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Protecting group-free immobilization of glycans for affinity chromatography using glycosylsulfonohydrazide donors

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ABSTRACT

A variety of applications in glycobiology exploit affinity chromatography through the immobilization of glycans to a solid support. Although several strategies are known, they may provide certain advantages or disadvantages in how the sugar is attached to the affinity matrix. Additionally, the products of some methods may be hard to characterize chemically due to non-specific reactions. The lack of specificity in standard immobilization reactions makes affinity chromatography with expensive oligosaccharides challenging. As a result, methods for specific and efficient immobilization of oligosaccharides remain of interest. Herein, we present a method for the immobilization of saccharides using *N*′-glycosylsulfonohydrazide (GSH) carbohydrate donors. We have compared GSH immobilization to known strategies, including the use of divinyl sulfone (DVS) and cyanuric chloride (CC), for the generation of affinity matrices. We compared immobilization methods by determining their immobilization efficiency, based on a comparison of the mass of immobilized carbohydrate and the concentration of active binding sites (determined using lectins). Our results indicate that immobilization using GSH donors can provide comparable amounts of carbohydrate epitopes on solid support while consuming almost half of the material required for DVS immobilization. The lectin binding capacity observed for these two methods suggests that GSH immobilization is more efficient. We propose that this method of oligosaccharide immobilization will be an important tool for glycobiologists working with precious glycan samples purified from biological sources.

1. Introduction

Affinity chromatography is a method for the purification of biomolecules based on specific and reversible binding of a receptor and its ligand.[1] We define the ligand as the binding partner that is immobilized onto an insoluble support and the target as the binding partner that is dissolved in a mobile phase. This methodology, developed almost 45 years ago, has revolutionized the fields of modern biology, chemistry, molecular biology, and biotechnology.[2] Affinity chromatography has often been employed for the purification of lectins using immobilized carbohydrates.[3,4] In contrast to protein-based ligands, there are specific issues for affinity chromatography related to the use of carbohydrate-based ligands. Carbohydrate ligands do not typically contain free amine groups, and chemistries that can form covalent linkages between the hydroxyl groups of the ligand and the solid support are required. The selection of the affinity matrix also contributes to the ligand immobilization strategy. The chosen matrix must have minimal non-specific interactions with the target, be macroporous to allow entry of large biomolecules, be physically and chemically stable, and have uniform characteristics.[1] Commercially available matrices used for affinity purification include agarose, cellulose, silica, polyacrylamide, polystyrene, and dextrose. Sepharose, an agarose-based (Gal-β1,4-[3,6]-anhydro-L-Gal) support, is often used for affinity chromatography and is tolerant of a large pH range and organic solvents.[1,5,6]

Linker chemistry for affinity chromatography should ideally be non-destructive to the binding epitope of the ligand and feature a defined point of attachment. For example, reductive amination can be used to immobilize glycans, but the attached product results in a ring-opened reducing end.[7] A variety of coupling methods for the immobilization of carbohydrate ligands to solid support have been developed,[6,8] but few feature defined linker chemistry for carbohydrates. Limited tools exist for affinity chromatography of complex oligosaccharides obtained from biological sources as free reducing sugars. We chose to compare some established methods for immobilization of carbohydrates to Sepharose with newly available protecting-group free glycosidation chemistries.[9]

Divinyl sulfone (DVS) is an electrophilic homobifunctional reagent capable of crosslinking hydroxyl nucleophiles. In an affinity chromatography experiment, DVS can be used to activate a hydroxyl-containing solid support through a Michael addition to one of the vinyl groups of the sulfone. Crosslinking can be minimized through control of stoichiometry.[10] A range of functional groups can react

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with DVS as nucleophiles, including amine and hydroxyl groups. For hydroxyl nucleophiles the reaction must be carried out at a pH greater than 10 to allow efficient formation of the ether linkage. DVS-coupled affinity ligands should not be exposed to conditions exceeding pH 8.5 to avoid potential retro-Michael degradation.[11] DVS activation has been used for immobilization of carbohydrates onto surfaces and solid supports for affinity chromatography.[3,12] Cyanuric chloride (CC) is a trifunctional, heterocyclic reagent which has been used for the immobilization of carbohydrates for affinity chromatography and binding assays.[13] The reactivity of CC is dependent upon substitution of the ring. The resulting linkage between a carbohydrate ligand and a solid support generated by CC coupling has increased stability at high pH relative to DVS-immobilized ligands.[14]

Recently, Nitz and coworkers have reported a method for protecting group-free glycosidation reactions based on the use of *N*′-glycosyltoluenesulfonohydrazide (GSH) donors.[9,15] This method results in selective reaction at the anomeric position of the donor in the presence of a slight excess of the acceptor. Additionally, in the case of 2-acetimido sugars, such as *N*-acetylglucosamine (GlcNAc), the reaction preferentially forms the β-anomer.[9] The conditions for the reaction are mild for the formation of the activated donor, as well as for the glycosidation reaction. We considered that this chemistry would be ideal for the immobilization of reducing oligosaccharides onto solid support. In this configuration, the hydroxy groups of a solid support would act as the glycosyl acceptor and the glycosyl hydrazide would act as the donor. This method should have several advantages over standard immobilization chemistries for carbohydrates. First, the method would not require an activation of the resin and could be performed under very mild conditions. Moreover, no capping or inactivation step would be required at the end of the reaction, reducing the likelihood of non-specific interactions. Most importantly, this strategy would offer the advantage of providing a regioselective and non-destructive immobilization chemistry for complex glycans obtained from biological sources.

Herein we develop a mild and protecting group-free protocol for the immobilization of carbohydrate ligands to a cross-linked agarose support (Sepharose CL-6B) using GSH donors. We envisioned that this method could provide similar levels of immobilization to existing chemistries but would be complimentary due to its regio- and stereo-selectivity. We compared our method with DVS and CC chemistries as a benchmark for existing methods used in lectin affinity chromatography. Each method was compared based on its immobilization efficiency and carbohydrate binding capacity of the resulting matrix. The immobilized ligands prepared by GSH were also characterized by high-resolution magic angle spinning (HR-MAS) ^{1}H NMR.

