycans solely in their closed-ring forms and in a low number of steps, we reappraised chemistry based on the use of *N*-substituted aminoxy linkers for glycan derivatization. Thereby, our goal was to develop a linker which (i) enables non-reductive reaction with non-protected saccharides, (ii) contains a fluorescent group for highly sensitive HPLC-based purification but with excitation and emission spectra different from those of labels used to detect lectin/antibody binding, (iii) features an azido- or amino-modified pentanoyl (C5) moiety for the immobilization onto solid supports via either alkyne-modified or *N*-hydroxysuccinimide (NHS)-activated surfaces, (iv) is easily detected by mass spectrometry and (v) is compatible with the use of glycosyltransferases. Indeed, all these characteristics were fulfilled; thereby, microarrays with glycans of different sizes (mono- to tetrasaccharides) were tested with plant lectins. We focused on mimicking selected aspects of mammalian glycosylation pathways especially *N*-acetylglucosamine and related antennal motifs (Cummins 2009); we also conjugated, immobilised and probed (with lectins and one antibody) a natural glycan derived from horseradish peroxidase (Yang, et al. 1996) to our bi-functional fluorescent linker in order to confirm its suitability for labelling and printing of natural glycans.

Results and Discussion

Chemical synthesis of fluorescent linkers

The linker design is based on three key elements: (i) a methoxylamino functionality for the chemoselective ligation with free reducing sugars and preservation of the closed-ring form, (ii) a fluorescent naphthalene ring to increase sensitivity during purification, and (iii) an azido- or amino-pentanoyl (C5) spacer for subsequent immobilization. The assembly of the fluorescent linker is shown in Scheme 1. The synthesis started with the commercially available diethylene glycol. First, the hydroxyl groups were tosylated to give compound **1** in high yield (90%). Then, **1** was treated with equimolar amounts of sodium azide to obtain the corresponding monoazide **2** in moderate yields (62%) with minimal formation of the diazido by-product (13%) and with recovery of the ditosylated starting material **1** (25%).

Subsequently, the bromide group was introduced by treatment of **2** with lithium bromide, which gave compound **3** in 82% yield. After Boc-protection of methoxyamine (Kawase, et al. 1989), the resulting *N*-Boc-methoxyamine **4** was alkylated with compound **3** to deliver compound **5**, which was subsequently reduced by catalytic hydrogenation to produce **6**. Subsequently, the fluorescent moiety was introduced by amide bond formation between compound **6** and 6-amino-2-naphthoic acid to give compound **7** in 78% yield. Two linkers with different functionalization were prepared. The azido-functionalized linker was synthesized by amide bond formation with 5-azido-pentanoic acid in 77% yield. The same

synthetic procedure was followed with Boc-5-aminopentanoic acid to prepare the amino-functionalized linker **8** in 92% yield. Eventually, cleavage of the Boc group delivered the azido-functionalized linker **11** and the amino-functionalized linker **12** in 92% and 94% yield, respectively. Hydrogenation of compound **9** before Boc deprotection gave the Boc-protected amino-functionalized linker **10** that is used afterwards for neoglycoconjugate quantification and as a reference surface on the glycan arrays.

Chemoselective neoglycosylation with chemically synthesized glycans

Optimization of reaction conditions with azido-functionalized linker 11—To find the best conjugation condition for the chemoselective ligation, several mono- and disaccharides were conjugated under different reaction conditions (Table 1). For those small sugar epitopes, the difference in hydrophobicity between the azido-functionalized linker **11** and glycoconjugates **13-21** enabled their purification by silica gel column chromatography.

Methoxyamine based neoglycosylation is often carried out under mildly acidic conditions, either in buffered aqueous (NaOAc/AcOH) solutions (Matsubara, et al. 2005) or in mixtures of organic solvents containing acetic acid (Langenhan, et al. 2013, Peri, et al. 2004). DMF/AcOH 3:1 has been frequently the choice due to its high dissolving power for both lipophilic and hydrophilic compounds. Therefore, we first performed the conjugation of linker **11** with GlcNAc under standard conditions in DMF/AcOH 3:1. Other parameters, such as temperature, incubation time, acidity and concentration of both reactants were screened in order to improve reaction yields (Table I, entries 1-4). In agreement with previously published results (Gudmundsdottir, et al. 2009, Munneke, et al. 2015), a considerably better yield was observed under highly concentrated solutions (1 M linker and 100 mM sugar). However, the limited solubility of complex glycans in DMF and the low pH led us to search for milder conditions compatible with natural glycans. Therefore it was necessary to find a solvent mixture, in which both the hydrophilic carbohydrate and the lipophilic linker were sufficiently soluble under highly concentrated solutions. A mixture of 1,4-dioxane/ ammonium acetate buffer 1:1 (v:v) pH 4.6 provided the best results with longer incubation times required for free and substituted GlcNAc. With the combination of a highly concentrated solution and mild reaction conditions, the reaction proceeded in high yields with a 10-fold improvement over our initial trials (Table I, entries 5-13). Thereby, our conjugation efficiency with GlcNAc (93% also taking into account the purification step) exceeds that of Munneke *et al* (Munneke, et al. 2015) in their report describing the use of sodium acetate buffer for conjugation at room temperature.

After purification by silica gel column chromatography, all glycoconjugates listed in Table 1 were characterized by NMR. In agreement with previously-published work, the stereochemistry is influenced significantly by the sugar unit at the reducing end (Langenhan, et al. 2011, Peri, et al. 1998). NMR data showed that the gluco-configured saccharides (Glc, GlcNAc, Lac, LacNAc, chitobiose) derivatives are found exclusively in β -pyranose form, whereas galactose derivatives (Gal, GalNAc) were present as a mixture of β -pyranose and furanose products. This high diastereoselectivity towards the β -pyranosides can be rationalized to unfavorable steric factors, such as 1,3-diaxial interactions, present in α -pyranosides which are not compensated by the weak anomeric effect exerted by the aminoxy

aglycon. Assignment of the β -anomeric configuration of the galactofuranose products was based on comparable published data (Langenhan, et al. 2011, Peri, et al. 1998) and additional NOESY experiments. The most complex mixture was observed for mannose (Man) products, with a mixture of α -pyranose (which corresponds to the natural eukaryotic O-Man-type linkage), β -pyranose and a furanose form. The values of the homonuclear $J_{H1,H2}$ coupling constant seen in anomeric mannofuranoses are not very different and thus not a reliable diagnostic feature to assign the anomeric configuration. Steric considerations would suggest assigning the α -anomeric configuration for the mannofuranosyl conjugate. By contrast to glucose or galactose derivatives, the axial 2-OH of Man promotes the formation of α -pyranoside via favorable dipole/dipole interactions with the anomeric center. For all glycoconjugates listed in Table 1, it was possible to separate the isomers by HPLC with no anomeric re-equilibrium observed by NMR over time. Based on the screen of conjugation reactions with different mono- and disaccharides, we can demonstrate the synthesis of biologically-relevant forms of key glycan elements which reflect O-mannosyl and collagen-type O-glycans (Praisman and Wells 2014, Spiro 1967), LacNAc-type (LN) N-glycan antennae (Cummings 2009) and the lactose core of lactosylceramides (Jennemann, et al. 2005). Most importantly, the ‘closed-ring’ β -linkage of N-glycans can be mimicked.

