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Immobilization of carbohydrate epitopes for surface plasmon resonance using the Staudinger ligation

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ABSTRACT

The detection of carbohydrate–protein interactions is often performed using techniques that require surface immobilization of the lectin or the glycan. A commonly used assay for lectin binding is surface plasmon resonance (SPR). We describe an implementation of the Staudinger ligation as a method to immobilize carbohydrate epitopes to a biosensor surface. This was accomplished by first introducing an azide functionality to a carboxymethyldextran surface, followed by reaction with a phosphane-modified carbohydrate ligand. The chemistry employed is extremely mild and was easily adapted to a commercial biosensor system. Using this approach, we investigated the binding of jacalin and wheat germ agglutinin (WGA) to galactose, lactose, and *N*-acetyl-lactosamine. We observed that WGA binding shows evidence of multivalent interaction with the surface. Additionally, we found that jacalin binding was influenced by the presence of a flexible and hydrophobic galactosyl aglycone.

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1. Introduction

Lectin–carbohydrate interactions are critical to a variety of biological recognition events. Among many known processes in humans, native lectins take part in inflammation, cancer, antigen recognition, and fertilization.[1–3] The measurement of lectin–carbohydrate interactions can be challenging, and a variety of methods have been employed including fluorescence spectroscopy,[4] isothermal titration calorimetry (ITC),[5] surface plasmon resonance (SPR),[6] and microarrays.[7–9] Solid-phase methods, including SPR and microarrays, are commonly employed due to their sensitivity and small sample requirements. For lectin–carbohydrate interactions, surface methods have the added advantage that surface density can be controlled to mimic multivalent presentations found in nature.[10] However, the application of solid-phase methods requires suitable surface chemistry that preserves the lectin–carbohydrate interaction under study. The development of improved, orthogonal, or more facile immobilization strategies is, therefore, of continued interest for assay development.

SPR has been an important technique for the analysis of lectin–carbohydrate interactions. Typical immobilization strategies exploit amine–carboxylate crosslinking of the lectin to the surface.[11] Alternative approaches have used biotin,[12] amine,[13] thiol,[14] or lipid tags[15] to generate carbohydrate surfaces. There has been a rapid expansion of bioorthogonal chemistry in recent years,[16] with a large number of groups developing selective strategies for protein or ligand immobilization.[17] An important subset of bioorthogonal reactions, those with excellent yields and substrate tolerance, is known as a 'click' reaction.[18] These reactions can provide useful alternatives to traditional immobilization strategies. The Staudinger ligation has been used to detect glycosyltransferase activity on microarrays[19] and to immobilize azide-labeled carbohydrates or proteins to a phosphane-derived surface.[20,21] A modification of this strategy, which used a traceless Staudinger ligation, has also been reported for generating self-assembled monolayer (SAM) and microarray surfaces.[22,23] Native chemical ligation of proteins to an SPR surface has also been described.[24] Microarrays used for lectin-binding studies have been generated using alkyne-modified carbohydrates and a sulfonyl-azide surface.[25] Carbohydrate surfaces suitable for SPR lectin-binding studies have been generated using azide-modified glycans and a SAM containing an alkyne.[26,27]

Although the Staudinger ligation has been applied for surface immobilization on SAM and microarray surfaces, there are no reports of adapting this chemistry to commercial SPR systems. Commercial SPR biosensors often use carboxymethyldextran (CMD) surfaces, and require relatively mild reaction conditions which

Abbreviations: CMD, carboxymethyldextran; DMSO, dimethyl sulfoxide; EDA, ethylenediamine; EDCI, *N*-ethyl-*N*′-(3-diethylaminopropyl)-carbodiimide; ITC, isothermal titration calorimetry; Lac, Galβ1,4-Glc; LacNAc, Galβ1,4-GlcNAc; NHS, *N*-hydroxy-succinimide; SAM, self-assembled monolayer; SPR, surface plasmon resonance.

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avoid organic solvents. Application of the Staudinger ligation should be compatible with these requirements. One potential limitation of this strategy is the sensitivity of the phosphane reagents to oxidation.[28] We considered that an azide-modified surface could be more robust than an immobilized phosphane and would reduce complications due to oxidation by only capturing intact groups. In previous work, we employed a synthetic strategy for generating phosphane-labeled carbohydrate ligands that could be used to generate glycoprotein conjugates.[29] We chose to adapt this strategy as a method to immobilize carbohydrate ligands to a CMD matrix. We found that Staudinger ligation to an azide surface provided an active and stable biosensor. Using this method, we investigated the binding of wheat germ agglutinin (WGA) and jacalin binding to Galβ, Galβ1,4-Glcβ (Lac), and Galβ1,4-GlcNAcβ (LacNAc).