2. Results

2.1. Functionalization of monosaccharides with DVS in solution

We first set out to compare the selected immobilization chemistries in solution to help establish reaction conditions and characterization data.[16,17] We took as a starting point the method reported by Fornstedt and Porath for the immobilization of D-mannose onto a solid support with DVS as a linker.[3] Initially we carried out the reaction using five equivalents of DVS to one equivalent of Gal (Fig. 1a). These conditions afforded a mixture of products that included mono, di, tri, and tetra-DVS-functionalized glycosides of Gal (**1**) in 60% yield. Using a similar methodology with reduced equivalents of DVS, we were able to isolate a mixture of modified glycosides of GlcNAc (**2**) in 51% crude yield (Fig. 1b). Lower ratios of DVS to monosaccharide (0.2:1) simplified the product mixture, likely by limiting the number of multiply-functionalized saccharides.[10] The ^{1}H NMR spectra of the mixture **2** revealed that H-1 shifted upfield by 0.15–0.20 ppm for both anomers (from 5.09 to 4.89 ppm for H-1α and from 4.57 to 4.42 ppm for H-1β). Due to overlap with the residual HOD signal, an accurate α/β ratio could not be obtained. These data suggest that substitution occurred primarily at O-1 of the monosaccharide, in addition to minor products resulting from reaction at other sites on the ring. This finding is consistent with observations from Cheng, et al.[12,18] and confirms that the promiscuity of the DVS reaction with glycans can result in complex mixtures.

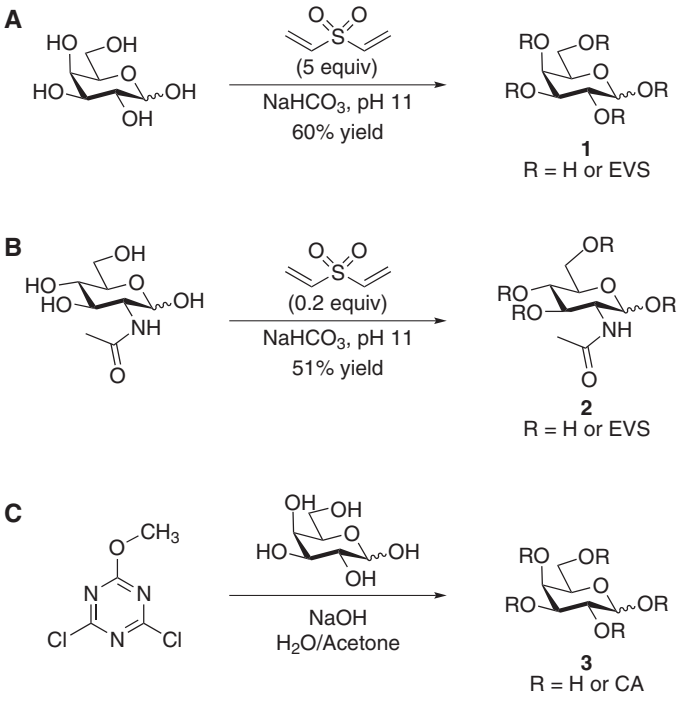

Fig. 1. DVS-functionalization of monosaccharides in solution. Monosaccharides (a) Gal and (b) GlcNAc were reacted with DVS in alkaline conditions to provide mixtures of ethyl vinyl sulfone (EVS) products (1 and 2). Using conditions previously reported,[13] we attempted derivitization of Gal with CC chemistry (c). The reaction afforded a mixture of mono- and di-substituted products (3).

2.2. Functionalization of monosaccharides with cyanuric chloride in solution

Cyanuric chloride (CC) has previously been used for immobilization of carbohydrates to solid support. We attempted to follow reported protocols;[19] however, in our hands these conditions were low yielding and resulted in hydrolysis of the cyanuric chloride to afford the corresponding cyanuric acid (CA). To investigate the site of attachment and the efficiency of the immobilization of carbohydrates using CC, we tested modified conditions (Fig. 1c).[13] The reaction afforded a mixture of products including the hydrolyzed, monosubstituted, and disubstituted triazine (**3**). The products were isolated using preparative TLC as described in Section 4. The ^{1}H NMR spectrum of the material shows a complex set of peaks between 5.5 and 4.4 ppm indicating substitution at more than one of the hydroxyl groups on the monosaccharide and consistent with previous reports.[13]

2.3. Glycosidations of N′-glycosyltoluenesulfonohydrazide donors in solution

Previous reports have used GSH donors for protecting group-free glycosidations in solution.[9,15] Using reported conditions, we generated glycosides using octanol as an acceptor. We generated the octyl glycosides of Gal, lactose (Lac), and GlcNAc (Fig. 2; compounds **5**, **7**, and **9** respectively).[9,15] The glycosidation reactions were

