A Simple Approach to Well-Defined Sugar-Coated Surfaces for Interaction Studies

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Protein–carbohydrate interactions play a crucial role in many relevant biological processes, and the development of simple and reliable tools for their study is a well-recognized need. Surface-based methods are particularly attractive because they i) can effectively mimic cell-surface recognition events, ii) allow the identification of low-affinity binders, iii) are easily adaptable to high-throughput screening, and iv) require minimal sample amounts. We describe here the design and synthesis of a peptide module that efficiently captures glycans through its reducing end, by oxime ligation. Immobilization to carboxyl-functionalized supports was thereby made possible. Chemically well-defined surfaces coated with selected glycan targets were generated by this approach for surface plasmon resonance (SPR) studies. The usefulness of the method was demonstrated in the analysis of interactions that covered a five-orders-of-magnitude affinity range; namely, the strong binding ($K_A \sim 10^9\,M^{-1}$) of a well-known lectin (wheat germ agglutinin) to chitopentose (GlcNAc$_5$), and that of the same sugar with a weak binder ($K_A \sim 10^4\,M^{-1}$), HEV32—the smallest hevein domain described.

Introduction

Specific protein–carbohydrate interactions regulate a wide variety of biological processes, ranging from fertilization, tissue maturation, and inflammatory[1,2] and immune response against pathogens[3] to tumor metastasis. Although awareness of the important role of carbohydrates has been slow to emerge,[4,5] their key role in the storage and transfer of biological information through lectins as signal decoding and transmitting molecules is now firmly established.[6] The study of lectin–carbohydrate interactions is particularly challenging because of the structural complexity of the glycans, the permissiveness and multiplexity of lectins, and the relatively low affinity constants involved. To overcome these drawbacks and gain information on the structural and energetic aspects of the interactive process, a great variety of biochemical and biophysical methods have been developed. X-ray crystallography and NMR have been widely used to study the structural details of the recognition event and—in conjunction with isothermal titration calorimetry (ITC) and fluorescence spectroscopy—to obtain significant thermodynamic data.[7,8]

Surface plasmon resonance (SPR) has proven particularly suitable for the study of lectin–carbohydrate binding[9] in a variety of studies.[10,11] Low sample demands and the ability to evaluate interactions under dynamic conditions have made SPR and other surface-based techniques especially relevant for carbohydrate–protein binding studies; for instance, in the study of cell adhesion and surface-interaction events, and in high-throughput screening for new lectins or protein-binding carbohydrates.[12,13]

A major issue in all surface-based techniques is the immobilization of the desired ligand onto a solid support. Covalent attachment is preferred over other specific interactions, such as hydrophobic immobilization[14] or biotin/streptavidin-mediated attachment,[15,16] as it allows an easily tunable control of the immobilization level. This is a main concern for several applications and specifically for SPR, in which homogeneous, low-density surfaces are required for reliable quantitative kinetic information. Furthermore, this approach renders a stable surface, a feature that is of utmost importance for reproducible comparative analyses.

Most SPR studies on sugar–protein interaction reported to date rely on lectin immobilization. The reverse format, that is, sugar immobilization, has been far less explored and in practically all cases requires rather demanding procedures (either technically or sample-wise).[9] Simple, efficient methods for surface capture of sugars would therefore be of considerable interest for identifying novel carbohydrate–binding proteins as well as for high-throughput lectin characterization. Additionally, a general sugar capture method would greatly facilitate profiling studies of complex carbohydrates in array type settings.[17,18]

We have devised an efficient, straightforward method of oligosaccharide immobilization on SPR sensor surfaces. It relies on a short peptide tag that can be easily and selectively coupled to glycans of diverse complexity through their reducing end—the usually preferred site for sugar capture.[19] The covalent linkage between the peptide N terminus and the oligosaccharide is created by well-established peptide chemical ligation methods.[20,21] Additional reactive amino functions on the Lys

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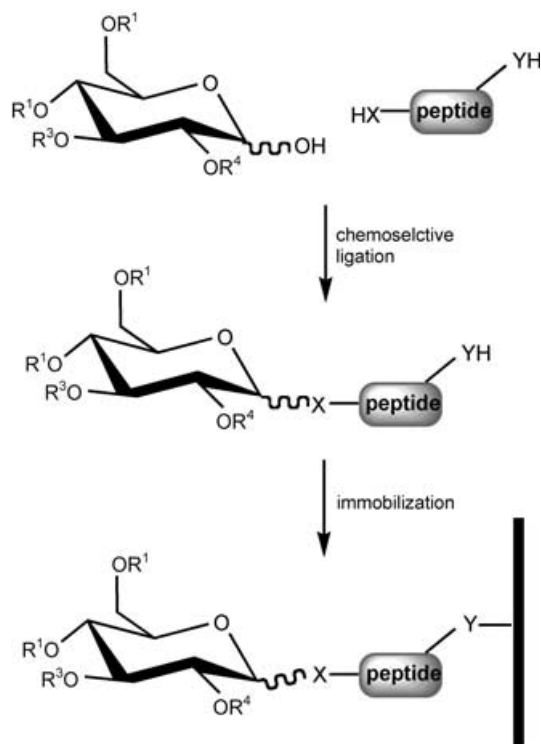
Scheme 1. Oligosaccharide immobilization by a peptide module that contains two chemoselective functionalities: X to bind to the carbohydrate reducing end and Y to react with sensitive groups on the chip surface. $R^{1,2,3} = H$ or any other substituent.

side chains of the peptide can then be used for orienting the immobilization by amide bonds to a carboxyl-activated sensor-chip surface (Scheme 1). A further advantage of this design is that the sugar–peptide probe can be readily isolated, purified, and thoroughly characterized by MS and HPLC prior to immobilization on the SPR surface.