2. Experimental

2.1. Synthetic reagents

2.1.1. Methyl 2-(diphenylphosphino)-4-(2-(2-(5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanoyloxy) ethoxy)ethylcarbamoyl)benzoate (1)

Biotin was coupled to a Boc-protected linker, *tert*-butyl-(2-(2-hydroxy ethoxy)-ethyl)carbamate **S-1**, to provide compound **S-2**.[30] Compound **S-2** (145 mg, 0.34 mmol) was then stirred in a 3 mL solution of 1:1 TFA–DCM for 3 h, then concentrated (see Supplementary data). The resulting amine was added to a solution of DCM (3.5 mL) containing Et_3N (56.8 μL, 0.41 mmol) and succinimidyl-3-diphenylphosphino-4-methoxycarbonylbenzoate (**S-3**, 189.1 mg, 0.41 mmol) and stirred overnight.[29,31,32] The solution was concentrated and purified by column chromatography (9:1 EtOAc–MeOH) to yield compound **1** (153 mg, 67%) as a yellow solid with minor amounts of the corresponding phosphine oxide (ca. ~4%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.06 (dd, J = 8.0, 3.6 Hz, 1H), 7.90 (dd, J = 7.9, 3.3 Hz, 0.1H), 7.81 (dd, J = 8.1, 1.4 Hz, 1H), 7.38 (dd, J = 3.7, 1.5 Hz, 1H), 7.36–7.26 (m, 10H), 6.91 (s, 1H), 5.85 (s, 1H), 5.27 (s, 1H), 4.63–4.38 (m, 1H), 4.33–4.11 (m, 3H), 3.72 (s, 3H), 3.69–3.43 (m, 6.7H), 3.12–3.07 (m, 1H), 2.87 (dd, J = 12.8, 4.8 Hz, 1H), 2.69 (d, J = 12.8 Hz, 1H), 2.42–2.20 (m, 2H), 1.64–1.61 (m, 4H), 1.47–1.33 (m, 2H); phosphine oxide: δ 8.36 (d, J = 13.7 Hz, 0.1H), 8.15 (d, J = 7.9 Hz, 0.1H), 7.68 (m, 0.5H), 7.52 (m, 0.3H), 7.45 (m, 0.5H), 3.40 (s, 0.3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.5, 166.8, 166.76, 166.6, 141.3 (d, J_{C-P} = 29.0 Hz), 137.3, 137.2 (d, J_{C-P} = 11.0 Hz), 136.7 (d, J_{C-P} = 19.0 Hz), 133.9 (d, J_{C-P} = 21.0 Hz), 132.9, 131.9, 130.8, 128.9, 128.6 (d, J_{C-P} = 7.0 Hz), 126.7, 69.3, 68.9, 62.8, 61.9, 60.1, 55.5, 52.2, 40.5, 39.8, 33.8, 28.2, 28.15, 24.7; ^{31}P NMR (162 MHz, $CDCl_3$): δ 32.90 (s, 0.04P), -2.39(s, 1.00P); HRESIMS: calcd for $C_{35}H_{40}N_3O_7PSNa$ $(M+Na)^+$ 700.2217; found 700.2214.

2.1.2. Phosphane compounds 2, 3, and 4

The syntheses of compounds **2**, **3**, and **4** were carried out as previously described.[29] Briefly, carbohydrate epitopes were generated with an amine-terminated ethylene glycol linker. The amine was then reacted with an *N*-hydroxysuccinimidyl-phosphane reagent, **S-3**, to provide the desired compounds.[31]

2.2. Biosensor surface preparation

Surfaces were prepared using a BIAcore 3000 and CM5 (carboxymethyldextran) sensor chip. Reactions were performed at a flow rate of 5 μL min^{-1} in order to maximize contact time. The chip was equilibrated and then reacted with the following solutions to generate an azide-modified surface: (1) 200 μL of a 1:1 solution of *N*-ethyl-*N*′-(3-diethylaminopropyl)carbodiimide (EDCI) (0.1 M in water) and *N*-hydroxysuccinimide (NHS) (0.1 M in water); (2) 325 μL of ethylenediamine (EDA) (1 M in water, pH 8.5); (3) 200 μL of azidoacetic acid-NHS ester **5** (50 mM in 10% DMSO, 90% 25 mM $NaHCO_3$, pH 8.5).[29,33] Carbohydrate or control surfaces were then generated by injection of the appropriate phosphane compound (**1**, **2**, **3**, or **4**) (200 μL 1.25 mM, in 6% DMSO–water). The change in RU of each surface after treatment with the phosphane compounds was as follows: **1**, 1760; **2**, 1970; **3**, 1120; and **4**, 860 RU.