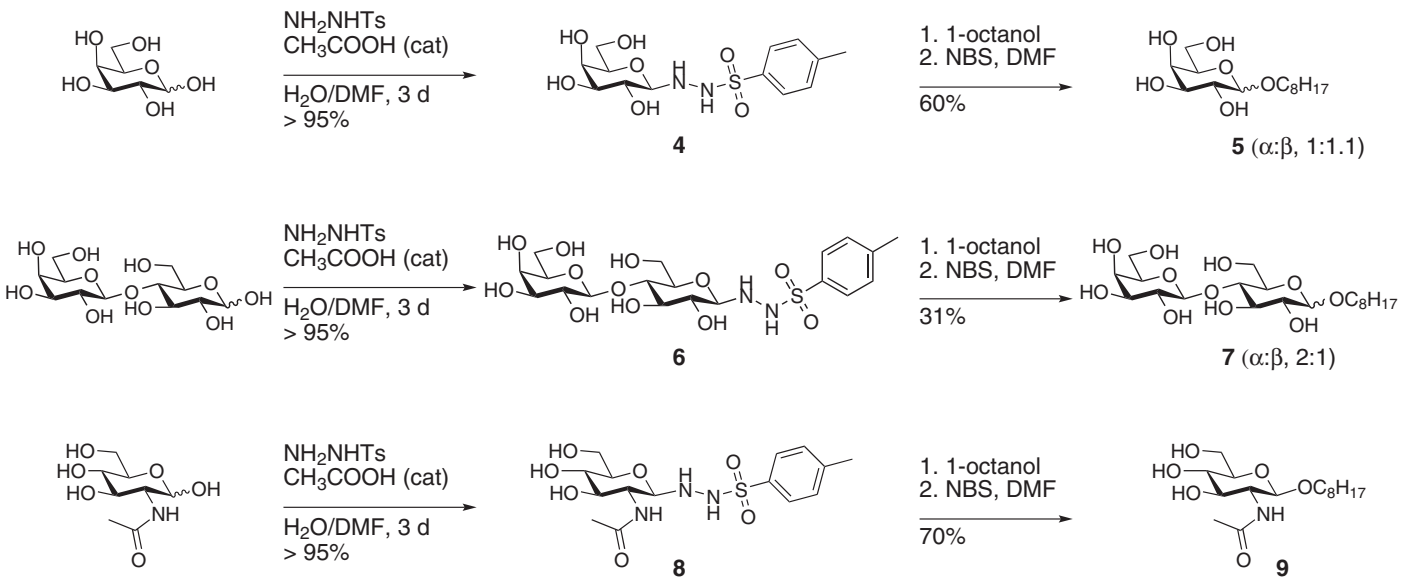

Fig. 2. Glycosidation reactions with GSH-donors in solution. Glycosidation of Gal, Lac, and GlcNAc using the corresponding GSH donors.

carried out under anhydrous conditions to minimize hydrolysis of the donor. The corresponding glycosides were obtained in good purity with yields ranging from 31 to 70%. The GSH glycosidations showed a small preference for the formation of the β-anomer in the case of Gal, and formed the β-anomer exclusively in the case of GlcNAc. It is possible that increased equivalents of acceptor would improve the yield of glycosylation.[20] The yields and anomeric selectivity obtained in these solution–phase reactions provided an indication of their suitability for carrying out the reaction in heterogeneous phase.

Examination of our results in solution phase confirmed that, in contrast to both DVS and CC chemistries, GSH donors were able to provide regioselective functionalization of the saccharides tested. We observed that the main disadvantage of the DVS method was the formation of polymers or polyfunctionalization of the glycans. In the case of CC, the method was low yielding in solution and resulted in multiple products. The GSH method was straightforward and provided moderate yields; its main disadvantage was the need for anhydrous conditions in order to preserve the glycosyl donor. However, GSH was unique among these methods in providing a single, regioselective product. Based on these observations, we expected the DVS and the GSH method to perform best in reactions carried out in heterogeneous phase.

2.4. *Immobilization of carbohydrate epitopes to solid support*

As discussed above, our aim in the current study was to compare methods of carbohydrate immobilization. We chose to use Gal, Lac, and GlcNAc saccharides as the ligands for our study and Sepharose as the solid support. The immobilization of carbohydrates for affinity purification using DVS or CC as linkers is well established, and we followed known protocols for their use.[3,19] For GSH immobilization we based our protocol around the solution studies described above with some minor modifications. The resin was transferred from a slurry in aqueous solution to dry DMF by washing with increasing concentrations of DMF in water. The *N′*-glycosyltoluenesulfonylhydrazide donor was then added to the mixture followed by activation using NBS, where the hydroxyl groups of the solid support act as the glycosyl acceptor (Fig. 3). The functionalized resin was then washed and used in further analyses.

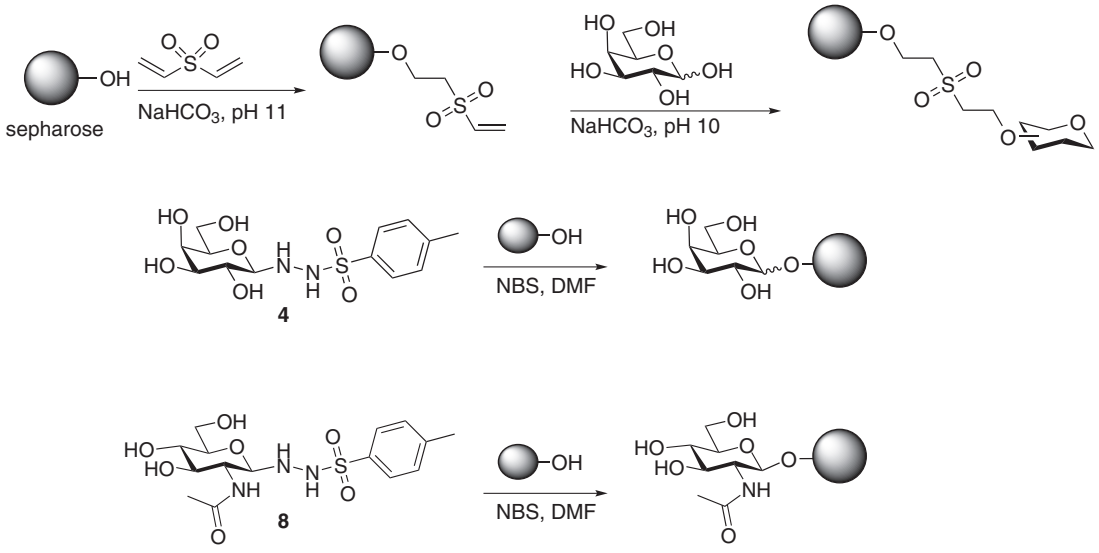

Fig. 3. Immobilization of galactose to Sepharose using DVS and GSH chemistries.

2.5. Quantification of immobilized carbohydrate using the phenol sulfuric acid assay

There are several methods for the determination of carbohydrate content in aqueous solutions. Among these, colorimetric methods are most commonly used because of their versatility and low cost.[21] Common coloring reagents for hydrolyzed monosaccharides include phenol,[22] alkaline ferricyanide ($K_4Fe(CN)_6$),[23] and anthrone.[16] We found that the anthrone method was difficult to implement due to interference from the Sepharose support. The phenol-sulfuric acid assay (PSA), which relies on acidic hydrolysis followed by dehydration to afford furfural degradation products, provided consistent determinations of modified Sepharose despite background from the support.[17,22] The reaction between the furfural and phenol in the PSA produces a yellow–orange color with maximum absorbance at 490 nm.[22,24]

We used the PSA to compare the efficiency of carbohydrate immobilization for each of the immobilization chemistries discussed above with three saccharides (Gal, Lac, and GlcNAc) (Table S1 and Fig. 4). The PSA data indicate that the GSH method gave the highest immobilization efficiency among the three methods tested. In comparison with the DVS method, GSH immobilized five to nine times more carbohydrate per milligram of resin, and approximately double the amount from CC immobilization. A possible explanation for the reduced efficiency of the DVS and CC method is consumption of activated sites due to the use of aqueous reaction conditions for immobilization, whereas the GSH method was carried out under anhydrous conditions. Analysis of GlcNAc-functionalized resins gave significantly lower loading capacity by PSA. This discrepancy is likely due to the reduced reactivity of amino sugars in colorimetric methods as compared to neutral sugars and likely is not an accurate measure of the amount of GlcNAc monosaccharide immobilized.[25,26] It is also possible that each linker chemistry has a different susceptibility to acidic hydrolysis conditions that could limit the release of immobilized carbohydrate.

