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Neo-glycopeptides: the importance of sugar core conformation in oxime-linked glycoprobes for interaction studies

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Abstract Carbohydrate binding proteins, such as lectins, are crucial in numerous biological recognition processes. While binding may be mediated by a single monosaccharide, several lectins have shown exquisite epimer and linkage recognition indicating that a larger structure is essential for optimal interaction. Several approaches have been described for their detailed study, including lectinosorbent assays, microarrays and surface plasmon resonance (SPR). Most of these approaches ignore that the aglycon-bound monosaccharide is often in a non-natural conformation that affects the occurring binding event. In this paper we demonstrate that oxime-bound glycans, employed in such approaches, occur predominantly in the open form (~70%). Through the use of a secondary amine, the aglycon-bound monosaccharide in the resulting neo-glycopeptide probe is forced into the ring-form. Resulting structures were analyzed by means of nuclear magnetic resonance and differential derivatization experiments. The impact of ring closure was further demonstrated through interaction studies using SPR and various lectins with distinct binding specificities.

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Introduction

The increasing recognition of the importance of post-translational modifications in the fine-tuning of protein function has brought about a re-acknowledgement of glycosylation. This truly consequential modification affects more than half of all known proteins, and its crucial role in protein folding, proteolytic resistance, or lysosomal traffic [1] has been conclusively established. Furthermore, and by virtue of their peripheral localization, particular oligosaccharide epitopes on proteins or lipids exert key functions in important intercellular communication processes such as fertilization [2], immune response [3], pathogen anchoring [4], or metastasis [5], to name just a few. These particular carbohydrate epitopes are recognized by membrane-anchored carbohydrate-recognition domains of different molecules such as receptors, enzymes, antibodies or lectins. The latter class comprises proteins of non-immune origin, frequently multimeric and rather ubiquitous, that are involved in many diverse biological functions and may exhibit exquisite epimer specificity (*e.g. Concanavalin A* in mannose *vs* galactose), or linkage (*Sambucus nigra agglutinin* and *Maackia amurensis agglutinin* in α2–6 *vs* α2–3). Some lectins, on the other hand, are relatively promiscuous in epitope recognition (*e.g. Lotus tetragonolobus agglutinin* recognizes fucose, has an increased affinity for blood group H, and binds best when this epitope is present in type-II glycans), which allows a certain degree of affinity ranking of the distinct epitopes and points to a possible role in the fine-tuning of biological function.

Due to their carbohydrate specificity, lectins are often used as tools for the structural and functional study of complex glycans [6] and as diagnostic markers in some diseases [6, 7]. In the discovery of new lectins and the study of their binding characteristics, the availability of the largest possible number of relevant structural sugar entities in sufficient amounts remains a main bottleneck. Although a study of the complex interaction between a glycoprotein and its counterpart would ideally require highly purified, well defined chemical species in sufficient amounts, the real situation is usually much less propitious. The amounts of naturally occurring glycans available, either as intact glycoprotein or as purified individual glycan (this one even more complicated, given the poor yields of both deglycosylation and ensuing purification) are unfailingly low. The alternative approach of chemical synthesis of complex oligosaccharides [8] remains a cumbersome and laborious affair, often requiring elaborate protecting-group strategies to access sugars with minimal (*e.g.* a single glycosidic linkage) structural differences. Further, for the sake of comparability the nature of the peptide moiety underlying the carbohydrate epitope is often ignored in interaction studies. Thus, in order to differentiate between different carbohydrate epitopes, or to enable the read-out of a particular interaction, non-biological platforms are often employed.

Several techniques have been developed over the last few years for the study of carbohydrate-lectin interactions, ranging from hemagglutination [9], enzyme-linked lectinosorbent assays [10] or, more recently, microarrays [11]. In the latter case, different chemistries for attaching oligosaccharides to a solid matrix have been described [12], ranging from reductive amination (with ring opening of reducing-end monosaccharides), to oxime ligation (with equilibrium between ring-opened forms) [13], or to hydrazide (with predominantly β-cyclic forms) [14]. Despite the obvious advantages of high-throughput microarray methods, the fact that non-labeled proteins (lectins) cannot be detected is a serious drawback. A different approach that does not require labeling for the measurements is based on surface plasmon resonance (SPR). In this technique, one of the two interacting entities (protein or sugar) is immobilized on the surface of a sensor chip, the other one is passed and the resulting read-out enables both quantitation and kinetic analysis of the interaction, without the need for large amounts of sample. The direct attachment of the sugar to the surface, however, suffers from the poor reactivity of the aldehyde function [15, 16]. Recently, we described an approach to overcome this limitation through a selective conjugation of glycans to a tailor-made peptide-module and efficient immobilization of the glycoprobe to the surface [17]. This approach, which relies on chemoselective oxime ligation to selectively target the reducing-end aldehyde of any unprotected sugar [18, 19], is adaptable to any peptide mimicking the original sequence of the protein. In a follow-up optimization study, the conjugation and purification conditions were made compatible with the usually small amounts of glycans available [20]. A remaining problem with this approach, however, is that when small (mono to trisaccharide) epitopes are the structural recognition motifs, the structure (open or closed) of the peptide-linked monosaccharide plays an important role. In this paper we have studied in detail the extent of this question—largely neglected in most array formats published thus far—by comparing peptide modules that induce a closed-ring structure upon conjugation with peptides of identical sequence but devoid of this feature. Structural analysis by nuclear magnetic resonance (NMR) and differential derivatization techniques have been employed to determine exactly the number and relative abundance of each isomer in both glycoprobes, and by SPR to demonstrate the effect of ring closure on the interaction with different lectins.