2.3. Lectin binding

All binding experiments were conducted on a BIAcore 3000 SPR system in HEPES running buffer (10 mM HEPES, 150 mM NaCl, 1 mM $CaCl_2$, 0.005% surfactant P20, pH 7.4). Experiments were performed at a flow rate of 10 μL min^{-1} (kinject). Data obtained from carbohydrate-modified surfaces were corrected by subtraction of the response in a control lane modified with compound **1,** which was run in parallel for all injections. Jacalin and WGA were obtained from US Biological (Swampscott, MA) and Sigma–Aldrich Chemical Co. (St Louis, MO), respectively. Stock solutions of each protein were made in running buffer, and concentrations were determined by A_{280} measurements. Equilibrium binding data were fit to either a single site,

$$RU = \frac{B_{\max}F}{K_d + F}, \quad (1)$$

or two-site model,

$$RU = \frac{B_{\max 1}F}{K_{d1} + F} + \frac{B_{\max 2}F}{K_{d2} + F} \text{ (see Ref. 34).} \quad (2)$$

2.4. Molecular modeling

Models of the interaction between jacalin and galactosyl residues containing a 3,5-dioxaheptyl aglycone were generated using a reported crystal structure (PDB ID: 1UH1).[35,36] The structure was modified using Macromodel (Schrodinger, Inc.) to incorporate either an α- or β-linked aglycone. The carbohydrate and protein residues within 10 Å of the ligand were then minimized and subjected to molecular dynamics for 10 ps, followed by an additional minimization (OPLS force field;[37] water solvent was modeled by a continuous dielectric).

3. Results and discussion

3.1. Preparation of the biosensor surface

We developed a surface immobilization protocol suitable for immobilization of Staudinger–Bertozzi reagents.[38] We first functionalized the CMD surface with ethylenediamine (EDA).[39] The amines on the surface could then be easily acetylated with an NHS ester of azidoacetic acid, **5** (Fig. 1).[29] We found that compound **5** required the inclusion of DMSO in the buffer (10%) for substantial modification of the surface, and that the compound was subject to hydrolysis if the solutions were not used rapidly. Once the azide was immobilized, the surface was stable and could be reacted with an appropriate Staudinger reagent as desired. We previously reported the synthesis of phosphane reagents that contained galactose (**2**, Galβ), lactose (**3**, Lac), and *N*-acetyl-lactosamine (**4**, LacNAc) moieties.[29] These compounds were used to derivatize the CM5 surface, after treatment as described above, to incorporate the azide functionality. Separate lanes of a single CM5 chip were then exposed to compounds **1**, **2**, **3**, and **4** to generate the desired surfaces (Fig. 2). The biotin-modified surface (compound **1**) was used for background subtraction of the other lanes during binding experiments.

To the best of our knowledge, this is the first report of an azide-modified surface used for SPR detection. However, it is important to note that work from the laboratories of Bertozzi and co-workers,[19] Waldmann and co-workers,[20] and Raines and co-workers[23] have