After purification of neoglycoconjugates (**13-21**), the azido group was reduced to the corresponding amino group by catalytic hydrogenation without the need of any further purification step. After hydrogenation, the amino-functionalized neoglycoconjugates (22-33) were ready for printing on commercially available NHS-activated glass supports. The stability of amino-functionalized neoglycoconjugate **24** was tested at different pH values ranging from 2.0 to 12.0 for 48 h. Under neutral to basic conditions (pH >6) the glycoconjugate was stable and no loss of the sugar unit was observed. In agreement with previous results (Gudmundsdottir, et al. 2009, Langenhan, et al. 2011), the glycoconjugate is rapidly hydrolyzed at pH below 6. After 24 h, 60% and 70% of the neoglycoconjugates lost the sugar unit at pH 4 and pH 2, respectively. The lowest degree of deglycosylation (5%) was observed at pH 6. In addition, the medium- and long-term stability in aqueous solution was studied for Boc-protected linker **10** and neoglycoconjugates **22** and **24**. For the three compounds, up to 80% is stable after 1-month storage at 4 °C or after 3-months storage at -20 °C.

Conjugation with amino-functionalized linker 12—With the aim of reducing the number of steps and facilitate the conjugation with larger and more complex glycans, the conjugation was carried out directly with the amino-functionalized linker **12**. Linker **12** was conjugated with lactose under optimal reaction conditions described above to give the corresponding glycoconjugate in quantitative yields (i.e., >95% yield). NMR spectra demonstrated that the reaction is completely selective for the methoxyamino functionality and no reaction with the amino-modified pentanoyl chain takes place.

In order to confirm the applicability of this approach to branched trisaccharide glycan structures, ABH blood group trisaccharides were conjugated to **12**. After clean-up with NH₂-SPE to remove partially the excess of linker, glycoconjugates were purified by NP-HPLC and characterized by MALDI-TOF MS and MS/MS (Figure 1, panel a-d). All fragment ions from glycoconjugates with hexoses at the reducing end retain the linker

moiety and the major ions are usually the result of glycosidic cleavages. By contrast, glycoconjugates with hexosamines at the reducing end results in $^{0,2}X_0$ cross-ring fragment ions containing the aglycon moiety in addition to B glycan fragment ions.

As shown in Figure 1 (panel e-g), commercially available sialyl- α 2,6-lactose was also conjugated to the amino-functionalized linker to test the suitability of this approach for acid-labile glycan modifications, such as sialic acid. After 72 hours of conjugation, glycoconjugates were cleaned up with NH₂-SPE, purified by NP-HPLC and analyzed by MALDI-TOF MS and MS/MS. The HPLC chromatogram showed that no desialylation occurred during conjugation (Figure 1, panel e).

In solution enzymatic modification of neoglycoconjugates

In a further application for the chemoselectively-synthesized neoglycoconjugates, the possibility of their modification with different natural or recombinant enzymes was explored. Beginning with the common core structure found in N-glycan antennae (i.e. β -GlcNAc $_p$) different glycan epitopes were enzymatically prepared. Due to the fluorescent naphthalene ring, the reactions could be monitored and quantified by HPLC equipped with a fluorescence detector. Glycoconjugates in the pmol range were straightforwardly monitored by HPLC prior to characterization by MALDI-TOF MS.

As shown in Figure 2, from compound **22** (with a β -GlcNAc $_p$ at the reducing end), the type 2 LN epitope (i.e. Gal- β 1,4-GlcNAc) was built using the commercially-available bovine milk β 1,4-galactosyltransferase (β 1,4-GalT). After full conversion, the type 2 LN epitope was further elongated on the Gal residue by a recombinant α 2,3-sialyltransferase from *Neisseria meningitidis* (α 2,3-SiaT) (Gilbert, et al. 1996). Furthermore, the complex tetrasaccharide sialyl Lewis X (sLeX) was prepared from the GlcNAc-neoglycoconjugate in two steps: i) one-pot multiple reaction of β 1,4-GalT and, α 2,3-SiaT to made the type 2 3'-SLN, and ii) sequential addition of recombinant fucosyltransferase from *Caenorhabditis elegans* (*Ce* FUT-6) (Yan, et al. 2013). The chromatograms and MS spectra showed that all reactions proceeded to completion.

Alternative starting points (see Supplementary Figure 1) were represented by the linkers displaying α Man $_p$ or β Gal $_p$ NAc at the reducing end; these could be respectively extended by a recombinant β 1,2-*N*-acetylglucosaminyltransferase (POMGnT1) (Akasaka-Manyu, et al. 2011) and β 1,4-GalT or by either bacterial CgtB β 1,3-galactosyltransferase (β 1,3-GalT) (Persson, et al. 2001) or α 2,6-sialyltransferase (α 2,6-SiaT; Pd2,6ST) (Yamamoto, et al. 1998). In these small-scale experiments, at least 50% conversion was achieved showing the compatibility of the linker with a range of glycosyltransferases.

Microarray analysis of neoglycoconjugates displaying mono- to tetrasaccharides

In order to test the applicability of these neoglycoconjugates for glycan arrays, those were immobilized onto NHS-activated glass slides and tested with several plant lectins. In these experiments, commercially available biotinylated lectins were employed, and the binding was detected after incubation with Alexa Fluor[®] 647-conjugated streptavidin. The naphthalene ring absorbs and re-emits at the UV light range (Ex 280/Em 380 nm). Therefore

its fluorescence activity does not interfere with the detection of binding in the red light range (Ex 620/Em 675 nm). First, typical mono- and disaccharide anomers (β -Gal p , α -Man p , β -Gal p NAc, β -Glc p NAc, β -Lac p and β -Lac p NAc) were spotted on the slide at three concentrations (33 μ M, 250 μ M and 1 mM) and the remaining free NHS-groups were blocked with ethanolamine. Subsequently, these glycan arrays were interrogated with lectins of different carbohydrate specificity at three concentrations (2, 10 and 20 μ g/mL).

As shown in Figure 3, positive binding was observed for different monosaccharides according to the carbohydrate specificity of lectins. *Ricinus communis* agglutinin I (RCA I), a β -Gal specific lectin (Wu, et al. 2006), was clearly binding to β -Gal p and β -Gal p NAc but not to Man or GlcNAc; this contrasts with its inability to bind reduced forms of galactose (Liu, et al. 2007). β -Gal p and β -Gal p NAc were also recognized by *Erythrina cristagalli* agglutinin (ECA), another β -Gal specific lectin (Iglesias, et al. 1982). However, the fluorescence intensity was lower in comparison to RCA, which confirms their different affinities (Itakura, et al. 2007). For both lectins, high lectin concentrations (i.e. 20 μ g/mL) or high immobilization levels (i.e. 1 mM) were necessary to detect lectin binding to monosaccharides. Only for Concanavalin A (ConA), a lectin generally considered specific for α -Man (Mandal, et al. 1994), bound well to α -Man p -glycoconjugate at 2 μ g/mL. In contrast, wheat germ agglutinin (WGA) binding to any monosaccharide conjugate was low. The absence of a signal for the control (immobilized linker alone) proved that lectins bind exclusively to their sugar epitopes and not to the linker, even at high lectin concentrations.

For RCA, ECA and ConA, higher binding intensities were observed for disaccharides than for monosaccharides at the same lectin concentration, whereas of the mono- and disaccharide conjugates tested, wheat germ agglutinin (WGA) only significantly bound the β -Lac p NAc disaccharide (Figure 3). Such results are not unexpected as the biological lectin-binding determinants are certainly more complex than a single residue and are in keeping with the older literature. For instance, RCA displays an increased affinity towards lactose as opposed to galactose (Podder, et al. 1974) or there are orders of magnitude difference in the inhibition of binding to WGA by GlcNAc di-, tri- and pentasaccharides as compared to GlcNAc alone (Allen, et al. 1973). This is reflected in more modern studies on WGA in which binding was observed to GlcNAc $_{2-5}$ on the Consortium array, while an interaction with the monosaccharide was highly dependent on the type of glycoconjugate used (Wang, et al. 2014). In the current study, good binding was observed to disaccharides printed at 33 μ M and detected with 20 μ g/mL lectin.