Materials and methods

Materials

Fmoc (N^{α}-(9-fluorenylmethyloxycarbonyl) protected amino acids were purchased from Senn Chemicals (Dielsdorf, Switzerland). Boc (*tert*-butyloxycarbonyl)-protected aminooxyacetic acid (Boc–Aoa) and Rink amide MBHA resin were from Novabiochem (Läufelfingen, Switzerland). 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) was obtained from Iris Biotech (Marktredwitz, Germany). *N*-acetylglucosamine (GlcNAc), *Erythrina cristagalli agglutinin* (ECA) and *Triticum vulgaris agglutinin* (WGA) were from Sigma-Aldrich (Madrid, Spain). *N*-acetyllactosamine (lacNAc) was purchased from Dextra laboratories (Reading, UK) and *Ricinus communis agglutinin* (RCA) was from Vector laboratories (Peterborough, England). *N*,*N*-diisopropylethylamine (DIEA) was from Merck Biosciences (Darmstadt, Germany), and high-performance liquid chromatography (HPLC)-grade acetonitrile (ACN), *N*,*N*-dimethylformamide (DMF), sodium dodecyl sulfate (SDS) and trifluoroacetic acid (TFA) were from (Peypin, France). CM5 sensorchips, *N*-ethyl-*N*'-(dimethylaminopropyl)carbodiimide (EDC), *N*-hydroxysuccimide (NHS), and HBS-P (0.01 M HEPES pH 7.4; 0.15 M NaCl; 0.005% *v*/*v* surfactant P20) buffer were from Biacore AB (Uppsala, Sweden).

Analytical and semi-preparative HPLC were run on Shimadzu equipment using columns packed with Luna C18 (3 µm, 50×4.60 mm) and (10 µm, 250×10.00 mm) for peptides and with Sphereclone C18 (5 µm, 250×4.60 mm) for glycopeptides. Linear gradients of buffer B into A for analytical (A: 0.045% TFA in H_2O and B: 0.036% TFA in ACN over 15 min) and for semi-preparative (A: 0.1% TFA in H_2O: B: 0.1% TFA in ACN over 30 min) scale were used. Mass spectra were obtained on a MALDI-TOF spectrometer (Voyager-DE STR Bio-spectrometry workstation (Applied Biosystems)). NMR spectra were recorded on a Varian Inova VXR-500 spectrometer in ^2H_2O. All SPR experiments were carried out on a BIAcore 3000 instrument.

Peptide synthesis

Solid-phase peptide synthesis was carried out in the manual mode on a Rink-amide *p*-MBHA resin. Fmoc-amino acids were coupled by means of HBTU and DIEA (threefold molar excess of each) in DMF. The aminooxy functionality was coupled as Boc–Aoa as previously described [21]. Full deprotection and cleavage were performed with TFA–water–triisopropylsilane (95:2.5:2.5 *v*/*v*, 90 min, rt). Peptides were isolated by precipitation with cold *tert*-butyl-methyl ether and separated by centrifugation, dissolved in water and lyophilized. The crude material was analyzed by analytical HPLC and characterized by MALDI-TOF mass spectrometry (MS). No further purifications were required as only peaks corresponding to the desired end-products were observed.

Peptide **1** $m/z: 663.4[M+H]^+, 685.4[M+Na]^+, 701.4[M+K]^+$.
Peptide **2** $m/z: 608.4[M+H]^+, 630.4[M+Na]^+, 646.4[M+K]^+$.

N-methylation of peptide **2** was done by the two-step protocol of Mutter [21]. The product was purified to homogeneity and characterized by MALDI-TOF mass spectrometry.

$N[\mathrm{Me}]-O-$peptide **2** $m/z: 622.3[M+H]^+, 644.3[M+Na]^+, 660.3[M+K]^+$.

Chemoselective ligation with GlcNAc and lacNAc

For each conjugation, the dry carbohydrate was dissolved in peptide solution (20 mM in 0.1 M NaOAc) to a 25 mM concentration. The reaction was allowed to proceed at 37°C and pH 3.5 or 4.6 for *N*-methylated or non-methylated peptides, respectively. After 72 h, the glycopeptides were purified by HPLC. Glycopeptide fractions of satisfactory purity (>95% by analytical HPLC, correct MALDI-TOF MS) were pooled and lyophilized.