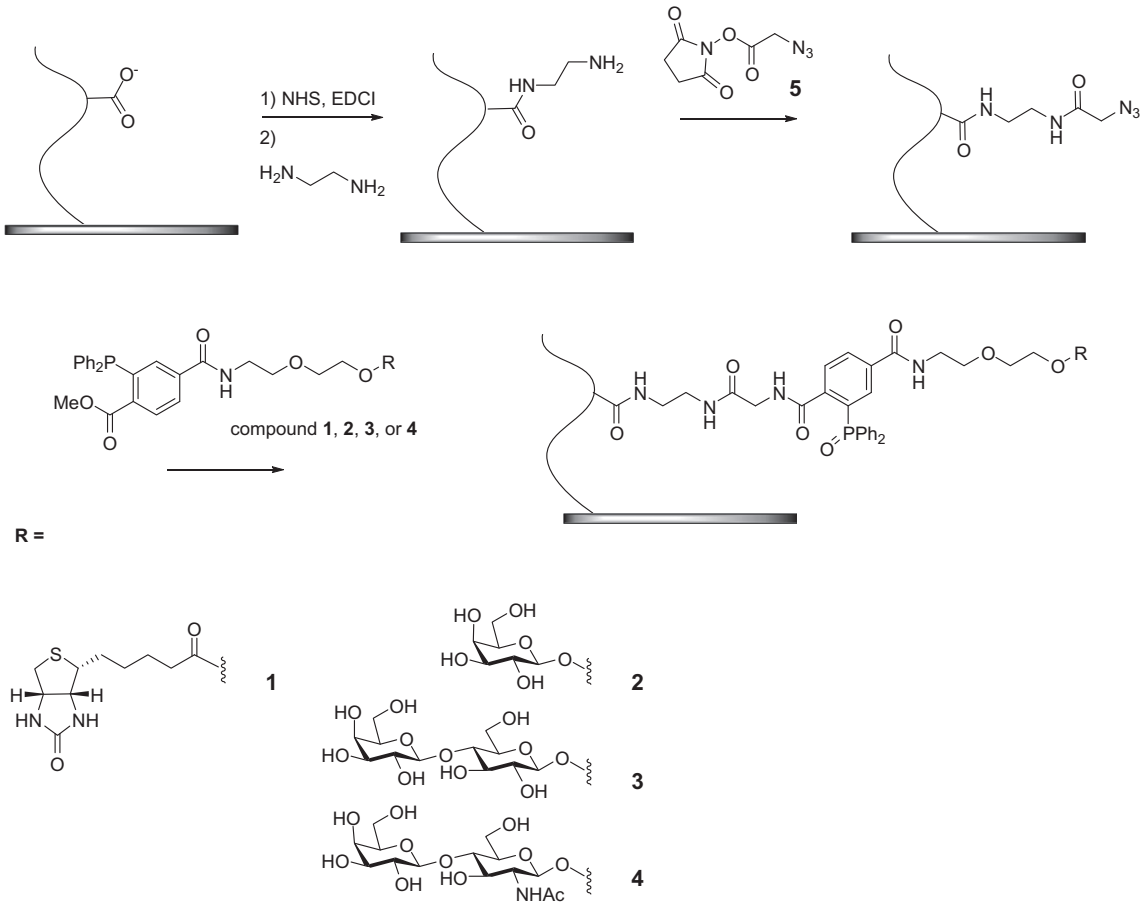

Figure 1. Conjugation of carbohydrate epitopes to a CMD surface. The preparations of compounds **2**, **3**, **4,** and **5** were carried out as reported.[29] Control surfaces were generated with a biotin-labeled phosphane **1**.

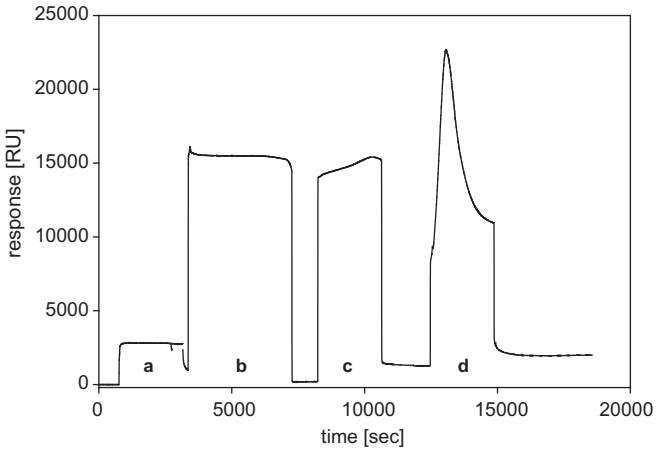

Figure 2. Modification of the biosensor surface with a carbohydrate ligand. A sensorgram is shown for modification of the sensor surface as described in Figure 1. The carboxymethyldextran was first activated with (a) EDCI/NHS (200 μL, 0.1 M) and then modified with (b) ethylenediamine (325 μL, 1 M, pH 8.5). The amine surface was then labeled with (c) NHS-azidoacetic acid (**5**, 200 μL, 50 mM, 10% DMSO). The azides on the surface were then allowed to react with (d) the phosphane-modified galactose reagent **2** (200 μL, 1.25 mM, 6% DMSO). The change in baseline RU was 1970 RU.

previously used variations of the Staudinger ligation as a surface immobilization strategy. In our formulation of the Staudinger ligation, we chose to use an azide surface to avoid any issues with oxidation of the phosphane component.[28] In this arrangement, oxidized phosphane would fail to react with the surface. By reversing this configuration to have the phosphane on the surface, any oxidation would reduce the number of available surface sites. Therefore, the azide-surface should provide higher capacity and may be advantageous if the surface requires storage or preparation separate from the immobilization step. Additionally, in this configuration the protocol requires less of the costly phosphane reagent.

3.2. Detection of lectin specificity for carbohydrate surfaces

To test the binding of lectins to the carbohydrate-modified surfaces, we injected solutions of jacalin (*Artocarpus integrifolia* agglutinin)[40] and wheat germ agglutinin (WGA)[41] over all lanes of the biosensor (Fig. 3). We observed that jacalin bound specifically to the galactose-containing compound **2**, and WGA bound only the GlcNAc-containing compound **4**. Neither lectin recognized the Lac-modified surface (compound **3**), and only bulk-response was observed for the control lane (compound **1**). WGA is known to bind specifically to GlcNAc mono- and oligosaccharides.[41] Jacalin is known to bind in preference to αGal; however, only weak binding to βGal would be expected (Table 1).[42] To explore the binding of each lectin in more detail, we proceeded to determine the affinity of the interaction using SPR.