The chemically synthesized neoglycoconjugate displaying α 2,6-sialylated lactose was also immobilized on the array at 250 μ M and interrogated with lectins that are specific for sialic acid. As shown in Figure 4a, clear binding was observed for *Sambucus nigra* agglutinin (SNA), a lectin that recognizes exclusively α 2,6 sialic acid (Shibuya, et al. 1987). By contrast, no binding was observed for *Maackia amurensis* agglutinin (MAA II) (Knibbs, et al. 1991) or WGA (Iskratsch, et al. 2009), which bind α 2,3-, but not α 2,6-linked sialic acid. In addition, partial loss of the RCA binding was observed when lactose is capped with a terminal sialic acid.

Neoglycoconjugates with branched ABH histo-blood group trisaccharide structures were also immobilized on the array and tested with several lectins. Binding of *Aleuria aurantia* lectin (AAL), a lectin with a broad affinity against fucosylated glycans, to all three ABH determinants corroborates that the methoxy group is not a hindrance for recognition of branched structures at the reducing end. By contrast, *Ulex europaeus* agglutinin I (UEA-I), a lectin with described high specificity to blood group H antigen (Matsumoto and Osawa 1969), showed a different binding profile for the three fucosylated trisaccharides, with the strongest affinity to blood group H, a weak affinity to blood group A and almost no binding to blood group B (Figure 4, panel b). Binding according to their carbohydrate specificity was observed for *Vicia villosa* agglutinin (VVA), an α -GalNAc specific lectin, and *Griffonia simplicifolia* agglutinin I (GSA-I), an α -Gal specific lectin. The microarray data presented here demonstrates that immobilized small sugar epitopes, such as di- and trisaccharides with either linear or branched structures, are recognized by relevant specific lectins.

To determine the utility of the enzymatically-modified neoglycoconjugates for array preparation, the enzymatic reactions (shown by HPLC and mass spectrometry to be quantitative) were desalted using mini-C18 columns and spotted directly onto the slide. These arrays were then tested with different lectins. Figure 4 panel c shows the binding observed towards derivatives of LacNAc for lectins, such as RCA I, MAA II and AAL (Itakura, et al. 2007, Kochibe and Furukawa 1980, Wang and Cummings 1988). RCA I bound strongly to the LacNAc-conjugate, but this binding is partially lost when the type 2 LN disaccharide is elongated with a terminal sialic acid in α -2,3 linkage (Green, et al. 1987). However, also in keeping with a previous study using remodeled glycoproteins (Iskratsch, et al. 2009), the binding is lost when the GlcNAc moiety is fucosylated. A similar effect was observed for MAA II that binds to the trisaccharide Neu5Ac- α 2,3-LacNAc but not to the fucosylated variants; this demonstrates that the binding of this lectin is indeed very context dependent (Geisler and Jarvis 2011) and is also compatible with results from an array based on reductive labelling (Byrd-Leotis, et al. 2014). On the other hand and as previously reported (Matsumura, et al. 2009), fucosylated epitopes (LeX and sLeX) were strongly recognized by the fucose-specific binding lectin AAL. The enzymatic modification performed on chip on GlcNAc-derivatized glycoconjugates also resulted in gain-of-epitope binding patterns (data not shown).

Conjugation and printing of a naturally derived glycan

Our final aim was to test whether this approach is suitable for labelling of natural glycans and preparation of natural glycan arrays. As an example, the major glycan structure of the horseradish peroxidase (HRP) glycoprotein (Yang, et al. 1996) was released by treatment with PNGase A and conjugated with the amino-functionalized linker under optimum conditions described before. After conjugation, excess of linker was partially removed by precipitation of the crude mixture in 90% MeCN. However, two additional purification steps (i.e. G-10 size exclusion chromatography and NP-HPLC) were necessary to obtain the pure glycoconjugate (Figure 5, panel a). After concentration, the glycoconjugate was characterized by MALDI-TOF MS (Figure 5, panel b and c); subsequently immobilized onto a glass slide and interrogated with either lectins or an antibody against HRP; the latter is a well-known polyclonal which detects core α 1,3-fucose and β 1,2-xylose on N-glycans of a

range of non-vertebrate organisms (Paschinger, et al. 2009). The larger size of the glycan chain allows lower immobilization level. As shown in Figure 5 (panel e), the glycan was strongly recognized by the antibody against HRP, even with an immobilization concentration of $\sim 30 \mu\text{M}$, which is lower than on other array platforms. Binding of AAL and ConA also demonstrated the ability of lectins to recognize either non-reducing or reducing parts of the immobilized glycan.

Conclusion

In this work, a derivatization strategy for free glycans is presented which combines, in the same straightforwardly-prepared linker, three important features not present together in other glycan array platforms: (a) one-step chemoselective reaction with free reducing glycans and preservation of the closed-ring form, (b) fluorescent derivatization for higher sensitivity and (c) a terminal azido- or amino- function for glycan immobilization on different surfaces. Several neoglycoconjugates covering the most common monosaccharides found in higher animals, as well as a natural glycan derived from HRP, were prepared, showing that the linker is compatible with both shorter and longer glycan chains. Not only were the conjugation conditions compatible with labile sugar residues (i.e., sialic acids), but the chemically-synthesized neoglycoconjugates could be extended enzymatically prior to testing with lectins. Upon testing the array, a not unexpected trend for lectins to bind more efficiently to di- or oligosaccharides was observed, an effect which can be examined in future experiments using natural N-glycans. With the ability to remodel, purify, characterize, immobilize specific small sugar epitopes and even natural glycans, we now have a tool, which promises to expand the existing range of glycan arrays to include structures previously inaccessible to such studies and so better reveal the variety of glycan-protein interactions in a range of biological systems.

Methods

Synthesis of linker and preparation of azido-functionalized neoglycoconjugates 13-21

Detailed synthetic procedures and characterization of linker (compounds 1 to 12) are described in the Supporting Information. For the conjugation with free reducing mono- and disaccharides, linker 11 (10 equiv.) and the sugar (1 equiv.) were placed in a reaction vial and dissolved in either DMF/AcOH mixture or dioxane/ammonium acetate buffer 1:1 (v:v) pH 4.6. The reaction mixture was stirred at rt, 40 or 60 °C for 24-72 h. The crude reaction was then concentrated under reduced pressure and purified by silica gel column chromatography. The following step gradients were used for purification: $\text{CHCl}_3/\text{MeOH}$ 20:1 for eluting the unreacted linker 11, $\text{CHCl}_3/\text{MeOH}$ 10:1 for eluting neoglycoconjugates 13-18 and $\text{CHCl}_3/\text{MeOH}$ 6:1 for eluting neoglycoconjugates 19-21. A second purification was needed for compound 14 ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 10:1) and compound 16 ($\text{CHCl}_3/\text{MeOH}$ 5:1) to separate isomers. The fractions containing the glycoconjugate were identified by TLC, combined and concentrated to yield the pure neoglycoconjugates as yellowish syrups. Pure neoglycoconjugates were quantified by fluorescence and characterized by NMR.