GlcNAc − peptide **1** m/z: $867.1[M+H]^+, 889.1[M+Na]^+, 905.1[M+K]^+$.
GlcNAc − peptide **2** m/z: $811.7[M+H]^+, 833.7[M+Na]^+, 849.7[M+K]^+$.
GlcNAc$-N[\mathrm{Me}]-O-$peptide **2** m/z: $825.6[M+H]^+, 847.6[M+Na]^+, 863.6[M+K]^+$.

lacNAc − peptide **2** m/z: $973.5[M+H]^+, 995.5[M+Na]^+, 1,011.5[M+K]^+$.
lacNAc$-N[\mathrm{Me}]-O-$peptide **2** m/z: $987.7[M+H]^+, 1,009.7[M+Na]^+, 1,025.7[M+K]^+$.

Derivatization of glycopeptides

GlcNAc-peptide 2 and GlcNAc-*N*[Me]-*O*-peptide 2 were acetylated with acetic anhydride and pyridine (1:1 *v*/*v*; 100 equiv) overnight at room temperature. Excess reagents were evaporated in a speed-vac and the resulting peracetylated glycopeptides were characterized by MALDI-TOF MS.

$(Ac)_n$[GlcNAc − peptide **2**] m/z:
(a) closed form : $1,021.8[M+H]^+, 1,043.8[M+Na]^+, 1,059.8[M+K]^+$.
(b) open form : $1,063.8[M+H]^+, 1,085.8[M+Na]^+, 1,101.8[M+K]^+$.
$(Ac)_n$[GlcNAc$-N[\mathrm{Me}]-O-$peptide **2**] m/z:
$1,035.6[M+H]^+, 1,057.6[M+Na]^+, 1,073.6[M+K]^+$.

NMR experiments

GlcNAc-peptide **1** was repeatedly exchanged in D_2O, with intermediate lyophilizations, and finally dissolved in 500 µl ^2H_2O. ^1H 1D and 2D NMR spectra were recorded on a Varian Inova VXR-500 spectrometer operating at 500 MHz. Chemical shifts (δ) were expressed in parts per million relative to D_2O (D_2O δ 4.754 at 25°C). Spectra were processed using MestRe-C software (version 2.0.0.1, MestreLab Research, Santiago de Compostela, Spain).

SPR analysis

For glycopeptide immobilizations, the carboxyl functionalities of the CM5 sensorchip were activated at a flow rate of 5 µl min^{-1} with a 10 min pulse of NHS and EDC (1:1 mixture), then a 1 mg ml^{-1} glycopeptide solution in 10 mM NaOAc, pH 6, was injected for approximately 12 min (depending on glycopeptide to ensure similar immobilizations levels), followed by blocking of the remaining

succinimidyl esters with 1 M ethanolamine hydrochloride, pH 8.5, for 12 min. Three surfaces were prepared: (1) a reference surface with *N*[Me]-*O*-peptide **2**, (2) lacNAc-peptide **2** and (3) lacNAc-*N*[Me]-*O*-peptide **2**. Immobilization levels of approximately 150 RUs were obtained for each surface.

All lectin binding experiments were performed at 25°C and a flow rate of 10 μl min^{-1}. Lectins ECA, RCA and WGA at 250 nM concentration were prepared in HBS-P buffer with $CaCl_2$ (5 mM) and $MnCl_2$ (1 mM). Then 3-min pulse lectin injections were passed over the surfaces. Surface regeneration was accomplished by injecting the complementary carbohydrates (lactose or GlcNAc) at 10 mM and 0.5 M concentrations, respectively. For kinetic data collection for ECA, RCA and WGA, several concentrations (5, 10, 20 and 60 nM for ECA and RCA; 3.75, 7.5, 15, 30 nM for WGA) were prepared by dilution of the original solution with running buffer. Each lectin solution was injected over the three channels at 20 and 40 μl min^{-1}. The specific response was obtained by subtracting from each channel response the reference channel response. Curve fitting of the sensorgrams was done by numerical integration algorithms in the BIAevaluation 3.0 software package.

Results and discussion

Our previous results [17] had shown that, with chitopentaose ($GlcNAc_5$) and a lectin with terminal sugar specificity, the conformation of the peptide-linked monosaccharide is not decisive for binding. However, in the case of complex glycans, composed of distinct monosaccharides, linkages, ramifications, etc., the precise study of lectin specificity and binding properties greatly benefits from the use of smaller sugar epitopes (mono- and disaccharide), and in these cases the cyclic structure of the peptide-linked monosaccharide is a prerequisite. In most of the approaches described for the generation of oxime linked-glycoprobes, it is not always clear whether the aglycon-bound monosaccharide is in the ring form (either pyranose or furanose) or in the open form. Most assumptions for these entities are based on hemiacetals behavior in aqueous solution, where the equilibrium is displaced towards the pyranose ring (both α and β anomers) conformation [22]. Therefore, for oxime-sugars several authors have suggested an equilibrium between the open structure and the ring form [23–27]. For small (mono- and disaccharide) epitopes, a closed structure of the peptide-linked monosaccharide is necessary for efficient and detectable binding. With a view to designing an appropriate glycoprobe for such small epitopes, the work shown in Fig. 1 was carried out. First, the relative abundance of each conformer (open and closed) in the glycopeptide probe was determined by NMR spectroscopy. Secondly, the Aoa residue was *N*-methylated so that, on conjugation to a monosaccharide, a cyclic structure was ensured [21]; this was corroborated in a differential derivatization experiment. Finally, a comparison between the two glycoprobes, Aoa-*N*-methylated and non-methylated using a variety of lectins, was done by SPR, which confirmed the importance of a correct conformational presentation of small sugar epitopes in interaction studies.