As a proof of principle, we demonstrate the feasibility of the method for studying the interaction between a chitopentose and a representative lectin (wheat germ agglutinin). We also show that our probe is useful for monitoring sugar binding to smaller, much weaker-binding ligands, such as a hevein derivative, HEV32, recently described as a minimal chitin-binding domain.[22]

Results and Discussion

Direct covalent immobilization of sugars, through their reducing end, on sensor surfaces is challenging because methods from protein chemistry are not easily adapted and most of those specifically designed for carbohydrates involve chemically demanding derivatization steps. Furthermore, carbohydrate epitopes involved in sugar–protein recognition tend to be low-molecular-weight motifs that do not produce large SPR signals and thus make reliable reaction monitoring difficult. Attempts to immobilize oligosaccharides directly on a sensor chip through hydrazide-mediated surface activation[23] have turned out to be impractical for SPR analysis of low affinity binders, such as HEV32 (see below). This is because i) no significant im-

mobilization occurs under the dynamic conditions available in a SPR apparatus (even when using very long reaction times and high concentrations), ii) immobilization of low-molecular-weight carbohydrates (i.e. < 1000 Da) is difficult to monitor by surface plasmon resonance, and iii) drifting baselines are often obtained. In view of these difficulties, we decided to design a scaffold to which almost any desired oligosaccharide could selectively be fastened through a clean and efficient reaction. An obvious choice was a peptide unit with additional reactive groups that allows immobilization onto the SPR sensor surface.
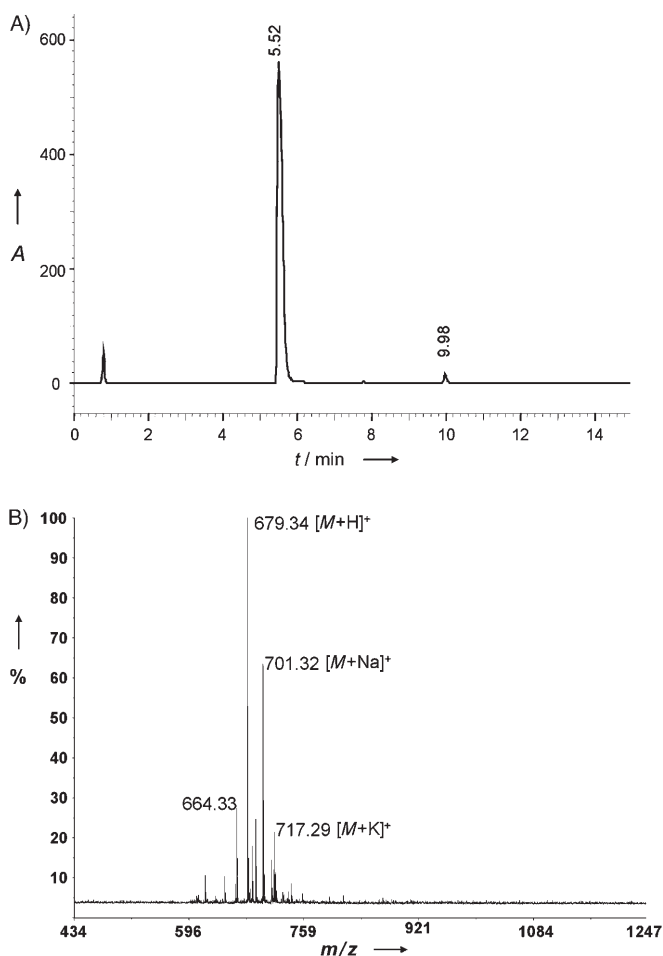
The oxime chemoselective ligation reaction[24] between the highly reactive amino group of an aminoxyacetyl (Aoa) containing peptide and the reducing end of an oligosaccharide has been demonstrated to be a powerful approach to glycoconjugates.[23,25–27] Therefore, we decided to use an Aoa residue as the glycosyl anchoring point of our probe. Unlike many other strategies, this capture procedure retains the reducing properties of the derivative; thus, the first monosaccharide of the conjugate preserves the naturally occurring closed-ring form in equilibrium with the open one.[23,28] Two Lys residues were incorporated into the peptide module to guide attachment to the sensor surface, after activation of the carboxyl-functionalized dextran matrix as N-hydroxysuccinimide (NHS) ester. The incorporation of Lys residues at the C terminus of the peptide ensures a homogeneous carbohydrate arrangement on the active surface. A Phe residue was also included in order to increase the hydrophobic character of the peptide and facilitate subsequent purification/isolation steps (Scheme 2). Given that steric hindrance is a major source of problems in the direct immobilization of small (short or highly branched) carbohydrates, the remaining Gly and Ala residues were added to provide flexibility and increase the distance between the chip surface and the glycosyl attachment point. Sequences longer than the Aoa–GFAKKG peptide (including one with an aminohexanoic acid spacer) were also explored with poorer results. This was probably because excess conforma-
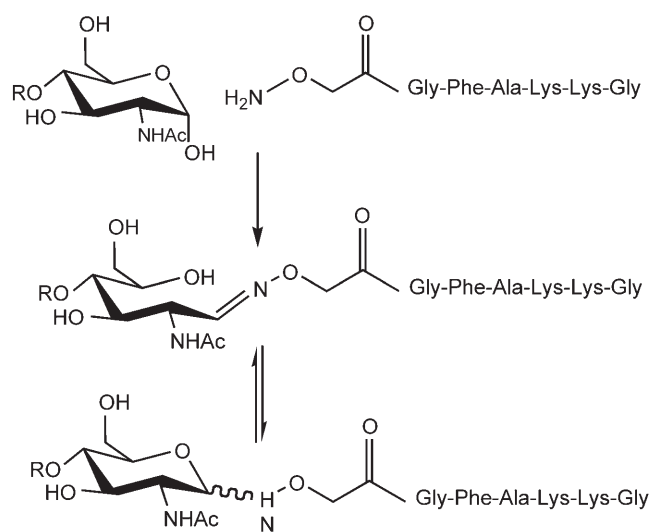
Scheme 2. Oxime chemical ligation reaction between the Aoa–GFAKKG peptide and an oligosaccharide. An equilibrium is established between the imino and amino (both α and β) forms.