In solution modification of neoglycoconjugates by glycosyltransferases

All enzymatic modifications started with a 25 μL solution of compound **22**, **27** or **29** (0.45 μg , 0.75 nmol) in 80 mM MES buffer pH 6.5 supplemented with 20 mM MnCl_2 . Nucleotide sugars (50 nmol) and the corresponding glycosyltransferase (3 μL , 3 μg) were added to the reaction mixture prior to overnight incubation at 37 $^\circ\text{C}$. Afterwards an aliquot (0.7 μL) was taken and diluted with MeCN for quantification by HPLC and characterization by MALDI-TOF MS.

Conjugation of amino-functionalized linker **12** with free reducing branched ABH blood group and sialylated trisaccharides

In a 1.5 mL Eppendorf tube, linker **12** (1.4 mg to 17.2 mg, 10 equiv., 1 M) in dioxane/ ammonium acetate buffer 1:1 (v:v) pH 4.6 was added to the dried free reducing glycan (0.2 mg to 2.8 mg, 1 equiv., 100 mM). The mixture was stirred and incubated at 40 $^\circ\text{C}$ in a heating block for 72 h. The crude reaction was concentrated under vacuum (SpeedVac) and cleaned up using NH_2 -SPE cartridges. An isocratic elution with 85% MeCN was used for branched ABH blood group trisaccharides and a gradient elution from 85% to 50% MeCN for sialylated glycoconjugates. Fractions containing the glycoconjugates were lyophilized, purified by NP-HPLC on a Tosoh Amide-80 column with flow rate 1 mL/min and a linear gradient from 75 to 40% B in A over 40 min (A= 10 mM ammonium formate, pH 7; B= 95% MeCN) and characterized by MALDI-TOF MS; 10 pmol of conjugate is estimated to result in a signal of 80 mV.

Conjugation of amino-functionalized linker **12** with naturally-derived HRP-glycans

PNGase A-released glycans from the horseradish peroxidase glycoprotein (HRP; starting with 4.5 mg glycoprotein) were conjugated with the amino-functionalized linker. In a 1.5 mL Eppendorf tube, linker **12** (1.6 mg, 1 M solution in dioxane/ ammonium acetate buffer 1:1 (v:v) pH 4.6) was added to the dried free reducing glycan (maximally 90 μg) and the mixture was incubated at 40 $^\circ\text{C}$ for 24 h. Then, the mixture was concentrated under vacuum (SpeedVac). The glycoconjugate was purified by precipitation with 95% MeCN following the protocol previously described (Song, et al. 2009a). It was necessary to repeat the precipitation step at least three times in order to remove the excess of linker. No glycoconjugate was observed in any of the supernatant fractions. The pellet was dissolved in water and further purified by gel filtration (Sephadex G10) chromatography. Fractions were lyophilized and analyzed by MALDI-TOF MS prior to being subject to NP-HPLC on a Tosoh Amide-80 column with 10 mM ammonium formate pH 7.0 as buffer A, and 95% MeCN as buffer B. For naturally-derived glycoconjugates, the gradient of buffer B was applied as follows: 0-5 min, 70% B; 5-10 min, 70-65% B; 10-15 min, 65% B; 15-20 min, 65- 60% B; 20-25 min, 60% B; 25-30 min, 60-50% B; 30-35 min, 50-45% B; 35-40 min, 45-40% B; 40-45 min, 40% B. The glycoconjugate was collected, lyophilized and analyzed by MALDI-TOF MS; an estimated 2 nmol of pure glycoconjugate was obtained.

Printing of arrays and lectin binding assay

Linker **10** and chemically synthesized glycoconjugates (**22**, **24**, **27**, **29**, **31** and **32**) were spotted at three concentrations (1 nl of 33 μM , 250 μM and 1 mM) on NHS-coated slides.

They were spotted in five replicates using a SpotBot3 Microarray (Arrayit, USA). Neoglycoconjugates with ABH trisaccharides, sialylated trisaccharides and chemo-enzymatically prepared neoglycoconjugates were spotted manually at 250 μ M in two replicates. The HRP-derived neoglycoconjugate was spotted at \sim 30 μ M in two replicates. The spot volume for the manually printing was 0.2 μ L. All samples were printed in 150 mM sodium phosphate pH 8.5 + 0.0025% Tween-20. After printing, the slides were incubated overnight at rt in a humidity chamber to allow amide coupling of free amine to the NHS groups. The slides were blocked with 50 mM ethanolamine in 50 mM sodium borate pH 9.0 for 1 h, and afterwards washed successively with PBS + 0.5% Tween-20, PBS and H₂O. For binding experiments, the slides were incubated with biotinylated lectins at 1-20 μ g/mL or with anti-horseradish peroxidase (1:300 of 10 mg/ml) in PBS + 0.05% Tween for 1 h (no cations were added to the buffer; however, considering Ca²⁺ and/or Mn²⁺ in some lectin preparations, dilution with PBS resulted in submicromolar concentrations of one or both of these ions being present and sufficient for binding in the cases of ConA, ECA, GSA-I, SNA, UEA, VVA and WGA). Afterwards, the slides were washed again and lectin binding detected by incubation with Alexa Fluor[®] 647-conjugated streptavidin at 2 μ g/mL for 1 h in the dark. Binding of anti-HRP antibody was detected using Alexa Fluor[®] 647-conjugated goat anti-rabbit IgG. Subsequently, the slides were washed as described above and scanned using the Agilent G2565CA Microarray scanner. The integrated spot intensities were determined using Imagene software (BioDiscovery; demonstration version) and converted from red colour to white. Dependent on the concentration of lectin used, the limit of detection of disaccharide conjugates is < 33 fmol for the robotically-printed array.

Supplementary Information

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

anti-HRP	anti-horseradish peroxidase
AAL	<i>Aleuria aurantia</i> lectin
ConA	concanavalin A
ECA	<i>Erythrina cristagalli</i> agglutinin
GSA-I	<i>Griffonia simplicifolia</i> agglutinin I
MAA	<i>Maackia amurensis</i> agglutinin

RCA I	<i>Ricinus communis</i> agglutinin I
SNA	<i>Sambucus nigra</i> agglutinin
UEA-I	<i>Ulex europaeus</i> agglutinin I
VVA	<i>Vicia villosa</i> agglutinin
WGA	wheat germ agglutinin

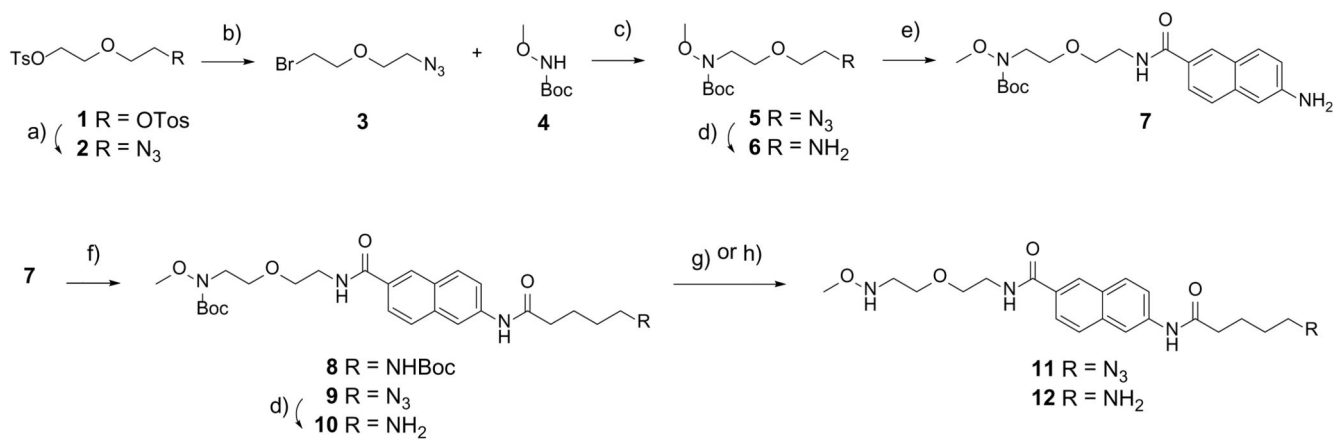
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Scheme 1. Synthesis of methoxy linkers