Conformation analysis of the sugar core by NMR

Due to the crucial importance of the cyclic structure for sugar epitope recognition, we analyzed the equilibrium of GlcNAc-peptide **1** by ^{1}H NMR. Figure 2 showed the ^{1}H 1D spectrum with resonance signals in the aromatic, bulk and aliphatic regions. The assignment of the signals corresponding to the amino acids was performed according to Wüthrich *et al*. [28] and is summarized on Table 1. The chemical shifts that were of particular interest corresponded to the GlcNAc-H1. The two down-field doublets at δ 7.62 ($^3J_{1,2}$ 5.86) and δ 6.86 ($^3J_{1,2}$ 7.32), separated only by the ring proton multiplet of the Phe residue (Fig. 2, a), were attributed to the imino proton of the *E* and *Z* isomers of the open monosaccharide oxime, respectively [29]. The two

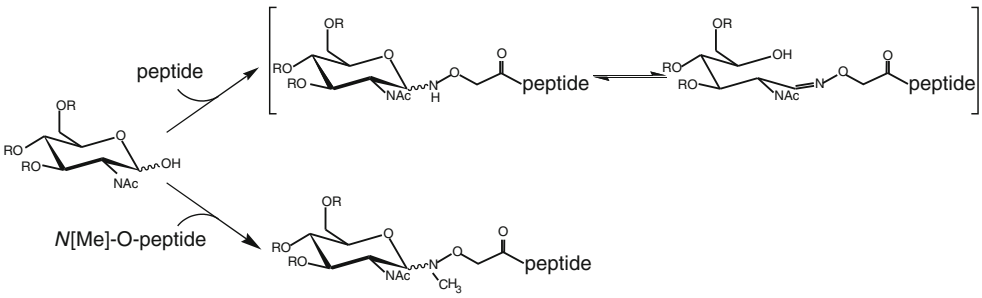

Fig. 1 General scheme of the oxime chemical ligation between an *N*-terminal, Aoa-containing peptide (both the primary and secondary amine) and a carbohydrate. Furthermore, a *table* depicting all the structures employed in the study is included

Name	Peptide sequence	Neoglycopeptide	Experiment
Peptide **1**	Aoa-Ahx-FKKG-amide	GlcNAc-Aoa-Ahx-FKKG-amide	NMR
Peptide **2**	Aoa-GFKKG-amide	GlcNAc-Aoa-GFKKG-amide	derivatization/MS
		lacNAc-Aoa-GFKKG-amide	SPR
N[Me]-peptide **2**	*N*[Me]-O-GFKKG-amide	GlcNAc-*N*[Me]-O-GFKKG-amide	derivatization/MS
		lacNAc-*N*[Me]-O-GFKKG-amide	SPR