2.6. HR-MAS ^{1}H NMR of functionalized Sepharose

HR-MAS NMR is a technique for obtaining NMR from solids.[27,28] We envisioned that HR-MAS NMR could be used to provide information about the regio- or stereochemistry of immobilized carbohydrate ligands on the solid support. We collected ^{1}H spectra of Sepharose samples using HR-MAS NMR and compared them to unmodified or control samples to look for resolved resonances that could be attributed to the immobilized ligands. One inherent challenge from this approach is that the Sepharose support is a carbohydrate polymer; thus, a considerable portion of the carbohydrate region in ^{1}H spectrum (4–2 ppm) was obscured. The DVS-activated resin samples show the appearance of vinylic protons attributed to the sulfone with good resolution at 6.8–6.3 ppm (Fig. S3). We next examined samples of DVS-activated resin reacted with Gal, and were pleased to observe the appearance of new peaks (δ = 5.19 and 4.51 ppm) close to the anomeric signals observed for Gal in solution (δ = 5.25 and 4.57 ppm). Notably, the chemical shift and coupling constants of H-1 and vinylic protons corresponded to those observed in solution phase NMR data. The HR-MAS ^{1}H NMR data were consistent with the immobilization of an anomeric mixture of Gal by DVS. Unfortunately, the H-1 peaks were not sufficiently resolved to allow integration. Analysis of Gal, Lac, and GlcNAc–Sepharose conjugates prepared using GSH glycosidation by HR-MAS ^{1}H NMR did not show the appearance of any substantial new peaks (Fig. S4). As a result, we could not confirm the anomeric selectivity of the GSH reaction with Sepharose. It is notable that HR-MAS ^{1}H NMR of the DVS-linked residues provided resolved peaks, while the glycosidation reaction did not. It is possible that the DVS linker provides sufficient spacing and flexibility from the matrix to allow detection by HR-MAS ^{1}H NMR.[29] In contrast, glycosidation with GSH directly links the residue to the solid support, which may result in more line broadening making signals from the carbohydrate epitope indistinguishable from that of the matrix.[30]

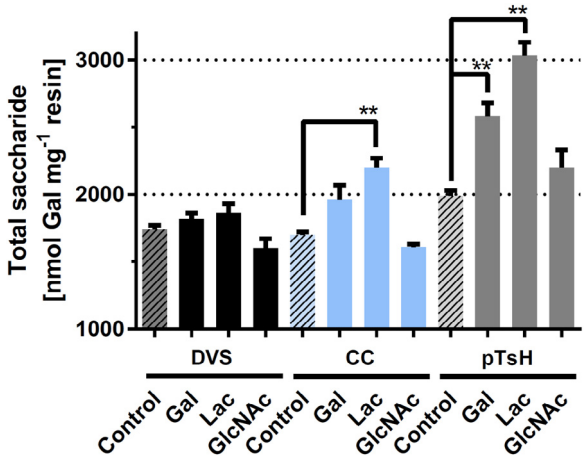

Fig. 4. Immobilization efficiency of Sepharose as determined by PSA. Results of the PSA were compared against the corresponding control for each chemistry (hashed bars) using a two-tailed Student's t-test (**$p < 0.001$). The control samples for DVS and CC were activated and quenched using 2-mercaptoethanol, the control for GSH was unmodified Sepharose. The results are presented as the mean ± SEM. All resin samples were 1 mg, and were prepared with 1.1 mmol of carbohydrate mL^{-1} of solid support.

2.7. Determination of lectin binding capacity

After quantifying the amount of carbohydrate immobilized by each method, we considered that different methods could give different amounts of active carbohydrate epitopes. For example, if a binding interaction is dependent upon the 6-OH of Gal, there may be fewer active sites in a DVS-immobilized sample than would be expected based on the mass of immobilized saccharide. Additionally, certain methods may provide for multiple sites of attachment to occur in a single ligand, which could lead to inactivation. Thus, we sought to use lectin binding to probe the availability of the immobilized epitopes.[31] We employed an immuno-precipitation assay, where a small amount of derivatized solid support was incubated with a known mass of protein in solution followed by elution and measurement of the bound protein using fluorescence spectroscopy. We selected two readily available lectins, Jacalin and the wheat-germ agglutinin (WGA), for our binding measurements. Jacalin is a lectin obtained from the seeds of *Artocaspus integrifolia*, commonly known as the jackfruit lectin or AIA, and is selective for Gal and GalNAc.[32] Jacalin binds preferentially to the α-anomer of Gal;[33] however, β-galactosides are known to bind.[34,35] WGA is a member of the cereal lectin family, and is a mixture of three isolectins.[32,36,37] The WGA lectin binds to GlcNAc containing glycans, such as *N*,*N*′-diacetyl-chitobiose, and has little preference for the anomeric configuration of its ligands.[36–38] Our binding results with immobilized carbohydrates are summarized in Tables S2 and S3, and Fig. 5.