Reagents and conditions: a) NaN_3 , DMF, 70 °C (62%); b) LiBr, DMF, 70 °C (82%); c) NaH, DMF (91%); d) H_2 , Pd/C 10% w/w, MeOH (74% for **6**, 99% for **10**); e) TBTU/HOBt/ Et_3N , THF/DMF (78%), f) HATU/DIPEA, DMF (92% for **8**; 77% for **9**); g) TFA, CH_2Cl_2 , rt (92%). h) 3 M HCl, dioxane (94%)

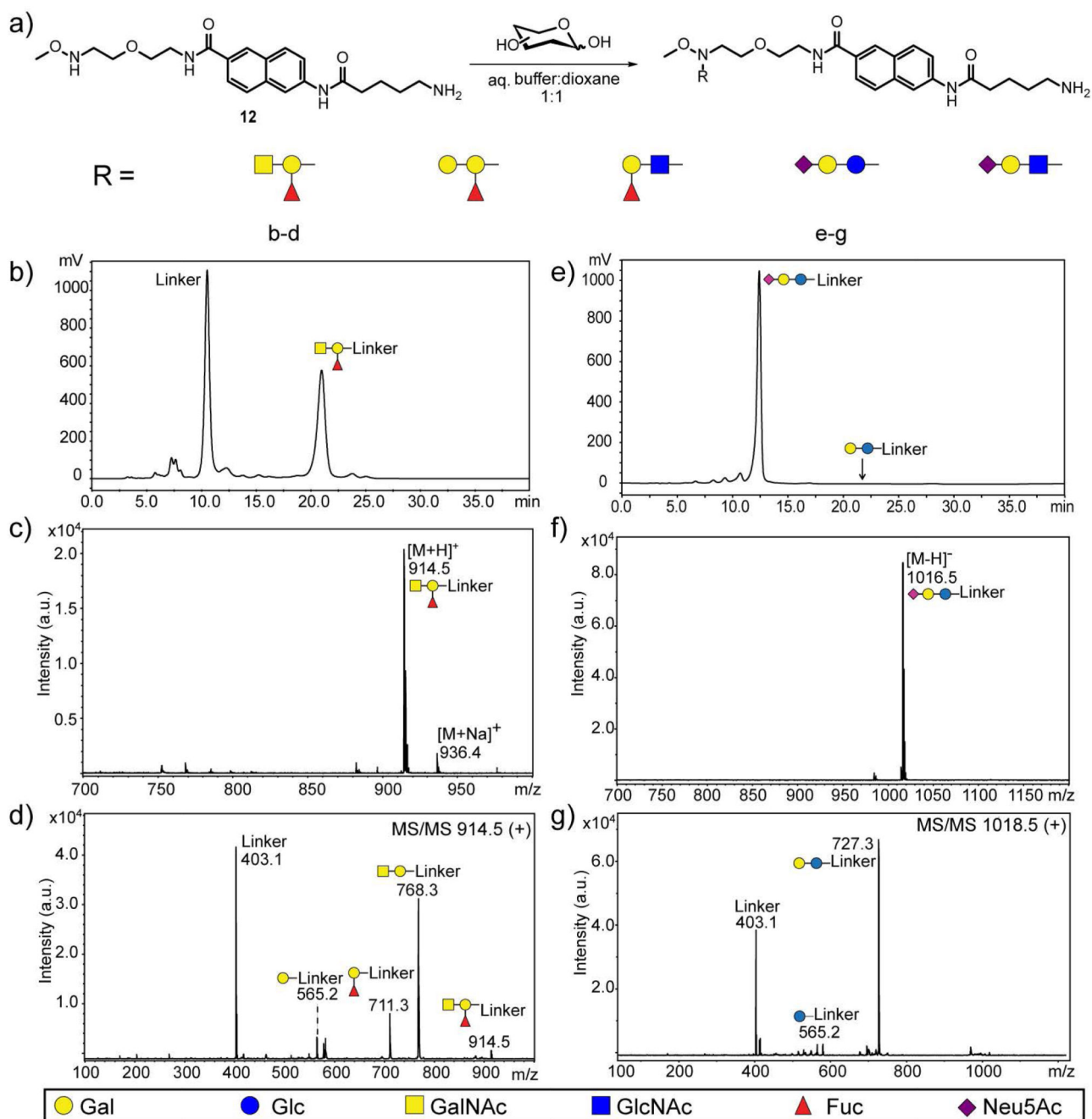


Fig. 1. Conjugation to amino-functionalized linker **12** with branched glycan structures and sialyl-containing trisaccharides. a) Schematic representation of conjugation and glycan structures tested. b-d) Conjugation with blood group A trisaccharide. (b) NP-HPLC chromatogram of crude mixture after NH₂-SPE clean-up. c-d) MALDI-TOF MS and MS/MS analysis of blood group A-linked glycoconjugate (GalNAc_{1,3}(Fuc_α1,2)Gal_β1-R). e-g) Conjugation with Neu5Ac- α 2,6-lactose (NeuAc_{2,6}Gal_β1,4Glc_β1-R). e) NP-HPLC chromatogram of crude mixture after NH₂-SPE clean-up. f-g) MALDI-TOF MS and MS/MS analysis of

Neu5Ac- α 2,6-lactose-containing glycoconjugate. Glycan structures are shown using the symbol nomenclature for glycans (SNFG) adopted by the Consortium for Functional Glycomics and for *Essentials of Glycobiology* (Varki, et al. 2015).