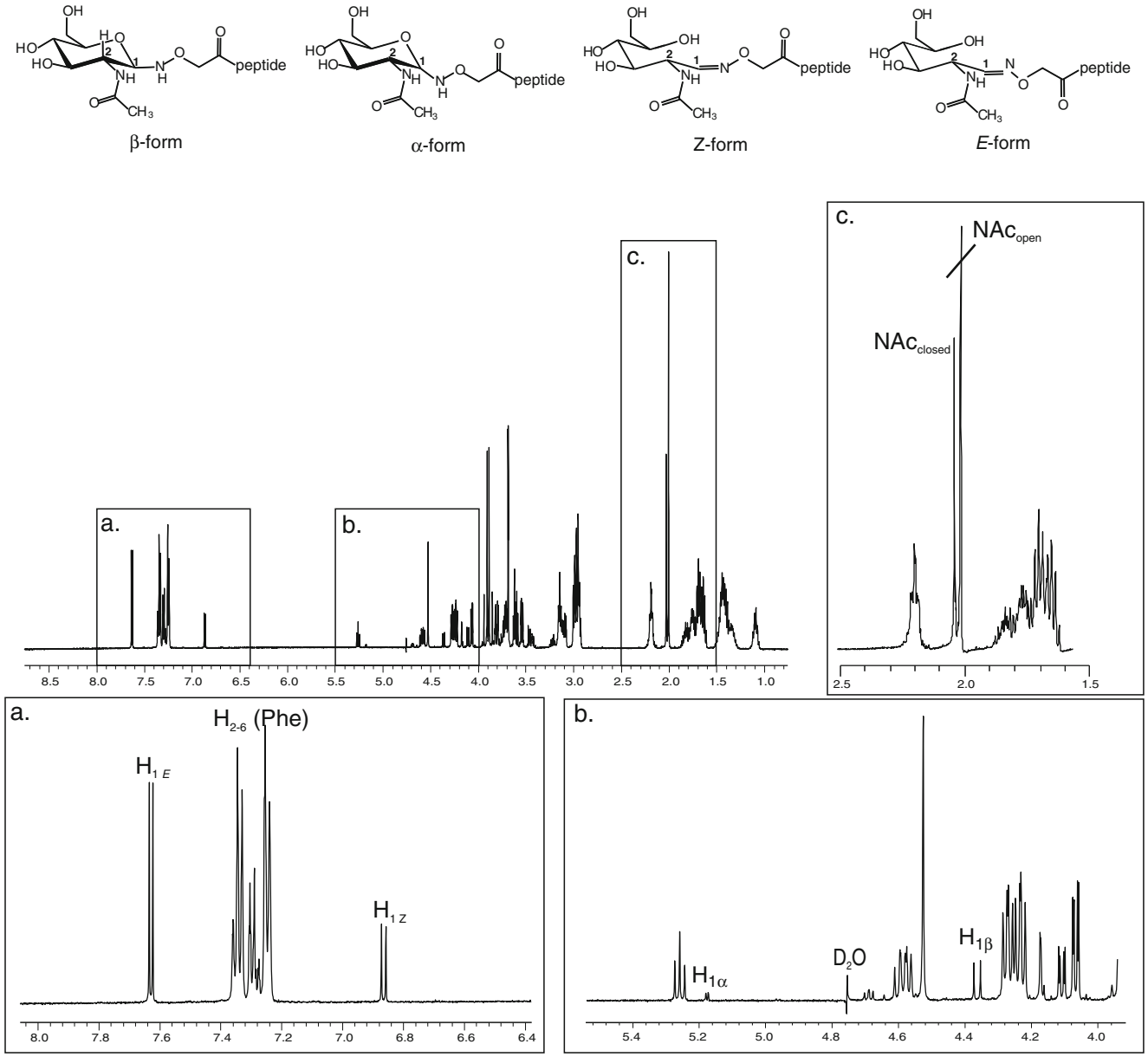

Fig. 2 ^{1}H 1D NMR spectrum (500 MHz) of GlcNAc-peptide **1** recorded in D_2O at 25 °C. In the *top panel* the four different structures are depicted. Enlarged areas of the full spectrum are indicated as *a*, *b*, and *c*

doublets at δ 5.18 ($^{3}J_{1,2}$ 3.42 Hz) and δ 4.36 ($^{3}J_{1,2}$ 9.77 Hz) were assigned to the anomeric protons of the α and β configuration of the closed ring, respectively [30]. The observation of four different signals for the anomeric proton indicated that both the open and closed ring forms exist. This was confirmed by the observation of two singlets at δ 2.03 and δ 2.00 for the methyl protons of the *N*-acetyl group of the closed and open forms, respectively. The relative ratios of each (*E*, *Z*) conformation and (α, β) configuration present in the sample were determined from peak integration as 45:25:6:24, respectively, taking the global area of the five aromatic protons of the Phe residue as reference. Thus, the global proportion of (open) oxime was 70%, *vs* 30% of closed ring form. Within this cyclic form, the β-anomer is the most abundant one by a factor of 4, as can be deduced from the integrals. This analysis, which represents the first accurate quantification of structural conformations present in these kinds of entities, strongly suggests that, for binding studies of unknown carbohydrate-binding entities employing relatively short (mono- to trisaccharide) sugar epitopes, careful evaluation of the results is mandatory, as the binding, or absence of binding, involves at least four different structures, two of which are usually not present in nature. Other approximations employing glycan-displaying platforms [13], where similar equilibriums occur should also take this aspect into due consideration.

Table 1 ^{1}H 1D NMR chemical shifts (500 MHz, at 25 °C) of exchangeable protons for the GlcNAc-peptide **1** in D_2O

Residue	Exchangeable proton	Chemical shift, ppm (coupling constant, Hz)	Signal integration
GlcNAc	$H_{1\ E}$	7.62 ($^{3}J_{1,2}$ 5.86)	0.44
	$H_{1\ Z}$	6.86 ($^{3}J_{1,2}$ 7.32)	0.25
	$H_{1\ \alpha}$	5.18 ($^{3}J_{1,2}$ 3.42)	0.06
	$H_{1\ \beta}$	4.36 ($^{3}J_{1,2}$ 9.77)	0.24
	$H_{2\ E}$	4.69	–
	$H_{2\ Z}$	5.26	–
	$H_{3\ E}$	4.06	–
	$H_{3\ Z}$	4.10	–
	$H_{2–6}$ (closed)	3.61–3.46	–
	CH_3 (open)	2.00	2
	CH_3 (closed)	2.03	1
Aoa	H_1	4.52	–
Ahx	H_1	3.16	–
	H_2	1.38	–
	H_3	1.09	–
	H_4	1.44	–
	H_5	2.18	–
Phe	$C^{\alpha}H$	4.36	–
	$C^{\beta}H$	3.76	–
	$H_{2,6}$	7.29	
	$H_{3,5}$	7.33	5
	H_4	7.24	
Lys	$C^{\alpha}H$	4.27; 7.23	–
	$C^{\beta}H$, $C^{\gamma}H$, $C^{\delta}H$	1.88–1.23	–
	$C^{\varepsilon}H$	3.14	–
Gly	$C^{\alpha}H$	3.68	–