Experiments with WGA found that both DVS and GSH methods provided similar binding capacity for immobilized GlcNAc. In our hands, CC immobilization did not provide detectable binding capacity for WGA or Jacalin. To explore the differences in binding capacity for DVS and GSH immobilization chemistry, we prepared a series of immobilized resin samples with different GlcNAc loading densities (Fig. 5a). After testing each of these resins for relative binding to WGA, we observed that GSH gave 2- to 3-fold improved

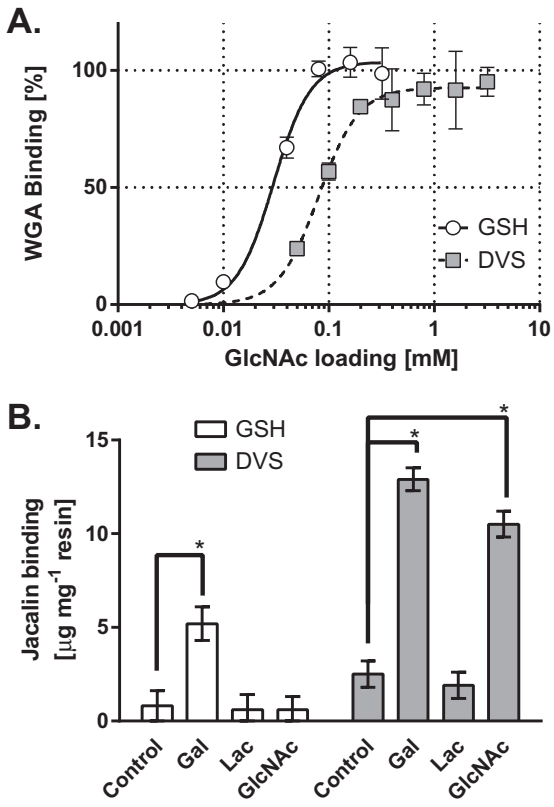

Fig. 5. Binding of lectins to immobilized carbohydrates. (a) The binding of FITC-WGA was determined for a series of resin samples functionalized with varying amounts of GlcNAc as indicated on the abscissa as the loading concentration. Data shown are from a representative run with three replicates for each point, error bars represent the standard deviation. (b) Three batches of resin functionalized with 1.1 mmol of carbohydrate mL^{-1} of solid support with the indicated carbohydrate were generated using DVS and GSH chemistry, and the binding capacity of each batch was measured in triplicate (N = 9). Binding of FITC-Jacalin was determined by incubating 1 mg of each resin sample with 75 µg mL^{-1} of lectin and measuring the reduction in fluorescence due to binding. The results are presented as the mean ± SEM. Samples were compared to the relevant control using a two-tailed Student's t-test (*$p < 0.05$).

immobilization efficiency for the same loading density of ligand over DVS.

We combined our measurements of immobilized monosaccharides with the binding capacity measurements obtained for WGA. To compare measurements across all the chemistries employed here, we used batches of resin immobilized with either 1.11 or 0.64 mmol of saccharide mL^{-1} of resin (Table 1). For determination of the ratio of protein bound $mmol^{-1}$ of carbohydrate used, data from Table S3 were converted to micrograms of WGA $mmol^{-1}$ of GlcNAc immobilized based on PSA and lectin binding results. The analysis presented in Table 1 supports the hypothesis that GSH immobilization was more efficient at lower loading (0.64 mmol), despite higher loading resulting in more immobilized carbohydrate. This finding may be an indication that higher loading of the resin results in steric crowding of binding sites reducing overall protein binding capacity.

Table 1
Relative efficiency of coupling methods. The efficiencies are expressed as a ratio of the µg of WGA bound $mmol^{-1}$ of carbohydrate used at the coupling step, and presented as mean ± SEM.

Coupling method	WGA bound [µg mg^{-1} resin]	GlcNAc loading [mmol mL^{-1} resin]	Ratio [µg WGA $mmol^{-1}$ of GlcNAc]
DVS	11.82 ± 0.05	1.11	600 ± 3.0
CC	0.8 ± 0.3	1.11	40 ± 20
GSH	9.8 ± 0.3	1.11	500 ± 20
	10.7 ± 0.4†	0.64	1000 ± 40

† Replicates for samples were N = 9 or 3.

Finally, we analyzed the binding of Jacalin to a series of immobilized saccharides using DVS and GSH strategies (Fig. 5b). As expected, both DVS- and GSH-immobilized Gal was able to bind to Jacalin. Similarly, lactose, a poor ligand for Jacalin binding, did not show appreciable binding for either strategy.[39] We were surprised to find that immobilized GlcNAc showed a large difference in binding capacity between the two strategies—with substantial binding capacity for DVS-immobilized GlcNAc, and insignificant biding for GSH-immobilized GlcNAc. We attributed this difference to the nature of the immobilization chemistries. The GSH strategy is expected to generate a β-linked GlcNAc residue.[9] Previous reports have found that GlcNAc-containing mono and oligosaccharides bind to Jacalin.[38,40] However, some terminal β-linked GlcNAc residues have been found to be poor ligands for Jacalin.[41] We propose that the binding epitope of the GSH-linked GlcNAc would be analogous to a terminal GlcNAc-β1,6-linked disaccharide (through reaction with the 6-OH acceptor of Gal found within the Sepharose polymer). GlcNAc residues found in a β1,6-linkage, such as in Core 6 glycopeptides, are known to be a poor ligands for Jacalin.[42] In contrast, DVS-immobilized GlcNAc will present a mixture of GlcNAc epitopes that include both anomers at O-1. Furthermore, the flexible sulfone linker may allow for the DVS-immobilized residue or the Sepharose support to gain favorable interactions with Jacalin subsites upon binding.[43]

3. Conclusions

Carbohydrate-binding proteins are often identified and purified using affinity chromatography, and improved methods are of continued interest to the field of glycobiology. We report the adaptation of protecting group-free glycosidation chemistry, using *N*′-glycosyltoluenesulfonylhydrazide donors, for use in affinity chromatography. Our results confirm that the GSH immobilization chemistry is more efficient than competing methods, and that it provides a more uniform linker chemistry to the solid support. The resulting affinity matrix is competent for binding of lectins and should provide a new mild alternative for the regioselective immobilization of complex carbohydrates purified from biological sources.

4. Materials and methods

4.1. General procedures

All reagents used were purchased from Sigma-Aldrich or Acros Organics and used without further purification unless otherwise noted. Reactions were monitored by analytical TLC on silica gel 60-F_{254} (0.25 mm, Silicycle, Quebec, Canada) and visualized under UV light (254 nm), or stained by charring with ceric ammonium molybdate (CAM) or potassium permanganate ($KMnO_4$). Organic solvents were removed under reduced pressure, and organic products were purified by flash column chromatography on silica gel (230–400 mesh, Silicycle, Quebec, Canada). ^1H NMR spectra were acquired on Varian 400, 500, or 600 MHz instruments as noted at 27 °C. ^{13}C NMR spectra were recorded at 125 or 150 MHz as noted at 27 °C. Electrospray-ionization mass spectra were recorded on an Agilent Technologies 6220 TOF instrument.