3.3. Determination of jacalin and WGA affinity

One of the advantages of a sensitive SPR assay is that it can be used to determine the affinity of biomolecular interactions by direct observation of association and dissociation kinetics. However,

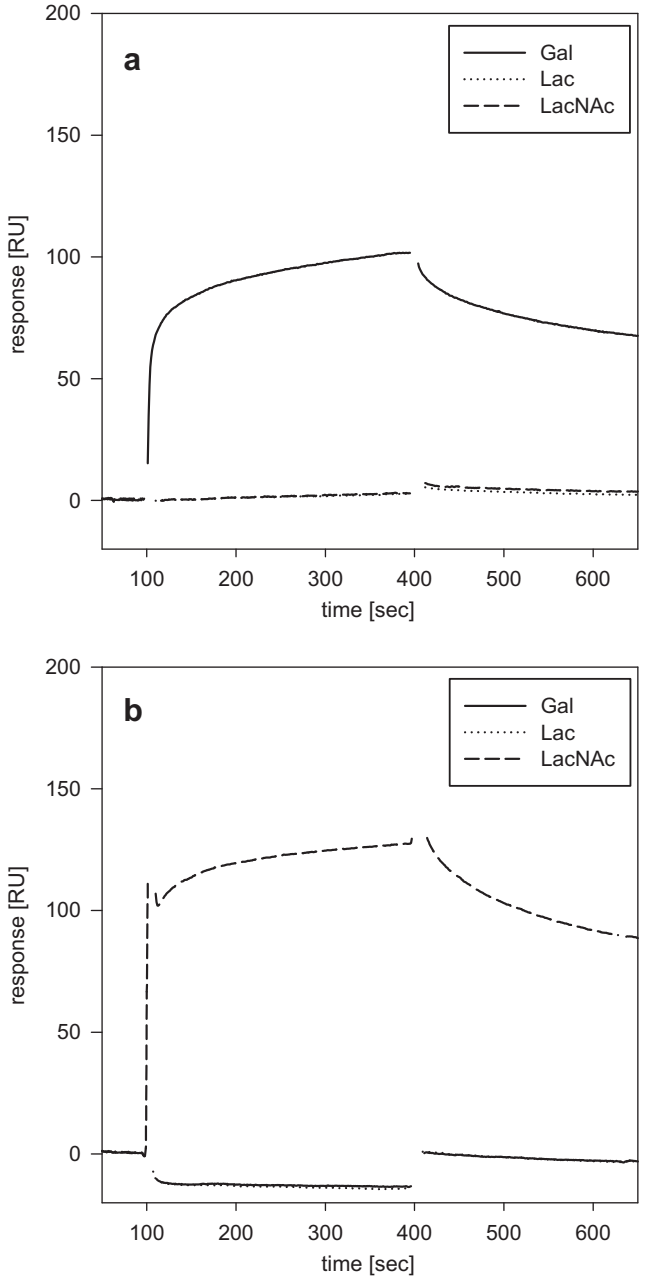

Figure 3. Specific binding of lectins to a carbohydrate-modified surface. (a) Jacalin (9.45 μM, 10 μL min^{-1}) and (b) WGA (21.1 μM, 10 μL min^{-1}) were injected over a sensor chip modified with compounds **2**, **3**, and **4**. Jacalin binds specifically to the galactose-containing compound **2**, and WGA binds specifically to *N*-acetyl-lactosamine compound **4**. Sensorgrams shown were reference subtracted using a surface modified with compound **1**. Spikes due to subtraction or injection artifacts have been removed for clarity.

kinetic determinations are inherently more challenging as a variety of artifacts may obscure an accurate measurement.[6] We injected a series of six concentrations of each lectin over the surfaces and analyzed the binding of jacalin to **2** and WGA to **4** (Fig. 4). Attempts to fit these data to a typical 1:1 binding model failed. More complex models, including bivalent binding and conformational change models, were unsuccessful, with all tested models giving poor fits.[43] These observations suggested that lectin binding to both surfaces followed complex kinetics. Therefore, we used equilibrium binding to determine the affinity of the lectins. A series of lectin concentrations were injected over the biosensor surface, and the response at steady-state binding was recorded. The resulting values were plotted versus the lectin concentration to provide a binding isotherm (Fig. 5).

Table 1
Lectin affinities determined for jacalin and WGA

Entry	Lectin (ligand)	Model or Ref.	(μM)		[RU]
1	Jacalin (Galα-OMe)	42	29		na
2	Jacalin (Galβ-OMe)	42	6250		na
3	Jacalin (Galβ1,3-GlcNAc)	35	25,000		na
4	Jacalin, (**2**)	One-site	K_d 800 ± 70	B_{max}	55 ± 1
5		Two-site	K_{d1} 200 ± 600	B_{max1}	19 ± 4
6			K_{d2} 1800 ± 2600	B_{max2}	40 ± 36
7	WGA (GlcNAc)	41	1300		na
8	WGA (GlcNAcβ1,4-GlcNAc)	41	200		na
9	WGA (Galβ1,6-GlcNAc)	46	1700		na
10	WGA, (**4**)	One-site	K_d 190 ± 40	B_{max}	111 ± 4
11		Two-site	K_{d1} 40 ± 80	B_{max1}	70 ± 40
12			K_{d2} 1300 ± 1800	B_{max2}	60 ± 30