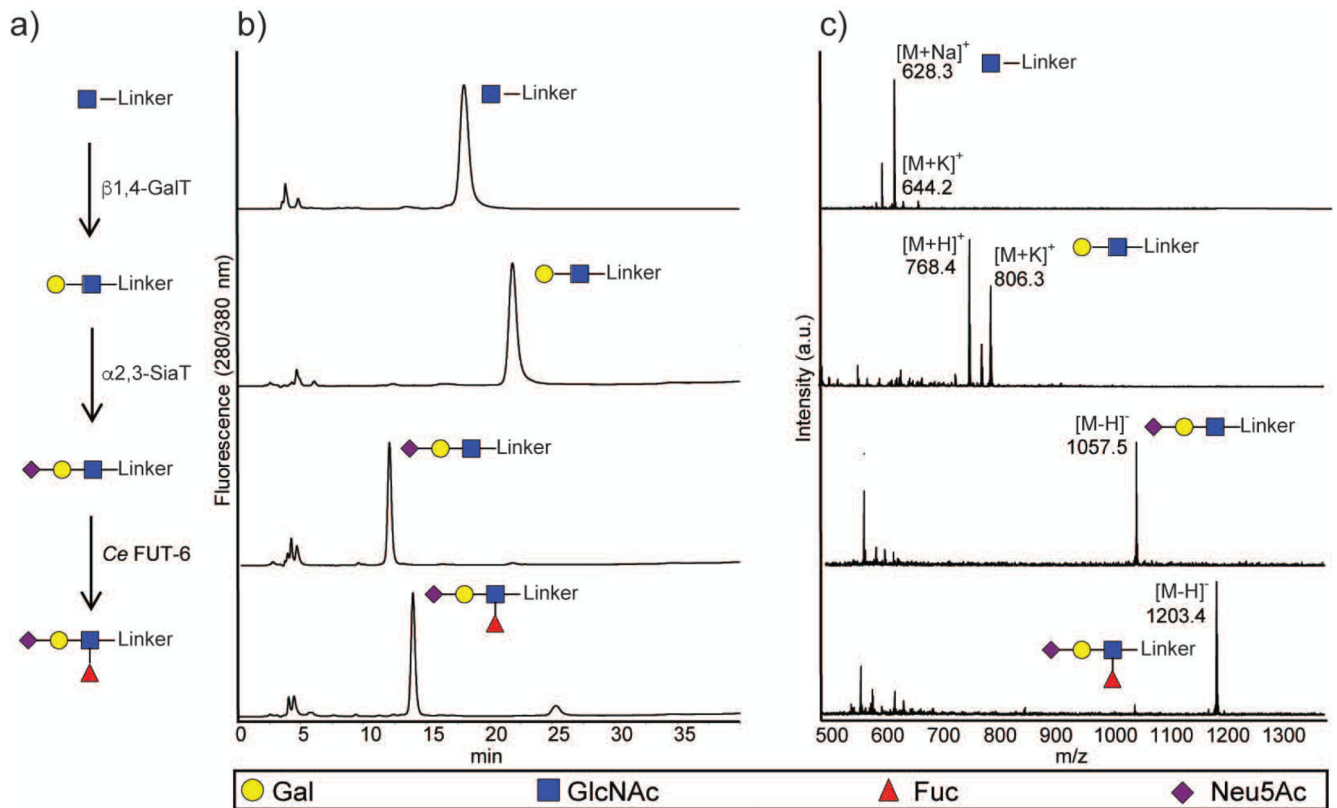


Fig. 2. *In solution* chemo-enzymatic synthesis of sLeX epitope. a) Schematic representation of enzymatic modification of GlcNAc-neoglycoconjugate (30 μ M) with bovine β 1,4-galactosyltransferase, *Neisseria* α 2,3-sialyltransferase and the *Caenorhabditis* FUT-6 α 1,3-fucosyltransferase. b) NP-HPLC analysis of starting material, intermediates and final sialyl Lewis X product NeuAca2,3Gal β 1,4(Fuca1,3)GlcNAc β 1-R with detection by fluorescence. c) MALDI-TOF analysis of crude reactions after each enzymatic modification in either positive or negative ion modes (a.u., arbitrary units). Symbols for each glycan are shown using SFNG nomenclature; glycosidic linkages and abbreviated names are given in Table 2.

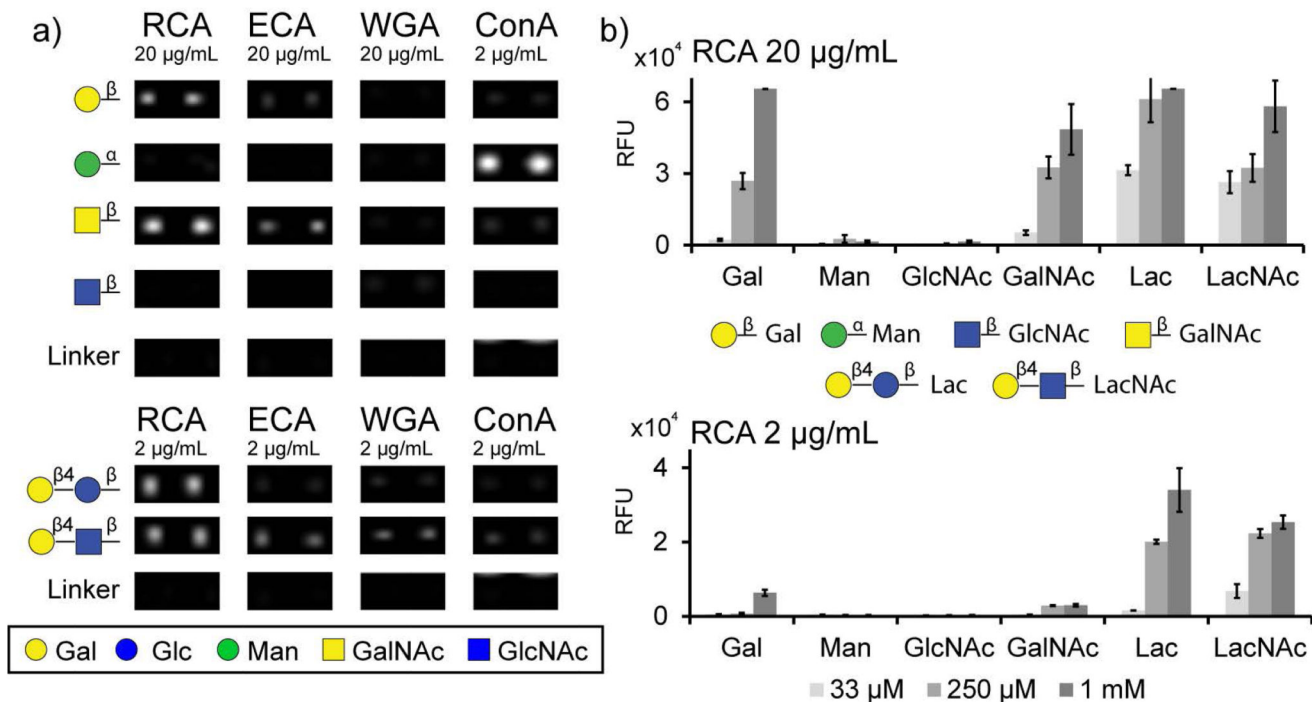


Fig. 3. Glycan microarray with chemically synthesized neoglycoconjugates displaying mono- and disaccharides. a) Image of lectin binding to robotically-printed glycoconjugates immobilized at 250 µM (1 nl; 100 µm diameter). Microarray was incubated with several biotinylated lectins, such as RCA, ConA, WGA and ECA at 2 and 20 µg/mL. Lectin binding was detected using Alexa Fluor[®] 647-conjugated streptavidin (at 2 µg/mL). Two representative spots from five replicates are shown. b) Histogram of quantified RCA binding at 2 and 20 µg/mL on different immobilization levels (33 µM, 250 µM, 1 mM). A full set of bar charts for ConA, ECA and WGA is shown in Supplementary Figure 2 (A-G).