4.2. Coupling of DVS with D-galactose in solution (**1**)

A sample of D-galactose (91 mg, 0.5 mmol) was dissolved in 1 mL of carbonate buffer (pH 11) and stirred until dissolved. In a glass vial, 295 mg (2.5 mmol) of divinyl sulfone was dissolved in 500 µL of carbonate buffer (pH 11) and then added dropwise to the solution

of galactose over 5 min, and reacted for 70 min. The mixture was concentrated to a final volume of approximately 0.5 mL and loaded onto a silica gel column and eluted with 25% CH_3OH in DCM. The fractions containing the product were concentrated *in vacuo* to afford a yellow oil in 60% yield. The product was a complex mixture of di-, tri-, and tetra-DVS substituted galactose. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 6.91–6.71 (m, *CH*═CH_2), 6.52–6.42 (m, CH═*CH_2*), 6.24–6.14 (m, CH═*CH_2*), 5.14–4.91 (m, H-1α), 4.47–3.18 (m, H-1β, H-2, H-3, H-4, H-5, H-6, H-6a, *CH_2*—SO_2, *CH_2*-O). Masses of the di-substituted ($C_{14}H_{24}O_{10}S_2Na$ M + Na^+) calcd. 439.0811 found 439.1, tri-substituted ($C_{18}H_{32}O_{13}S_3Na$ M + Na^+) calcd. 575.1005, found 575.1 ($C_{22}H_{40}O_{16}S_4$ M + Na^+) calcd.: 711.1199, found: 711.1.

4.3. Coupling of DVS with N-acetyl-D-glucosamine (**2**)

N-Acetyl-D-glucosamine (40 mg, 0.18 mmol) was dissolved in 1 mL of carbonate buffer (pH 11) and stirred until dissolved. In a glass vial, 4.47 mg (0.036 mmol) of divinyl sulfone was dissolved in 500 μL of carbonate buffer and then added to the glucosamine solution drop-wise over 5 min, and reacted for an additional 70 min. The mixture was concentrated to a final volume of approximately 0.5 mL and loaded onto a Sep-Pak C18 cartridge and eluted with 25% CH_3OH in H_2O. The fractions containing the product were concentrated *in vacuo* to afford an off-white solid with an isolated yield of 57%. ^{1}H NMR (400 MHz, CD_3OD): δ = 6.98–6.78 (m, *CH*═CH_2), 6.41–6.26 (m, CH═*CH_2*), 6.24–6.10 (m, CH═*CH_2*), 4.88–4.84 (m, H-1α, overlaps with residual HOD peak), 4.42 (d, 1H; $^3J_{H\text{-}1,H\text{-}2}$ = 8.4 Hz, H-1β), 4.29–4.05 (m, *CH_2*—SO_2, *CH_2*—O), 4.02–3.22 (H-3β, H-4, H-5, H-6, H-6a), 3.93–3.87 (m, H-2α, from COSY), 3.68 (m, H-2β, from COSY), 3.41 (m, H-3β, from COSY), 2.01–1.96 (m, Ac). ESI HRMS m/z ($C_{12}H_{21}NO_8SNa$ M + Na^+) calcd. 362.1, found 362.1.

4.4. 2-Galactopyranosyl-6-hydroxy-4-methoxy-[1,3,5-triazine] and 2,4-digalactopyranosyl-6-methoxy-[1,3,5-triazine] (**3**)

D-Galactose (80 mg, 0.44 mmol) was dissolved in 6 mL of water, followed by addition of NaOH (26.4 mg, 0.66 mmol). 4,6-Dichloro-6-methoxy-[1,3,5-triazine] dissolved in 1 mL of acetone (80 mg, 0.45 mmol) was added drop wise to the reaction mixture and stirred for 8 h. The mixture was purified by preparative TLC using 12:1:0.4 isopropanol:CH_3OH:H_2O as eluent. The product was recovered with CH_3OH and dried under reduced pressure to afford a mixture of the mono and di-substituted products. ^{1}H NMR (400 MHz, CD_3OD): δ = 5.23–5.07 (m, H-1α), 4.64–3.39 (m, H-1β, H-2, H-3, H-4, H-5, H-6, H-6a, O*CH_3*). Masses of the mono-substituted ($C_{10}H_{15}O_8N_3Na^+$) calcd. 328.0757, found 328.0754, di-substituted ($C_{16}H_{25}O_{13}N_3Na^+$) calcd. 490.1285, found 490.1283

4.5. N′-(β-D-Galactopyranosyl)-p-toluenesulfono-hydrazide (**4**)

Compound **4** (1.95 g, 5.6 mmol) was prepared as previously reported.[15] Isolated yield was 99%, and only the β-anomer was observed. ^{1}H NMR (500 MHz, CD_3OD): δ = 7.79 (m, 2H, Ar), 7.37 (m, 2H, Ar), 3.75 (dd, 1H; $^3J_{H\text{-}4,H\text{-}5}$ = 1.0 Hz, $^3J_{H\text{-}3,H\text{-}4}$ = 3.5 Hz, H-4), 3.71 (dd, 1H; $^3J_{H\text{-}5,H\text{-}6}$ = 7.6 Hz, $^3J_{H\text{-}6,H\text{-}6a}$ = 11.4 Hz, H-6), 3.71 (dd, 1H; $^3J_{H\text{-}5,H\text{-}6a}$ = 4.4 Hz, $^3J_{H\text{-}6,H\text{-}6a}$ = 11.4 Hz, H-6a), 3.62–3.55 (m, 2H, H-1 [3.61 ppm from HSQC], H-2 [3.58 ppm from HSQC]), 3.40 (dd, 1H; $^3J_{H\text{-}3,H\text{-}4}$ = 3.5 Hz, $^3J_{H\text{-}2,H\text{-}3}$ = 9.0 Hz, H-3), 3.71 (ddd, 1H; $^3J_{H\text{-}4,H\text{-}5}$ = 1.0 Hz, $^3J_{H\text{-}5,H\text{-}6a}$ = 4.4 Hz, $^3J_{H\text{-}5,H\text{-}6}$ = 7.6 Hz, H-5), 2.42 (apparent s, 3H, Ph*CH_3*) ppm. A starting material impurity was detected at δ = 7.53, 7.22, and 2.34 ppm. ^{13}C NMR (125 MHz, CD_3OD): δ = 145.1, 137.4, 130.6, 129.1, 92.3, 78.0, 75.2, 70.6, 69.4, 63.0, 21.5 ppm. ESI-HRMS m/z calcd. for $C_{13}H_2N_{20}O_7SNa^+$ (M + Na^+) 371.0883, found 371.0878.