tional mobility hampers an optimal arrangement for interaction under dynamic conditions.

The peptide probe was readily assembled by standard solid-phase synthesis protocols and purified to homogeneity (Figure 1). Particular care was taken to minimize exposure of

Table 1. Optimization of ligation conditions.[a]

	[sugar] /mM	[peptide] /mM	buffer	Yield /%
A	1	1.2	92% ACN[b]	13.5
B	0.125	0.200	92% ACN	3.2
C	0.250	0.400	92% ACN	0
D	1	1.2	0.1 M AcONa[c]	12.7
E	1	2	0.1 M AcONa	21
F	2.4	2.5	0.1 M AcONa	15
G	25	2.5	0.1 M AcONa	91
H	25	21	0.1 M AcONa	80
I	10	5	0.1 M AcONa	82
J	5	10	0.1 M AcONa	85

[a] All ligations carried out at 37°C. [b] 92% ACN/H$_2$O. [c] 0.1 M AcOH pH 4.6

Figure 1. A) HPLC analysis of the purified Aoa-peptide. The peak at 9.98 min results from a side reaction between the aminoxy function and acetone traces in ACN. B) MALDI-TOF analysis of the purified Aoa–peptide. The spectrum corresponds to the peak at 5.52 min. The *m/z* 664.33 peak corresponds to a fragment ion produced by cleavage of the N—O oxime bond upon MALDI ionization.[26]

the N-terminal aminoxy function to aldehyde and ketone impurities that are present in the acetonitrile? (ACN) used for RP-HPLC purification, as undesirable by-products could arise. In particular, a by-product with a molecular weight (MW) consistent with the formation of a Schiff base between the purified Aoa–peptide and acetone impurities (from HPLC-grade ACN) could be detected after prolonged standing of the reaction mixture in solution. The problem was prevented by fast pooling of the HPLC-purified peptide-containing fractions, followed by rotary evaporation to remove ACN and volatile impurities.

Several ligation conditions between the peptide module and a panel of oligosaccharides (lactose, cellopentose, chitopentose, and chitohexose) were explored[26, 29] (Table 1 summarizes results obtained with chitopentose). Since the Aoa–peptide is soluble in a wide variety of solvents from aqueous to highly organic media, selection of an appropriate buffer is particularly crucial for dissolving the carbohydrate at high concentrations that significantly improve the yields. Roughly equimolecular conditions and high concentrations of sugar and peptide (entry H, Table 1) allow the ligation reaction to proceed with high yields and without waste of reagents. This is interesting for costly carbohydrates and also for facilitating subsequent purification steps. On the other hand, large excesses of peptide (entry J Table 1) only slightly improve the yield and entail a purification step to remove large amounts of nonglycosylated peptide, which would compete (i.e., give rise to unproductive binding sites) with the lectin probe for surface binding. Accordingly, ligation between the peptide and glycan is best carried out at 21 and 25 mM, respectively, in AcONa (0.1 M, pH 4.6) at 37°C for 72 h. Thereby, a major product with a MW consistent with the expected glycopeptide is obtained (Figure 2). Unreacted aminoxy groups on the peptide were capped by Schiff base formation with excess acetone (2 equiv) prior to purification. This capping step increases the hydrophobicity of the by-product and facilitates reversed-phase separation. It also averts the undesirable acylation of the highly reactive Aoa–peptide by the active esters on the sensor-chip surface. Alternatively, an aldehyde-containing resin can be used to trap the Aoa–peptide (data not shown), and the residual carbohydrates need not to be removed as they will not react with the amino-capturing surface.