Conformation analysis of the sugar core by derivatization and MALDI-TOF MS analysis

As mentioned above, *N*-methylation of the Aoa residue should exclude the possibility of an open oxime form (Fig. 1, bottom) [21, 31, 32]. To corroborate this point, both GlcNAc-peptide **2** and GlcNAc-*N*[Me]-*O*-peptide **2** were peracetylated and analyzed by MALDI-TOF MS. Both glycopeptides have in common two Lys side chains that can be acetylated, but depending on whether the sugar moiety is in open or closed structure, the number of free hydroxyl groups is different, giving a total of six or five labile hydrogens for the open and closed forms, respectively. Figure 3a shows the mass spectra of GlcNAc-peptide **2** and its acetylation product. Two product clusters can be observed in the second spectrum and readily assigned to the peracetylated versions of the glycopeptide with both open and closed forms of GlcNAc. A rough estimation of the peak areas of both species corroborated the NMR findings, *i.e.*, 25% in the closed form and 75% in the open form. In contrast, Fig. 3b shows a much simpler spectrum for the peracetylated derivative of GlcNAc-*N*[Me]-*O*-peptide **2** as the peaks correspond to the closed form of GlcNAc only and thus confirm the hypothesis. The use of a secondary amine in the aminooxy functionality ensures that the conjugation to a monosaccharide renders this residue in a structural form that is stable (no ring opening occurs) and essential for binding to carbohydrate-binding entities.

Effect of Aoa *N*-methylation on the yield of lacNAc-peptide **2** conjugation

In line with previous work [17], the conjugation between *N*-[Me]-*O*-peptide **2** and the disaccharide lacNAc was tried first at pH 4.6, with a similar conjugation of peptide **2** as control. After 72 h, the reaction was almost quantitative for peptide 2 (87% yield), but not for *N*-[Me]-*O*-peptide **2** (4%). This can be explained by the poor reactivity of a secondary relative to a primary aminooxy group. To optimize the reaction, various pHs in the 3.0–5.0 range were screened, with pH 3.5 yielding the best conjugation (40%). Even though this yield is still significantly lower than that obtained with the non-methylated peptide (~87%) one needs to consider that the new product contains only functional carbohydrates (*vs* the ~30% in the non-methylated peptide) and consists of a single product (*vs* at least two in the non-methylated peptide). In conclusion, both a reduction in structural heterogeneity as well as a ~25% yield increment was achieved.

A further observation was that the conjugates of *N*-[Me]-*O*-peptide **2** and various sugars were not stable at pH 2, *i.e.*, typical peptide HPLC purification conditions where approximately 0.05% (*v*/*v*) TFA-containing solutions are used for elution. Under these conditions for a few hours, the glycopeptide underwent acid-catalysed hydrolysis [33] to the non glycosylated *N*-(Me)-*O*-peptide **2**. In order to prevent this side reaction, glycopeptide pools from HPLC fractions were immediately neutralized to pH 5, frozen and lyophilized.

Functionality of the glycan-*N*[Me]-peptide **2** by SPR

Having assured a closed-ring form through the use of a secondary amine in the aminooxy functionality, as well as optimized the preparation yields of these neo-glycopeptides, the following step was verifying that the closed-ring conformer indeed showed an improved behavior in the recognition by carbohydrate binding entities. Both neo-glycopeptides (with and without *N*-methylation) were immobilized to a CM5 sensor chip as described. The immobilization levels were of similar magnitude (~150 RUs) in order to enable accurate comparison. Following immobilization and surface conditioning, three lectins (ECA, RCA and WGA) with distinct carbohydrate requirements (mono- or disaccharide) were passed at a concentration of 250 nM (Fig. 4). The SPR sensorgrams correspond to the differential