The equilibrium binding data were fit to a single (Eq. 1) or two-site (Eq. 2) binding model to obtain the affinity of each interaction (Table 1). When the data were plotted in semi-log form or as a Scatchard plot (Fig. S2), it could be seen that jacalin binding was well described by a single-site model, giving a dissociation constant of 800 ± 70 μM (r^2 = 0.98). Fitting the same data to a two-site model gave an affinity for **2** of 200 ± 600 μM at the first site, and 1800 ± 2600 μM for the second site (r^2 = 0.99). Although this model provides a slightly improved fit, the lack of a concave Scatchard plot suggests that the two-site model should not be used. Based on the single-site affinity, the binding of a βGal derivative appears to be remarkably high. Previous reports have determined that the affinity of βGal to jacalin is close to 5 mM, while the αGal anomer has much higher affinity.[42] Hagiwara et al. have found that Galβ-*p*-nitrophenyl has greater affinity to jacalin than to GalNAc, suggesting an ability for the lectin to accommodate a hydrophobic β-linked aglycone.[40] Additionally, Smith et al. have reported that αGal derivatives with a hydrophobic aglycone have slightly improved affinity to jacalin.[14] Comparison of the single-site affinity measured here for a βGal derivative falls between the expected affinity of a GalαOMe and GalβOMe, suggesting that **2** has higher than expected affinity for the lectin (vide infra).

The data obtained for binding of WGA to compound **4** were partly described by the single-site model (190 ± 40 μM, r^2 = 0.82). However, a Scatchard plot of these data gave a concave-up curve, suggesting heterogeneity in the binding data.[44,45] Consistent with this finding, a fit of the data to the two-site model (r^2 = 0.88) showed improvement over the single-site model, and gave values of 40 ± 80 μM and 1300 ± 1800 μM for the two affinity constants. The WGA lectin is known to be specific for GlcNAc-containing saccharides; however, its affinity for LacNAc has not been previously determined. Measurement of WGA affinity for GlcNAc using isothermal titration calorimetry (ITC) found a value of 1.3 mM.[41] Larger oligosaccharides of β1,4-linked GlcNAc gave tighter binding; for example, the dimer gave an affinity of 200 μM. The affinity of an isomeric disaccharide of LacNAc, Galβ1,6-GlcNAc, for WGA has been determined to be 1.7 mM.[46]

The binding of WGA to **4** is, therefore, of unusually high affinity. When the data were analyzed using the single-site binding model, the observed affinity of **4** is similar to the GlcNAc dimer. However, when the non-linearity of the Scatchard plot is taken into account, a more complex model is required. We interpret the non-ideal Scatchard plot as an indication of heterogeneous binding sites on the surface. This heterogeneity is likely the result of high affinity sites that arise from carbohydrate epitopes on the surface that are close enough to bridge multiple sites of a single WGA tetramer.