As a further demonstration that the ligation reaction is completely selective for the aminoxy functionality and that no reaction with Lys side chains takes place, the glycopeptide (GlcNAc$_5$–peptide) was sequenced by tandem mass spectrometry (MS/MS; Figure 3). Fragmentation spectra acquired under high collision energies (CEs) produced y ions that allowed both peptide sequence and ligation site assignment. This proved that the glycan unit was joined to the peptide N terminus by the Aoa residue. Moreover, lower CEs produced significant y and b ions from the fragmentation of the oligosaccharide section. This allowed the sequencing of the monosaccharide units and corroborated the integrity of the captured sugar. These

A)

B)
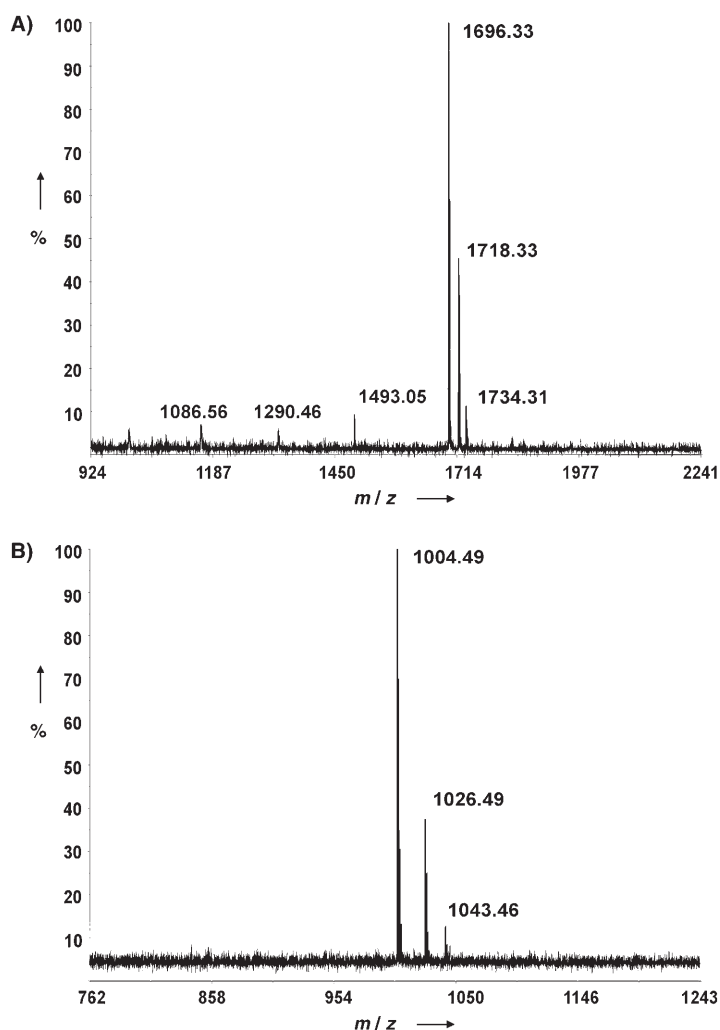
Figure 2. MALDI-TOF analysis of A) GlcNAc$_5$– and B) lactose–Aoa–GFAKKG conjugates. Both glycopeptides were easily detected by using standard reflector mode and positive detection. Peaks from carbohydrate fragmentation (loss of GlcNAc, 203 Da) were also observed for the chitopentose probe.

results clearly suggest that this approach can be expanded to carbohydrate tagging and structural characterization by MS/MS.[30]

In order to demonstrate the feasibility of our approach to oligosaccharide immobilization we chose chitin and wheat germ agglutinin (WGA) as a model sugar–lectin system and monitored their interaction by SPR. A carbohydrate probe displaying penta-*N*-acetyl-glucosamine (GlcNAc$_5$), a representative chitin structure, was synthesized as described above and immobilized in one of the flow channels of a CM5 sensor chip. Analogously, a lactose-containing glycopeptide was used to generate a reference surface for subtracting matrix related interactions. WGA was injected over the two surfaces, with Ca^{2+} added to both sample and running buffers, and the variation in SPR response over time on each surface was recorded.

Sensorgrams (Figure 4 A) clearly show that WGA specifically interacts with the chitopentose while no interaction is detected with the reference cell. This proves that the carrier peptide does not interfere in the recognition event. To further confirm

the specificity of the interaction, *N*-acetyl-glucosamine, a known low-affinity ligand for WGA, was injected over the flow cell that contained the WGA lectin bound to the immobilized GlcNAc$_5$. The resulting sensorgram (Figure 4 B) clearly shows that the monosaccharide displaces WGA from the chitopentose surface until the initial baseline level is recovered. In order to establish optimal conditions for analysis, two surfaces with different immobilization levels of chitopentose (100 and 1500 resonance units (RU)) were prepared. The resulting sensorgrams (Figure 5) show that low-density surfaces, generally preferred for kinetic studies, also provide higher sensitivity; however, densely populated arrays hamper interaction, possibly due to steric hindrance.