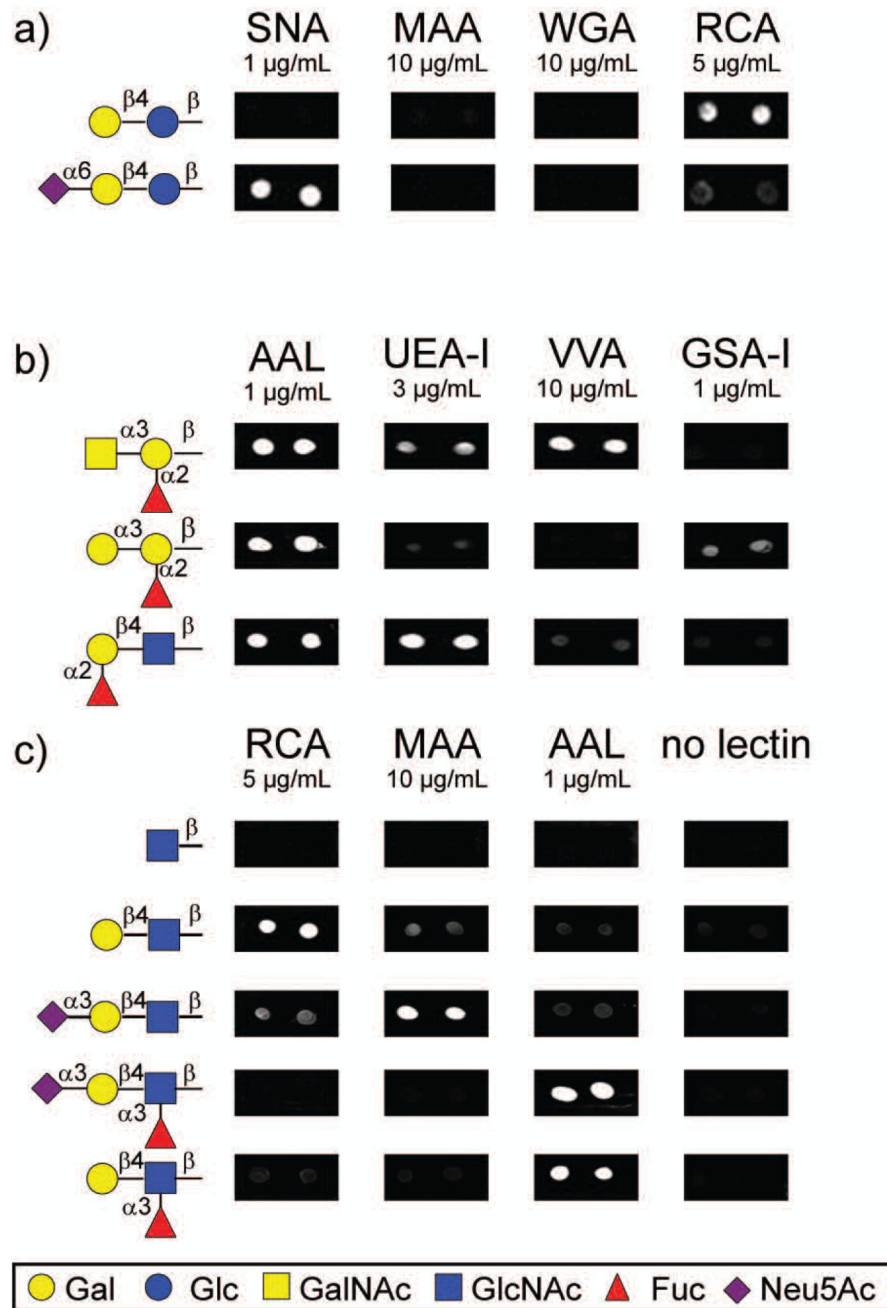
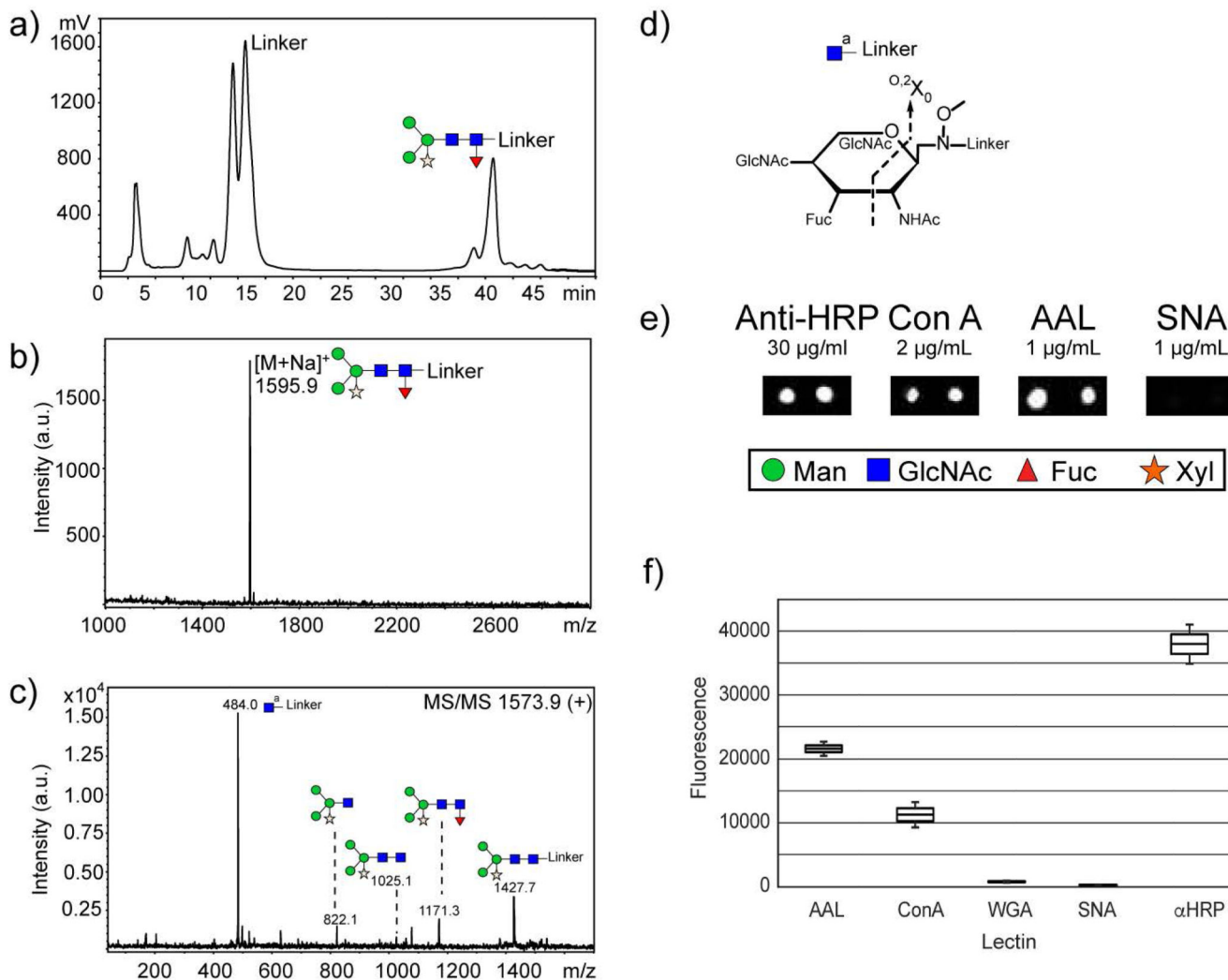