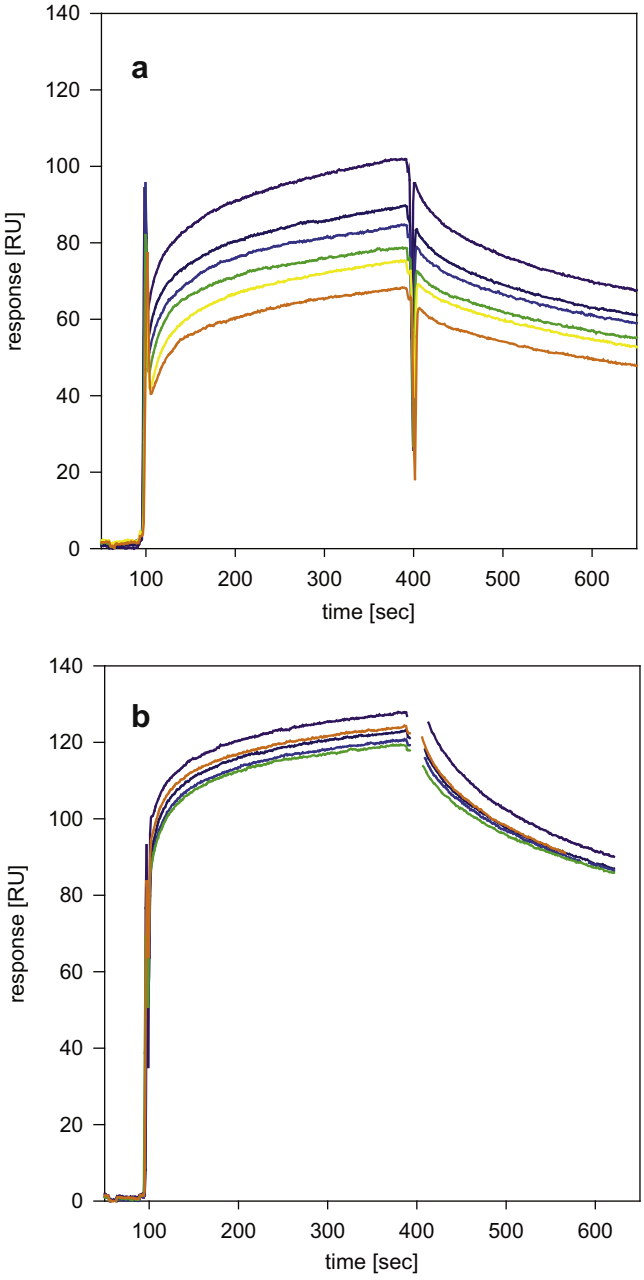

Figure 4. Binding of lectins to galactose and *N*-acetyllactosamine surfaces. (a) Jacalin was injected over the galactose surface (9.5, 5.7, 4.7, 3.5, 2.8, 2.4 μM, 10 μL min^{-1}). (b) WGA was injected over the *N*-acetyllactosamine surface (21.2, 12.7, 10.6, 7.9, 6.4, 5.3 μM, 10 μL min^{-1}). Sensorgrams shown were reference subtracted using a surface modified with compound **1**. Spikes due to subtraction or injection artifacts have been removed for clarity.

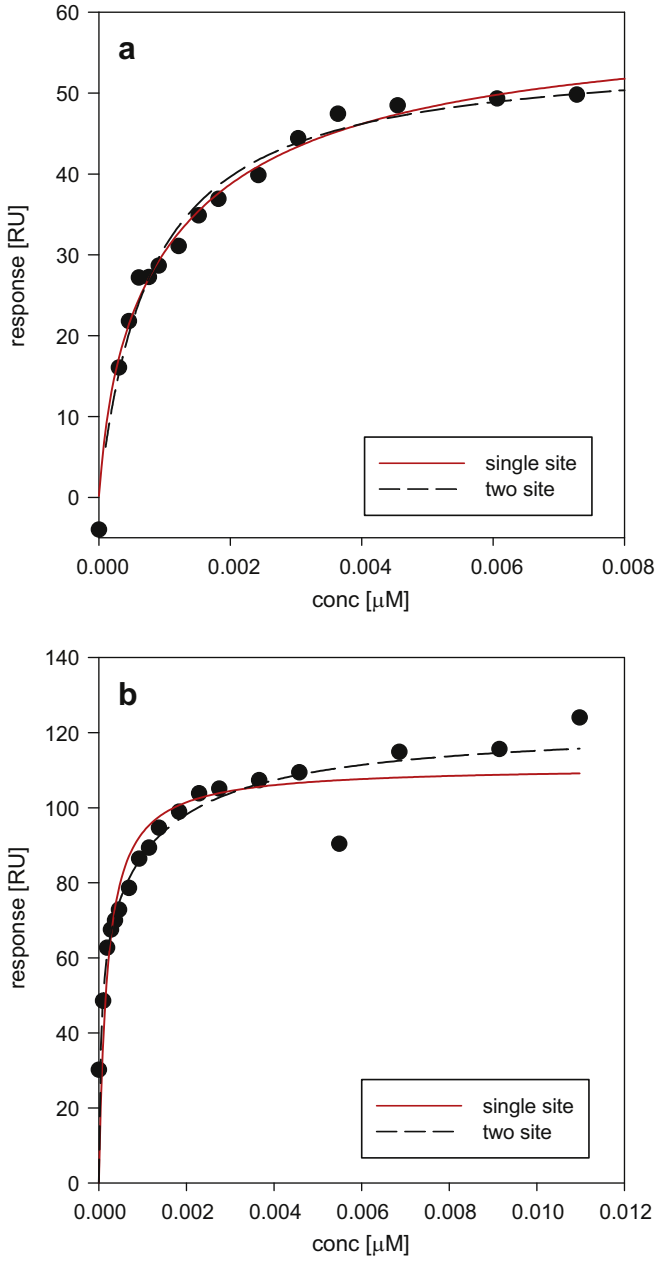

Figure 5. Binding isotherms of Jacalin and WGA. The binding of (a) jacalin to the galactose-modified surface (7.3, 6.1, 4.5, 3.6, 3.0, 2.4, 1.8, 1.5, 1.2, 0.91, 0.76, 0.61, 0.46, 0.30 μM) and (b) WGA to the *N*-acetyl-lactosamine surface (11, 9.1, 6.9, 5.5, 4.6, 3.7, 2.7, 2.3, 1.8, 1.4, 1.1, 0.92, 0.69, 0.46, 0.37, 0.27, 0.19, 0.09 μM) are shown. Each point is a steady-state RU value at the end of the association phase. See Supplementary data for semi-log and Scatchard analysis (Fig. S2).