To characterize the chitin–WGA interaction quantitatively, seven different WGA concentrations were injected over a sensor surface that contained 100 RU of immobilized GlcNAc$_5$. Sensorgrams were recorded and corrected by subtraction of the signal from the reference cell (Figure 6). Kinetic analysis was performed by separated numerical integration of the association and dissociation phases by using a Langmuir (1:1) model. Kinetic and thermodynamic parameters (Table 2) are similar to those reported by Zeng et al.[31] for the binding of WGA to a polymer functionalized with GlcNAc$_2$. However, the results are considerably better (K_A two orders of magnitude higher) than those reported by Nahálaková et al.[32] for the interaction of WGA and an GlcNAc monosaccharide derivative. The difference between our (and Zeng and co-workers') results and the values described for monosaccharides underline the well-known avidity of chitin-binding lectins towards polysaccharides.

Once our approach was shown to be useful for monitoring sugar–lectin binding in a well-known system, such as chitin–WGA, we tested the same carbohydrate probe on a more challenging chitin-binding protein. HEV32 is a hevein-derived peptide that has been postulated to be a minimal chitin-binding domain[22] with much lower molecular weight and weaker affinity for carbohydrates than other known lectins. HEV32 was injected at seven different concentrations on the chitin-surface and sensorgrams were recorded as described (Figure 7). Results again clearly show a specific interaction between the peptide and the immobilized chitopen-

Table 2. Kinetic and thermodynamic data of WGA binding to chito-oligosaccharides.

	K_a [M^{-1} s^{-1}]	K_d [s^{-1}]	K_A [M^{-1}]	χ^2
GlcNAc$_5$	3.58×10^5	1.27×10^{-4}	2.26×10^9	0.301
GlcNAc$_n$[a]	6.9×10^5	6.5×10^{-4}	1.1×10^9	–
GlcNAc[b]	4.67×10^4	4.96×10^{-4}	9.42×10^7	–

[a] Polymer containing GlcNAc$_2$ units.[31] [b] 2-acetamido-2-deoxy-β-D-glucopyranosylmethylamine.[32]
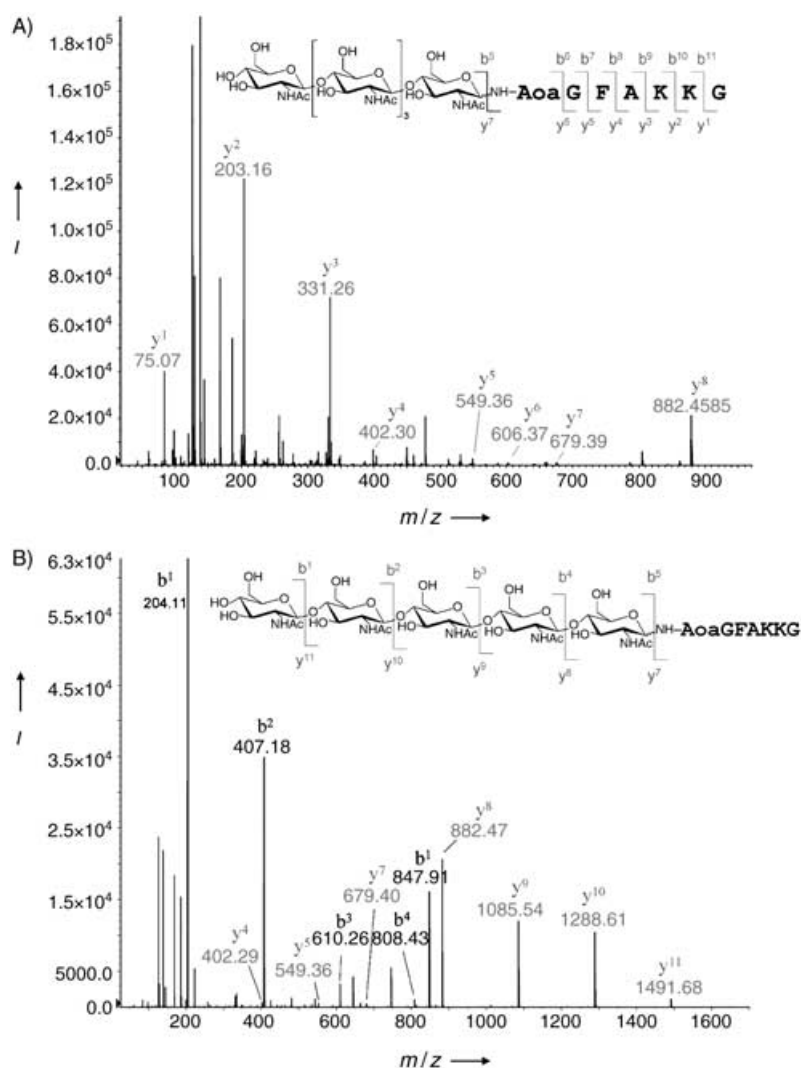
Figure 3. Q-TOF MS/MS spectra of the GlcNAc$_5$–Aoa–GFAKKG glycopeptide by using different CEs. A) Fragmentation spectrum obtained under high CE displays characteristic y ions, which result from breakage at the peptide linkages. This confirms that the oligosaccharide is attached to the aminoxy group. B) Low CE provides a fragmentation spectrum with valuable information on the glycosidic part (both b and y series observed) of the carbohydrate probe.