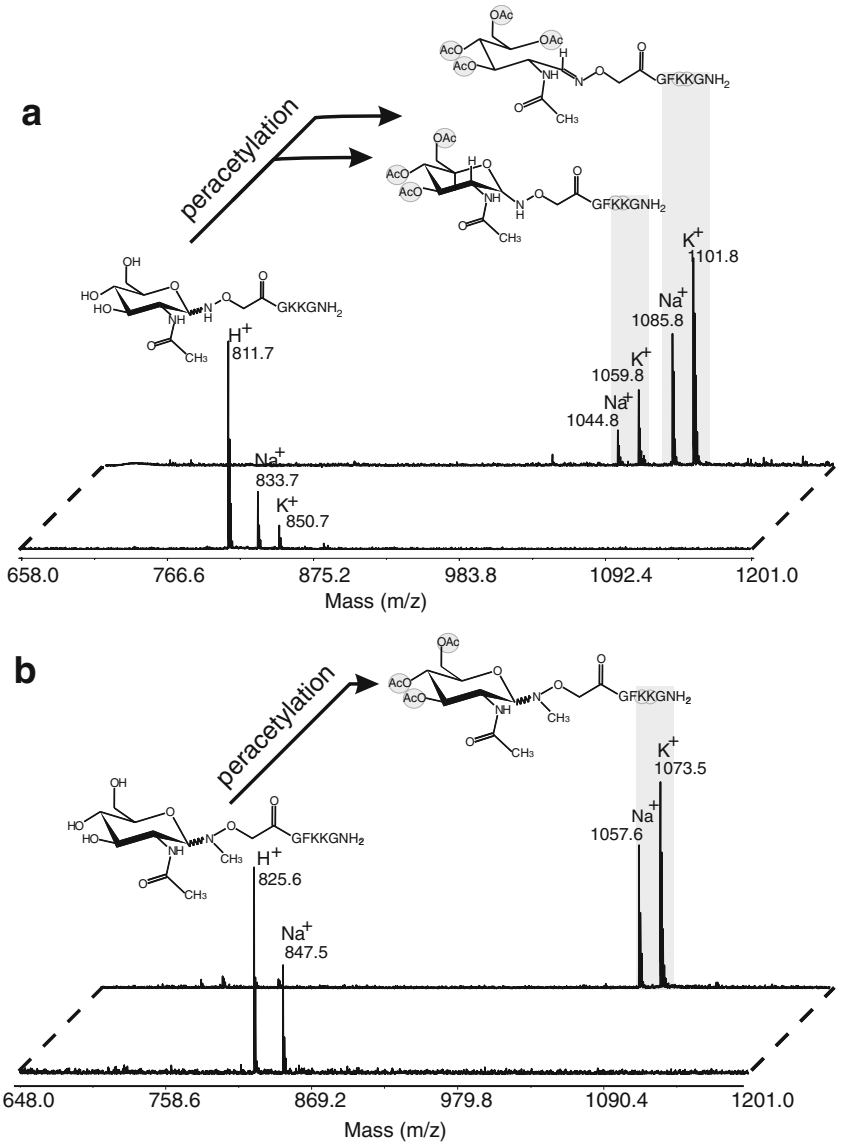

Fig. 3 MALDI-TOF MS spectra of glycopeptides (*front trace*) and their peracetylated equivalents (*back trace*) of: **a** GlcNAc-peptide **2**, **b** GlcNAc-*N*[Me]-O-peptide **2**

curves, where a correction for matrix effects was carried out using the *N*-methylated peptide without carbohydrate epitope (immobilized in flow cell 1). For each lectin a different sensorgram as well as a clearly different response between the two glycoprobes was observed.

First, for ECA (Fig. 4a), a lectin described to interact strongly with lacNAc and requiring the entire disaccharide in a ring-closed Gal(β1–4) configuration for binding, was passed. This lectin was found not to interact with a galactopyranosyl-peptide (data not shown). Whereas with the non-methylated neo-glycopeptide (discontinuous trace) a fairly subtle response was observed that corresponded to the closed structure, the response for the methylated neo-glycopeptide (continuous trace) was very clear-cut. Measurements just before dissociation showed the closed ring structure to have approximately threefold higher absolute response values (~460 *vs* ~180 RUs), corroborating the data from both the NMR and the differential derivatization experiments. Importantly, the methyl-group at the exo-anomeric center does not interfere with the binding. In an attempt to obtain similar response levels for both neo-glycopeptide probes, a higher immobilization level for the non-methylated probe (calculated to require three to fourfold surface density) was effectuated. However, with such high immobilization (~400 RU), lectin binding (ECA 250 nM) was completely abolished. Evidently, at these surface densities, steric hindrance plays a significant role, resulting in impeded binding. Thus, it may be anticipated that for all type of carbohydrate array platforms, similar effects will play a role and that surface verification, or even validation with a standardized lectin assay, will be of utmost importance to accurately interpret binding results.

Beyond the straightforward binding experiments for both glycoprobes, the kinetic association and dissociation constants