In support of this model, the lower affinity site should yield the expected solution affinity of GlcNAc for WGA. This is indeed the case when the data are fit to the two-site model. This interpretation suggests an approximately 30-fold enhancement of affinity at sites that can engage the lectin in a chelated binding mode.[47] Non-ideal effects due to surface density or multivalent binding are well-known in SPR measurements, and these results allow us to quantify one mechanism of the enhancement of lectin affinity.[10,45,48]

3.4. Structural model of jacalin binding

Our determination of the affinity of jacalin for compound **2** gave an unexpectedly high value for a β-galactoside. Previous work with ITC has found that Galβ-OMe has significantly weaker binding than the Galα-OMe derivative. Structural determinations of galactoside binding to jacalin have concluded that the key H-bond interactions between the protein and the glycoside are at O-4, O-6, and O-3. The methyl aglycone of the α-galactoside has been shown to project over the face of Y122, while the aglycone of the β-galactoside results in unfavorable steric interactions with the same tyrosine residue. This steric interaction resulted in a decrease in affinity of approximately 100-fold.[49] Previous observations by Smith et al. and Hagiwara et al. suggest that α- and β-galactosides with a hydrophobic aglycone retain tight affinity to jacalin.[14,40] In light of these structural data, we were intrigued at our result. We considered that a possible explanation for these findings was that a

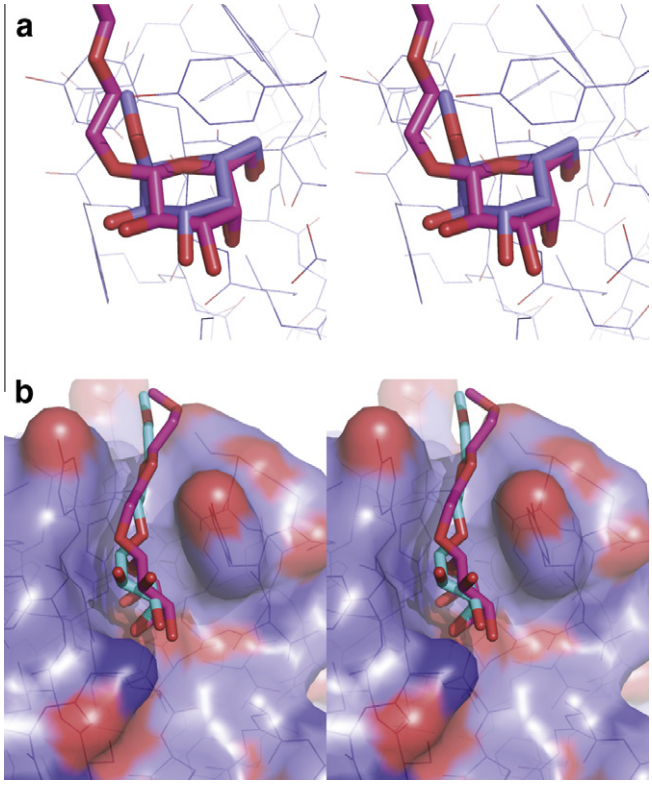

Figure 6. Molecular modeling of jacalin binding. (a) Models of a β-galactoside with a PEG aglycone (magenta) were generated, and then minimized (see Section 2). The critical H-bond contacts at O-4 and O-6 are maintained, although the orientation of the ring was slightly twisted from that of the α-galactoside (blue). (b) The aglycone may occupy secondary site A, which is a relatively hydrophobic channel formed by Y78 and Y122. Both the α and the β anomers with a flexible aglycon are able to take advantage of contacts in secondary site A; however, an additional carbohydrate residue would be too bulky to be accommodated.

flexible and hydrophobic linker could impart additional specificity to the glycoside. To examine this hypothesis in more detail, we used molecular modeling to examine the binding of α- and β-galactosides with a short flexible linker (Fig. 6). We found that a modified α-galactoside could maintain an almost identical orientation of the saccharide in the binding site as compared to the orientation of the free saccharide. The flexible linker of the aglycone could insert into a short hydrophobic channel formed by Y78 and Y122 on the protein. This region of the binding site was referred to as 'secondary site A' by Jeyaprakash et al.[35] In the case of the β-galactoside, we found that in order to accommodate the β-linked aglycone, the orientation of the ring in the active site was slightly altered. Importantly, the position of the major H-bond contacts at O-3, O-4, and O-6 was maintained. We also confirmed that the aglycone of the β-galactoside was able to gain favorable interactions at secondary site A. Thus, our model suggests that additional hydrophobic interactions at secondary site A can explain previous results with synthetic galactosides.[14,40] Additionally, the model suggests that tighter binding ligands for jacalin could be developed by exploiting these contacts.

4. Conclusions

We describe an implementation of Staudinger reagents for the immobilization of carbohydrates to a biosensor surface. To avoid issues of phosphorus oxidation, we have used an azide-derivatized surface, which is then reacted with a soluble phosphane. Using this method, we are able to detect the specificity of lectins for carbohydrate-modified surfaces. We confirmed that expected lectin–carbohydrate interactions, such as WGA binding to GlcNAc, could be observed and quantified. We are also able to quantify an enhancement of WGA binding due to multivalent binding of the lectin with a high-density GlcNAc surface.[47] Additionally, we observed an unexpected interaction of a β-galactoside with jacalin. We propose a structural model for the interaction of the galactosyl aglycone with secondary subsite A of the protein. Our model helps to rationalize our binding results, as well as previous reports of jacalin binding to synthetic substrates.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2010.09.020.

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