tose. In this case, none of the available mathematical models for numerical integration could be fitted to the data and thus kinetic and thermodynamic constants could not be derived. This could be explained by very fast association and dissociation rates for the sugar–protein complex, and/or by the formation of multivalent complexes, such as those observed by NMR for HEV32 (and also native hevein) and large polysaccharides like GlcNAc$_5$.[8,22] Therefore, affinity constants were calculated by using a general steady state model that assumes the system achieves equilibrium during sample injections. This is the case in our experiments, as a plateau is reached before the dissociation phase. A plot of the response signal at equilibrium as a function of concentration is adjusted to a hyperbolic equation (Figure 8) from which the thermodynamic affinity constants can be obtained. The goodness of the fitting was assessed by the r^2 and χ^2 values, 0.9996 and 1.98, respectively. A K_A of 2.31×10^4 M^{-1} was obtained, which is in good agreement

with previously reported fluorescence and NMR data for HEV32 binding to other chitooligosaccharides.[22]
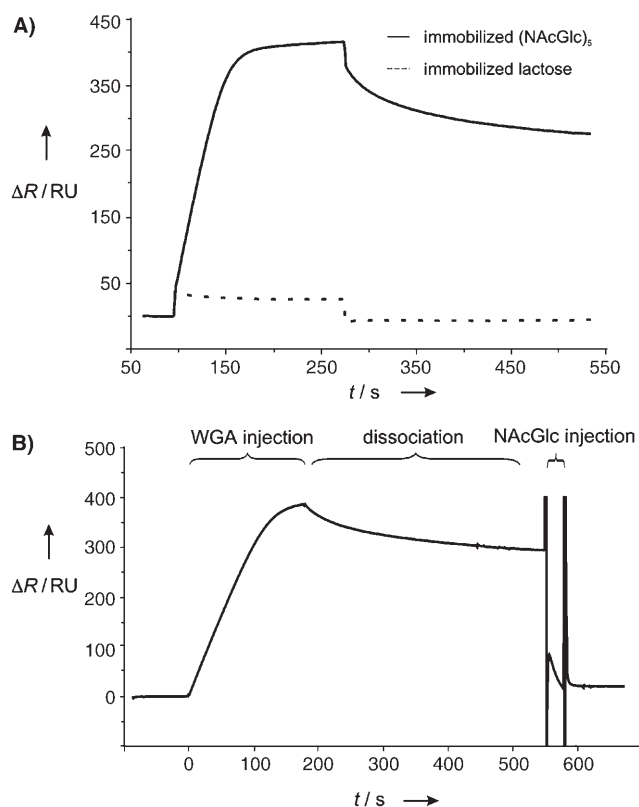
Conclusion

Efficient and selective methods for oligosaccharide immobilization are a pressing requirement for gaining insight into protein–carbohydrate interactions[33] and deciphering the sugar code.[6] This work shows that an Aoa-functionalized designed peptide can be used to selectively capture carbohydrates neatly and efficiently through their reducing end to give a native-like glycoconjugate. Oxime ligation between the Aoa–peptide and various glycans is efficient and simple, and no relevant by-products are generated. It could thus be used to capture complex carbohydrates that can only be obtained in minute amounts. The resulting glycopeptide can then be immobilized under strictly controlled conditions and used as a carbohydrate surface probe for SPR kinetic studies or high-throughput screening of novel carbohydrate-binding proteins. Our approach provides an alternative to the recently described preparation of oligosaccharide microarrays based on neoglycolipid technology.[34,35] The orientation and accessibility of the sugar moiety is a key issue in surface-based carbohydrate-recognition systems. We are reasonably confident that tethering the sugar to the chip surface through our designed peptide module favors optimum sugar display. Experiments with WGA show that the peptide moiety does not interfere with protein–carbohydrate binding, and that accurate kinetic and thermodynamic data (consistent with previously reported results) can be obtained. The method has also proven useful for evaluating the interaction of carbohydrates with small, low-affinity binders, such as the HEV32 peptide. Thus, this method constitutes a general tool for the investigation of protein–carbohydrate interactions.

Experimental Section

Materials: Fmoc (*N*-α-(9-fluorenylmethyloxycarbonyl)) protected amino acids were purchased from Senn Chemicals (Dielsdorf, Swit-

Figure 5. Binding of WGA (250 nм) on low (100 RU) and high (1500 RU) density surfaces of immobilized carbohydrate probe.
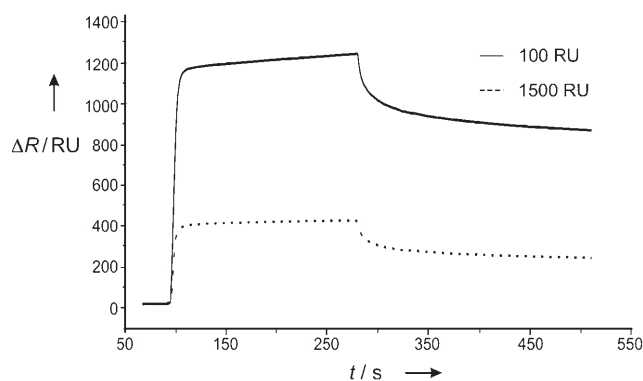