4.6. Octyl-D-galactopyranoside (**5**)

Compound **4** (35 mg, 0.10 mmol) was dissolved in 1 mL of DMF, and octanol (150 μL, 0.124 mmol) was added to the solution. *N*-Bromosuccinimide (36 mg, 0.2 mmol) was then added to the solution, and after 25 min, Amberlite resin (OH^-) was added to quench the reaction and the solution was stirred until the yellow color disappeared. The resin was filtered and washed with CH_3OH, and the solvent was removed under reduced pressure. The residue was purified by flash chromatography to afford a white solid (18 mg, 0.062 mmol) in 62% isolated yield. (α:β, 1:1.1). ^{1}H NMR (500 MHz, CD_3OD): δ = 4.79 (d, 1H; $^3J_{H\text{-}1,H\text{-}2}$ = 3.3 Hz, H-1α), 4.19 (d, 1H; $^3J_{H\text{-}1,H\text{-}2}$ = 7.6 Hz, H-1β), 3.91–3.65 (m, 11H, H-2α, H-3, H-4, H-6, H-6a, O*CH_2*), 3.56–3.39 (m, 5H, H-2β, H-5, O*CH_2*) 1.69–1.54 (m, 4H; $OCH_2CH_2(CH_2)_5CH_3$), 1.43–1.23 (m, 20H; $OCH_2CH_2(CH_2)_5CH_3$), 0.89 (t, 6H; ^3J = 7.0 Hz; $O(CH_2)_7CH_3$) ppm. ^{13}C NMR (125 MHz, CD_3OD): δ = 105.0, 100.3, 76.6, 75.1, 72.6, 72.4, 71.6, 71.1, 70.9, 70.3, 69.2, 62.8, 62.5, 33.0, 30.9, 30.6(2), 30.6(0), 30.4, 27.4, 27.2, 23.7, 14.4 ppm. ESI-HRMS m/z calcd. for $C_{14}H_{28}NaO_6$ (M + Na^+) 315.1778, found 315.1774.

4.7. N′-(β-D-lactopyranosyl)-p-toluenesulfono-hydrazide (**6**)

The *N*′-(β-D-lactopyranosyl)-*p*-toluenesulfono-hydrazide (**6**) was prepared as previously reported,[15] and obtained in 97% yield. Spectral data match those reported.[15] ESI-HRMS m/z calcd. for $C_{19}H_{30}NaO_{12}S$ (M + Na^+) 533.1412, found 533.1401.

4.8. Octyl-D-lactopyranoside (**7**)

Compound **6** (51 mg, 0.10 mmol) was dissolved in 1 mL of dry DMF, and octanol (150 μL, 0.124 mmol) was added to the solution. *N*-Bromosuccinimide (36 mg, 0.2 mmol) was added, and after 25 minutes Amberlite resin (OH^-) was added to quench the reaction and the solution was stirred until the yellow color disappeared. The resin was filtered and washed with CH_3OH, and the solvent was removed under reduced pressure. The residue was purified by flash chromatography to afford a white solid (14 mg, 0.031 mmol) in 31% isolated yield (α:β, ~2:1). ^{1}H NMR (500 MHz, CD_3OD): δ = 4.75 (d, 2H, $^3J_{H\text{-}1,H\text{-}2}$ = 3.8 Hz; H-1α, overlaps CD_3OD), 4.36–4.32 (m, 3H, H-1′), 4.27 (d, 1H, $^3J_{H\text{-}1,H\text{-}2}$ = 8.0 Hz; H-1β), 3.90–3.63 (m, 41H, H-2α, H-3, H-4, H-5, H-6, H-6a, H-2′, H-3′, H-4′, H-5′, H-6′, H-6a′, O*CH_2*), 3.23 (apparent t, 1H, $^3J_{H\text{-}1,H\text{-}2}$ = $^3J_{H\text{-}2,H\text{-}3}$ = 8.0 Hz; H-2α, overlaps *CD_3*OD), 1.68–1.55 (m, 6H; $OCH_2CH_2(CH_2)_5CH_3$), 1.43–1.23 (m, 30H; $OCH_2CH_2(CH_2)_5CH_3$), 0.89 (t, 9H; ^3J = 6.8 Hz; $O(CH_2)_7CH_3$) ppm. ^{13}C NMR (125 MHz, CD_3OD): δ = 105.1, 99.9, 81.1, 77.1, 74.9, 73.5, 73.3, 72.6, 72.1, 70.3, 69.4, 62.5, 33.1, 30.6, 30.4, 27.4, 23.7, 14.4 ppm. ESI-HRMS m/z calcd. for $C_{20}H_{38}NaO_{11}$ (M + Na^+) 477.2306, found 477.2306.

4.9. N′-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-p-toluenesulfono-hydrazide (**8**)

Compound **8** (1.24 g, 2.4 mmol) was prepared as previously reported,[9] with an isolated yield of 99%. Spectral data match those reported,[9] and only the β-anomer was observed by NMR. ESI-HRMS m/z calcd. for $C_{15}H_{23}N_3NaO_7S$ (M + Na^+) 412.1149, found 412.1147.

4.10. Octyl-2-acetamido-2-deoxy-β-D-glucopyranoside (**9**)

Compound **9** was prepared as previously reported, and isolated as a white solid in 70% yield.[9] Spectral data match those reported,[9] and only the β-anomer was observed by NMR. ESI-HRMS m/z calcd. for $C_{16}H_{31}NO_6Na$ (M + Na^+) 356.2044, found 356.2044.

4.11. Divinyl sulfone immobilization of glycans to Sepharose

Carbohydrate-modified Sepharose gel was prepared as previously reported.[3] Settled Sepharose CL-6B (1 mL; GE Healthcare Life Sciences, Piscataway, NJ) was thoroughly washed with water in a sintered funnel and then re-suspended in 0.5 M carbonate buffer (pH 11) with 100 μL of divinyl sulfone. The mixture was agitated for 70 min, after which the resin was transferred to a sintered funnel and extensively washed with water. The moist cake was suspended in a 1 mL solution of the indicated carbohydrate (1.11 mmol mL^{-1} in 0.5 M carbonate buffer, pH 10) and left agitating for 18 h. The resin was washed again with distilled water over a sintered funnel, and the moist cake re-suspended in carbonate buffer (1 mL, 0.5 M, pH 8.5) and 2-mercaptoethanol (6 μL). After 2 hours the sample was washed with distilled water and stored in 20% ethanol.