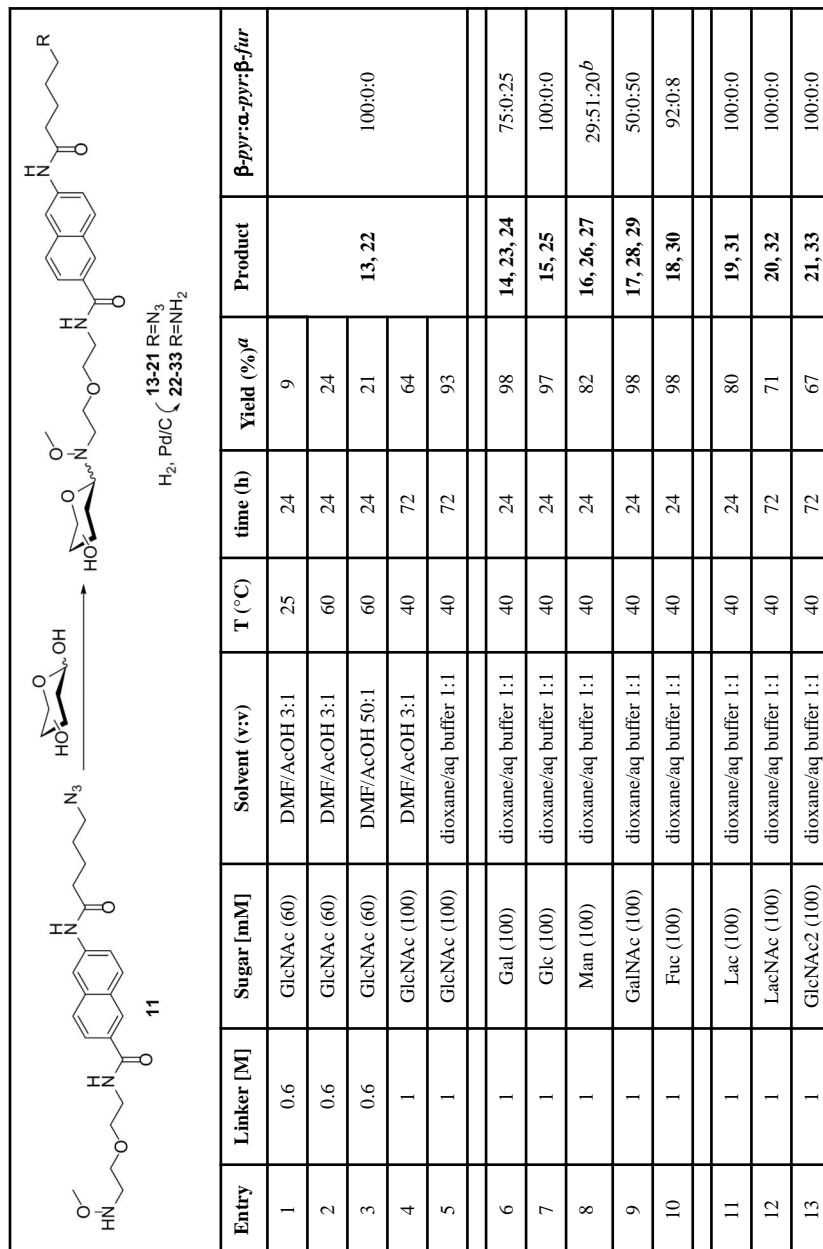
Fig. 4. Further examples of microarrays prepared in this study. Chemically-synthesized neoglycoconjugates (a, b) and chemo-enzymatically modified neoglycoconjugates (c) were immobilized at 250 µM on NHS-activated slides (0.2 µl hand-printed; 1 mm diameter). After blocking with ethanolamine, microarrays were interrogated with several lectins at different concentrations. Glycan-protein binding was detected using Alexa Fluor® 647-conjugated streptavidin (at 2 µg/mL). The structures are summarised in Table 2 and a full set of bar charts is shown in Supplementary Figure 2 (H-I).

**Fig. 5.**

Conjugation of amino-functionalized linker **12** with major HRP-derived glycan. a) NP-HPLC chromatogram of an aliquot of the crude reaction of the linker with the PNGase A-released glycan after G-10 size-exclusion chromatography. b) MALDI-TOF MS spectrum of pure glycoconjugate. c) MALDI-TOF MS/MS analysis of HRP-containing glycoconjugate. d) Schematic representation of the observed cross-ring cleavage. e) A natural array was prepared with neoglycoconjugate immobilized at $\sim 30 \mu\text{M}$ on NHS-activated slides ($0.2 \mu\text{l}$ hand-printed; 1 mm diameter) and incubated with biotinylated lectins and a rabbit anti-HRP antibody. Binding was detected using Alexa Fluor[®] 647-conjugated streptavidin (at $2 \mu\text{g/mL}$) or Alexa Fluor[®] 647-conjugated goat anti-rabbit IgG (at $2 \mu\text{g/mL}$); SNA was used as a negative control. f) Box and whisker plot of the array data.

Table I












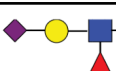

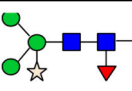
Neoglycosylation conditions and glycoside stereochemistry



Entry	Linker [M]	Sugar [mM]	Solvent (v:v)	T (°C)	time (h)	Yield (%) ^a	Product	β -pyr: α -pyr: β -fur
1	0.6	GlcNAc (60)	DMF/AcOH 3:1	25	24	9		
2	0.6	GlcNAc (60)	DMF/AcOH 3:1	60	24	24		
3	0.6	GlcNAc (60)	DMF/AcOH 50:1	60	24	21	13, 22	100:0:0
4	1	GlcNAc (100)	DMF/AcOH 3:1	40	72	64		
5	1	GlcNAc (100)	dioxane/aq buffer 1:1	40	72	93		
6	1	Gal (100)	dioxane/aq buffer 1:1	40	24	98	14, 23, 24	75:0:25
7	1	Glc (100)	dioxane/aq buffer 1:1	40	24	97	15, 25	100:0:0
8	1	Man (100)	dioxane/aq buffer 1:1	40	24	82	16, 26, 27	29:51:20 ^b
9	1	GalNAc (100)	dioxane/aq buffer 1:1	40	24	98	17, 28, 29	50:0:50
10	1	Fuc (100)	dioxane/aq buffer 1:1	40	24	98	18, 30	92:0:8
11	1	Lac (100)	dioxane/aq buffer 1:1	40	24	80	19, 31	100:0:0
12	1	LacNAc (100)	dioxane/aq buffer 1:1	40	72	71	20, 32	100:0:0
13	1	GlcNAc2 (100)	dioxane/aq buffer 1:1	40	72	67	21, 33	100:0:0

^aEstimated by quantification of fluorescence intensity after purification.^bThe anomeric configuration could not be definitely proven.

Table II
Conjugate structures tested on arrays

Structure	Depiction	Figure	Reactivity
Gal β 1-R		3	RCA+
Man α 1-R		3	ConA+
GalNAc β 1-R		3	RCA+
GlcNAc β 1-R		3, 4	n.d.
Gal β 1,4Glc β 1-R (Lac)		3, 4	RCA+
Gal β 1,4GlcNAc β 1-R (LacNAc)		3, 4	RCA/ECA/WGA+
NeuAca2,6Gal β 1,4Glc β 1-R (6'-SialylLac)		4	SNA+
GalNAca1,3(Fuca1,2)Gal β 1-R (Blood group A)		4	AAL/UEA/VVA+
Gala1,3(Fuca1,2)Gal β 1-R (Blood group B)		4	AAL/GSA+
Fuca1,2Gal β 1,4GlcNAc β 1-R (Blood group H)		4	AAL/UEA+
NeuAca2,3Gal β 1,4GlcNAc β 1-R (3'-SialylLacNAc)		4	MAA+
NeuAca2,3Gal β 1,4(Fuca1,3)GlcNAc β 1-R (SialylLeX)		4	AAL+
Gal β 1,4(Fuca1,3)GlcNAc β 1-R (LeX)		4	AAL+
Man α 1,6(Man α 1,3)(Xyl β 1,2)Man β 1,4GlcNAc β 1,4(Fuca1,3)GlcNAc β 1-R		5	AAL/ConA/ α HRP+