Figure 4. A) Selective interaction of WGA (7.81 nм) with the GlcNAc$_5$–peptide surface. No interaction was observed with the reference cell which contained immobilized lactose. B) Competition experiment for WGA. A lectin solution (60 μL, 3.91 nм) was injected over a flow cell that contained immobilized GlcNAc$_5$, at 20 μL min^{-1}. This was then replaced by running buffer and the carbohydrate–lectin complex was allowed to dissociate. Finally, a GlcNAc solution (10 μL, 0.5 м) was injected to promote the displacement of bound WGA and the recovery of the baseline. Thus the specificity of the measured interaction with the sensor surface was demonstrated.
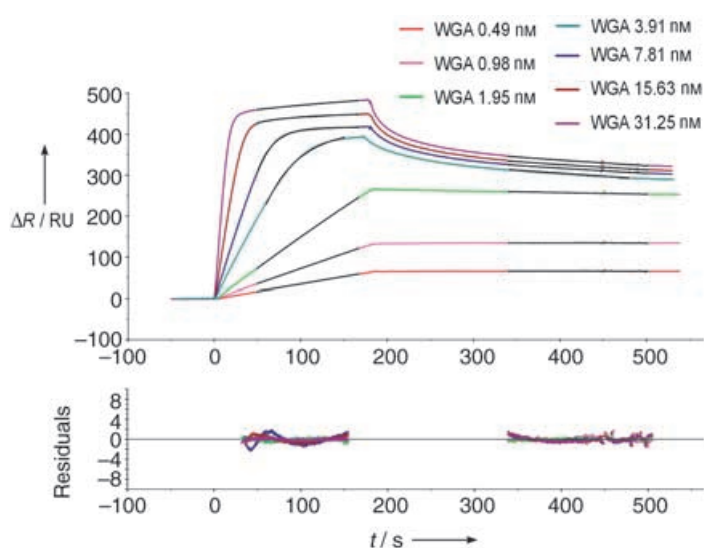
3 equiv of amino acid, HBTU, and HOBt were used in the presence of 6 equiv of DIEA. The peptide was fully deprotected and cleaved from the resin by treatment with cocktail R (TFA/anisole/thioanisole/EDT, 90:2:5:3 *v/v/v/v*) for 2 h at RT. The Aoa-containing peptide was isolated by precipitation with cold *tert*-butyl methyl ether and centrifugation, then taken up in acetic acid (0.1 м) and lyophilized. For preparative HPLC purification, a linear gradient from 0 to 15% of ACN in TFA/water (0.1%) on a Phenomenex Luna C8 column (10 μm, 1.0×25 cm) was used, at a flow rate of 5 mL min^{-1}, in a Shimadzu LC-8 A instrument. Fractions judged to be homogeneous by analytical HPLC were promptly pooled together, rotary evaporated to remove all ACN and volatile impurities that could react with the free aminoxy functionality, and lyophilized (see Results and Discussion). The purified peptide module was further characterized by MALDI-TOF and ESI MS and quantified by amino acid analysis. HEV32 was synthesized as previously described.[22]

zerland) and *tert*-butyloxycarbonyl–Aoa (Boc–Aoa) from Novabiochem (Läufelfingen, Switzerland). *p*-Methylbenzhydrylamine (MBHA) resin and 2,4-dimethoxy-4′-(carboximethyloxy)-benzhydrylamine (Rink amide) linker were from Bachem (Bubendorf, Switzerland). 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and *N*-hydroxybenzotriazole (HOBt) were from Albatross Chem (Montreal, Canada). Chito- and cello-oligosaccharides and lactose (Gal(β1–4)Glc) were from Toronto Research Chemicals (Toronto, Canada) and wheat germ agglutinin (WGA) from BioChemika (Madrid, Spain). HPLC-grade ACN, peptide synthesis-grade *N,N*-dimethylformamide (DMF), *N,N*-diisopropylethylamine (DIEA) and trifluoroacetic acid (TFA) were from SDS (Peypin, France). Other reagents were from Sigma–Aldrich (Madrid, Spain).

Peptide synthesis: The carrier peptide, Aoa–GFAKKG, was manually assembled as a C-terminal carboxamide on a Rink-amide *p*-MBHA resin (0.67 mmol g^{-1}) by using standard Fmoc solid-phase synthesis protocols[36] at the 0.1 mmol scale. Lysine side chains were protected with Boc groups. Couplings were performed with Fmoc–amino acid, HBTU, and HOBt (5 equiv each), and DIEA (10 equiv) in DMF. For the coupling of the Boc–Aoa,
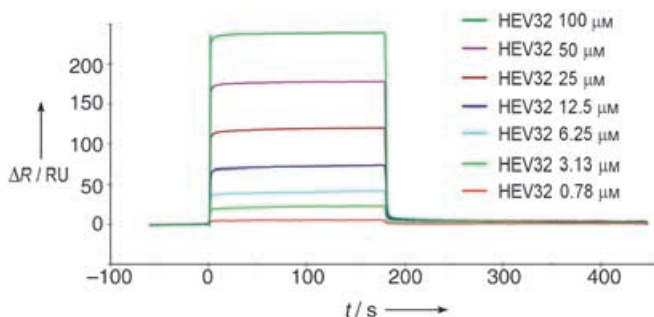

Figure 6. Binding of WGA to immobilized GlcNAc$_5$ at seven different concentrations. A) Numerically fitted association and dissociation curves using a Langmuir model are shown in black. B) A plot of the residuals (difference between experimental and fitted data) as a function of time. Residuals below 2 for all curves ensure the good quality of the fitting ($\chi = 0.301$). A sensorgram obtained for 7.81 nм WGA was removed from the calculations, as its residual distribution was nonrandom.