4.12. Cyanuric chloride immobilization of glycans to Sepharose

The procedure of Finlay et al. was adapted.[19] Briefly, 3 mL of settled Sepharose CL-6B was extensively washed with distilled water in a sintered glass funnel. The resin was transferred to organic phase by washing with 9 mL of increasing concentrations of acetone in water (25, 50, 75, and 100%). A final wash with acetone was carried out twice, and the resin was re-suspended in 3 mL of acetone and transferred to a three-necked-round-bottom flask with a condenser attached. The mixture was heated to 50 °C and slowly agitated while adding *N*,*N*-diisopropylethylamine (600 μL of a 2 M solution in acetone). After 30 min, a solution of trichloro-*S*-triazine (600 μL of a 1M solution in acetone) was added drop-wise and mixed for 1 hour at 50 °C. The resin was washed thoroughly with acetone in a sintered glass funnel, and re-suspended in acetone (3 mL) followed by addition of aniline (60 μL, 2 M in acetone) and incubated for 30 min. The resin was filtered and washed thoroughly with acetone and then transferred back to aqueous phase by washing with decreasing concentrations of acetone in water (75, 50, and 25%), followed by two washes with distilled water. The activated resin was re-suspended in a solution containing 20 mg mL^{-1} of the indicated carbohydrate in bicarbonate buffer (pH 10) and reacted for 18 h, followed by extensive washes with distilled water.

4.13. N′-glycosyltoluenesulfonohydrazide immobilization of glycans to Sepharose

Settled Sepharose CL-6B gel (1 mL) was extensively washed with distilled water in a sintered glass funnel. The resin was transferred to an organic phase by washing with 5 mL of increasing concentrations of DMF in water (25, 50, 75, and 100%). A final wash with 100% DMF was carried out twice, and the resin was re-suspended in 1 mL of DMF and transferred to a dry round bottom flask. The mixture was agitated slowly in an orbital shaker, and the indicated hydrazide was added (dissolved in 1 mL DMF) and allowed to mix for 5 minutes. The flask was then charged with *N*-bromosuccinimide (2.5 mmol in 1 mL DMF) by drop-wise addition to the reaction mixture. Evolution of N_2 gas was observed during the reaction, and after 30 min the resin was transferred to a sintered glass funnel and washed with 5 mL of DMF followed by 5 mL of decreasing concentrations of DMF in water (75, 50, and 25%), followed by two washes of distilled water. All resin samples were re-suspended in water and freeze-dried for storage.

4.14. HR-MAS ^{1}H NMR

HR-MAS ^{1}H NMR experiments were performed using an Agilent/Varian VNMR three-channel 600 MHz spectrometer equipped with a Varian gHX nano-NMR probe. Spectra from 40 μL samples were spun at 2 kHz and recorded at 27 °C. The experiments were recorded with suppression of water signal at 4.75 ppm and a spectral width of 12,000 Hz. The spectra were obtained using 2048 transients with an acquisition time of 3 s. The resin was freeze-dried three times from D_2O before use in NMR experiments. Resin (3 mg, dry powder) was re-suspended in 100 μL of D_2O and mixed to form a suspension. An aliquot of the gel (40 μL) was transferred to a 4 mm sample tube using a micropipette.

4.15. Phenol sulfuric acid assay

The procedure of Masuko et al. was adapted by changing the order of addition of the phenol solution and the sulfuric acid.[14] Briefly, 5 mg of dry resin was weighed into a 10 mL round bottom flask, and 5 mL of 2.5 M H_2SO_4 was added followed by reflux at 110 °C for 6 hours. After hydrolysis, samples were allowed to cool for 30 min with the condenser attached. The flask was mixed vigorously and 1.5 mL of the suspension was transferred to a 2 mL Eppendorf tube. Tubes were centrifuged at 10,000 × *g* for 10 minutes to precipitate the resin. Taking care to not disturb the pellet, 100 μL of the supernatant was transferred and diluted with 900 μL of distilled water in triplicate. The samples were thoroughly mixed, and 200 μL of the solution was transferred to a fresh Eppendorf tube followed by the addition of 120 μL of 5% phenol solution in water (freshly prepared), and 600 μL of concentrated H_2SO_4 was added immediately. Samples were mixed and then incubated for 5 min at 95 °C in a heating block. Samples were allowed to cool for 5 min, then 230 μL of the solution from each tube was transferred to clear-bottomed 96-well polystyrene microplate and the A_{490} was measured in triplicate using a Spectra Max M2 plate reader. The absorbance data collected were converted into μmol Gal mg^{-1} of resin using a previously determined calibration curve.

4.16. Lectin binding assays with immobilized glycans

Resin samples were prepared using the methods above with either Gal or GlcNAc to test the ability of soluble lectins to recognize the immobilized epitope. Sepharose resins were prepared using either DVS or GSH strategies, typically with 1.1 mmol of saccharide per mL of swelled resin. For quantitation, a range of saccharide concentrations were used with separate samples made for each point. Functionalized Sepharose (1 mg) was weighed as a dry powder into 0.5 mL Eppendorf tubes. Binding buffer was added to each tube (50 μL, 10 mM HEPES, 0.15 M NaCl, pH 7.5, 0.1 mM $CaCl_2$). The samples were mixed for 30 min at room temperature to allow the resin to swell and equilibrate. A solution of FITC-labeled Jacalin or FITC-labeled WGA (Vector Labs, Burlington, Ontario; 200 μL of a 75 μg/mL solution) was added to transfer 15 μg of total protein per tube. Triplicate samples were incubated at room temperature for 3 hours and left mixing protected from light. After the incubation period, the samples were centrifuged at 10,000 × g for 10 minutes to precipitate the resin. The supernatant (40 μL) was transferred to a 384-well black-bottomed microplate. Fluorescence was measured with λ_{ex} = 490 nm, λ_{em} = 518 and a λ_{cutoff} = 515 nm using a Spectra Max M2 plate reader. The fluorescence data were converted to micrograms of Jacalin or WGA bound per milligram of resin using a calibration curve determined with a solution of the labeled lectin.

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Appendix: Supplementary material

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