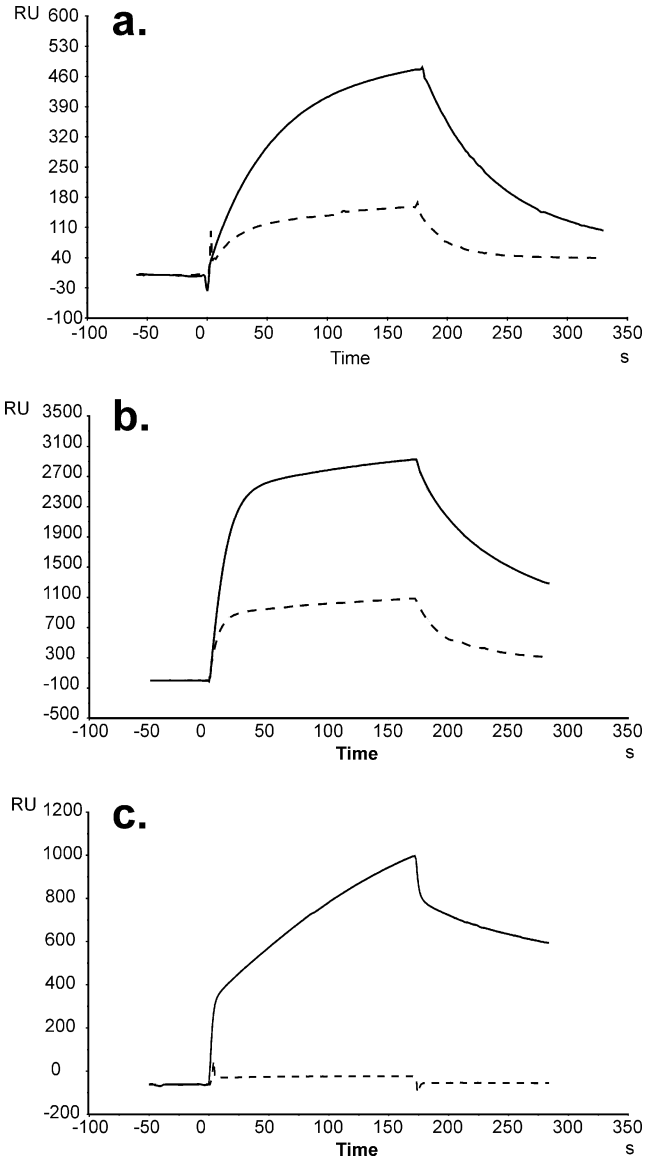

Fig. 4 SPR sensorgrams of the binding of different lectins (**a** ECA; **b** RCA; **c** WGA) at 250 nM to lacNAc-glycoprobes. lacNAc-peptide **2**: *discontinuous trace* and lacNAc-*N*[Me]-peptide **2**: *solid trace*

with ECA were determined at two different flow rates (Table 2). As can be observed, higher association and lower dissociation rates were obtained for the methylated neo-glycoprobe. This indicates that, at least for this lectin, also the dynamic character of the non-methylated neo-glycoprobe negatively affects the binding behavior, leading to inaccurate data.

The binding of another lectin (RCA) to the different probes is depicted in Fig. 4b. This lectin has a described specificity for terminal galactose [34], and displayed significant binding to the non-methylated neo-glycoprobe (discontinuous trace). Nonetheless, with the methylated variant, the response levels went up threefold, reaching more than 3,000 RU absolute binding. In effect, RCA binds specifically to galactose but this binding is enhanced when the monosaccharide is engaged in a type-II structure. Again, kinetic parameters for this lectin were determined with both neo-glycoprobes (Table 2), with higher association and lower dissociation rates being also observed for the methylated variant.

As a final test, WGA was passed across the surface (Fig. 4c). It has been reported that WGA binds specifically to GlcNAc [35], preferentially when terminally exposed, though the binding cleft of this lectin is capable of harboring also non-terminal residues. Figure 4c shows the sensorgram for WGA (discontinuous for the non-methylated, continuous for the methylated neo-glycoprobe). In this case a spectacular difference was observed, namely zero-binding to the non-methylated variant and clear and tight interaction to the methylated one. Whereas the binding in the ring variant demonstrated that the probe is perfectly recognized by the lectin, the absolute lack of recognition in the other case was surprising, as ~30% of the structure should be in closed ring form at a given time. The only plausible explanation is that the dynamic character of the non-methylated neo-glycopeptide renders the interaction that unstable that no net binding can be observed. In any case, the observation is of extreme importance when related to the application of these or similar probes for screening purposes, as a zero binding with a non-natural probe could lead to a false interpretation.

Concluding remarks

In this paper we have addressed the unresolved issue of whether oxime-conjugated sugars maintain a closed ring structure. The observation that the equilibrium is shifted to

Table 2 Association (K_A) and dissociation (K_D) constants of lectins (ECA, RCA, WGA) to lacNAc-peptide **2** and lacNAc-*N*[Me]-peptide **2** determined by SPR at 25 °C

Lectin	Immobilized ligand	K_A (M^{-1})	K_D (M)
ECA	lacNAc-peptide **2**	1.93×10^{6}	5.19×10^{-7}
	lacNAc-*N*[Me]-peptide **2**	5.85×10^{6}	1.71×10^{-7}
RCA	lacNAc-peptide **2**	4.75×10^{7}	$2{,}11\times10^{-8}$
	lacNAc-*N*[Me]-peptide **2**	5.29×10^{7}	1.89×10^{-8}
WGA	lacNAc-peptide **2**	–	–
	lacNAc-*N*[Me]-peptide **2**	2.31×10^{7}	4.33×10^{-8}

the open structure prompted a change in our strategy, namely using a secondary amine to ensure the closed ring form of the sugar, which in turn required an optimization of synthesis conditions. With these improvements, we have demonstrated by means of surface plasmon resonance that the new probes yield better and, what is more important, more reliable information on the interaction between carbohydrates and sugar-binding molecules.

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