Figure 7. Binding of HEV32 at seven different concentrations over immobilized GlcNAc$_5$. Signal from a lactose–peptide reference cell was subtracted.
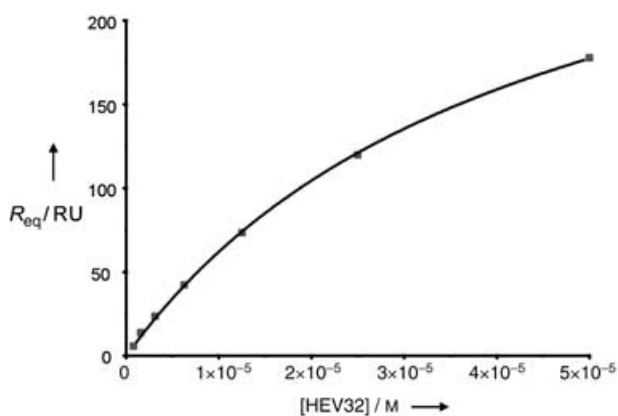
Figure 8. Equilibrium response as a function of HEV32 concentration. Experimental data (black squares) were fitted by using the steady state model to obtain values for the thermodynamic association and dissociation constants.

Carbohydrate probe synthesis and characterization: Several reaction conditions were explored. In our optimal protocol, peptide solution (21 mM) in AcONa (0.1 M, pH 4.0), was added to the dry oligosaccharide to give a final concentration of 25 mM. The reaction proceeded at 37°C for 72 h and its progress was efficiently monitored by measuring the decrease of the peptide signal by HPLC. Once the reaction was complete, the glycopeptides were purified by preparative HPLC, as described above. Homogeneous fractions containing the desired product (confirmed by MALDI-TOF MS) were pooled and lyophilized. In order to confirm that the ligation site was between the reducing end of the oligosaccharide and the aminoxy functionality of the peptide, the resulting glycopeptide was sequenced by MS/MS in a Q-Star-Pulsar nanospray instrument (Applied Biosystems, Foster City, CA, USA). Different collision energies were used to obtain optimal fragmentation of the two regions of the molecule: 50, 45, and 40 eV for the glycosidic and 70, 65, and 60 eV for the peptide moieties. Analysis of the fragmentation spectra by means of the Analyst software package (Applied Biosystems, Foster City, CA, USA) allowed the determination of the peptide and glycosidic sequences and the ligation site.

Surface plasmon resonance studies: All SPR measurements were carried out on a BIAcore 3000 instrument (Biacore, Uppsala, Sweden) by using a CM5 sensor chip and HBS-P (0.01 M HEPES pH 7.4, 0.15 M NaCl, 0.005% Surfactant P20) as running buffer. For lectin–carbohydrate binding experiments, CaCl$_2$ was added to both sample and running buffers to give a final concentration of 5 mM.

All reagent solutions were freshly prepared, filtered through a 0.22 μm filter, and degassed by sonication.

Immobilization of carbohydrate probes to CM5 chip: Carboxyl groups on the chips' dextran matrix were activated by the formation of an NHS ester by using the standard procedure recommended by BIAcore. The surface was activated by injecting a freshly made solution of NHS (0.05 M) and EDC (0.2 M) in water (35 μL), at 5 μL min^{-1}. Then the glycopeptide probe was dissolved in running buffer (up to 100 μg mL^{-1}) and injected over the activated flow cell during 14 min, at a flow rate of 5 μL min^{-1}. For kinetic experiments, an immobilization level of 100 RU was aimed at. Unreacted active esters were neutralized by injection of ethanolamine (1 M, pH 8.5; 35 μL) at a flow rate of 5 μL min^{-1}. Finally, the surface was equilibrated by repeated injections of HCl (10 mM) and NaCl (0.5 M) until a stable baseline was obtained. The reference flow cell was analogously obtained by immobilization of a lactose-carrying glycopeptide on the first flow cell of the sensor chip (Fc1).

Lectin binding experiments: WGA solutions in the 0.48–250 nM concentration range were prepared in HBS-P buffer that contained CaCl$_2$ (5 mM), by dilution from a protein stock solution (58 mM) in PBS. The dilutions were injected (60 μL) at 20 μL min^{-1} over the active surface. After protein injection, sample solution was replaced by running buffer and the carbohydrate–lectin complex allowed to dissociate for 4 min. The active surface was regenerated by two series of GlcNAc (0.5 M) and HCl (10 mM) injections (10 μL each) at 20 μL min^{-1}. Kinetic data were obtained by consecutive injections of WGA solutions (as above) over an active surface with immobilized GlcNAc$_5$. The signal of the reference flow cell was subtracted with an automated method. HEV32 binding experiments were performed by injecting peptide samples in the 0.78–100 μM range dissolved in HBS-P, as described for WGA.

Sensorgrams were analyzed by curve fitting by using numerical integration algorithms in the BIAevaluation 3.0 software package.

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