

Novel Dextran-Supported Biological Probes Decorated with Disaccharide Entities for Investigating the Carbohydrate–Protein Interactions of Gal-3

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The quest for novel natural-like biomolecular probes that can be used to gain information on biological recognition events is of topical interest to several scientific areas. In particular, the recognition of carbohydrates by proteins modulates a number of important biological processes. These molecular recognition events are, however, difficult to study by the use of naturally occurring oligosaccharides and polysaccharides owing to their intrinsic structural heterogeneity and to the many technical difficulties encountered during the isolation of sufficient quantities of pure material for detailed structural and biological stud-

ies. Therefore, the construction of homogenous biomolecular probes that can mimic both the biophysical properties of polysaccharide backbones and the properties of bioactive oligosaccharide fragments are highly sought after. Herein, synthetic methodology for the construction of well-defined bioconjugates consisting of biologically relevant disaccharide fragments grafted onto a dextran backbone is presented, and a preliminary NMR spectroscopy study of their interactions with galectin-3 as a model lectin is conducted.

Introduction

Carbohydrates are the key molecules of life. They are found as structural molecules in biological membranes, are used by living cells as energy-storage molecules, and play various roles in molecular-recognition events.^[1,2] Understanding the roles of

carbohydrates in these processes requires access to natural-like well-defined biomolecular probes. Such probes could either be isolated from natural sources or be created by chemical methods. Working with natural saccharides is challenging, as these molecules are intrinsically heterogeneous and their isolation in pure form and their structural characterization are difficult, which thus affects the reproducibility and reliability of conducted biological studies. On the other hand, using the carbohydrate probes described in the literature, for example, carbohydrate ligands grafted onto backbones such as polymers, peptides, nanoparticles, and liposomes,^[3–5] does not sufficiently mimic the biophysical properties of the polysaccharide backbones to which biologically active carbohydrate fragments are attached in nature, and therefore, the research data obtained with such molecules might differ from the interactions taking place in nature. The use of carbohydrate ligands grafted onto carbohydrate backbones would be an accurate representation of natural polysaccharides, although this approach does not account for other types of glycoconjugates such as glycoproteins and glycolipids. The chemical reaction technologies required for the construction of such homogenous biomolecular probes with well-defined structural parameters are, however, rare in the scientific literature. To the best of our knowledge, only one similar approach was reported previously by Sato et al., who grafted sialic acids on a dextran backbone.^[6] Most other structural analogues reported to date are carbohydrate-based dendrimers^[7–9] and carbohydrates on cyclodextrin supports,^[10] which differ from polysaccharides in their biophysical properties (Scheme 1).

Therefore, we decided to investigate the possibility of creating polysaccharide–disaccharide-based biomolecular probes.

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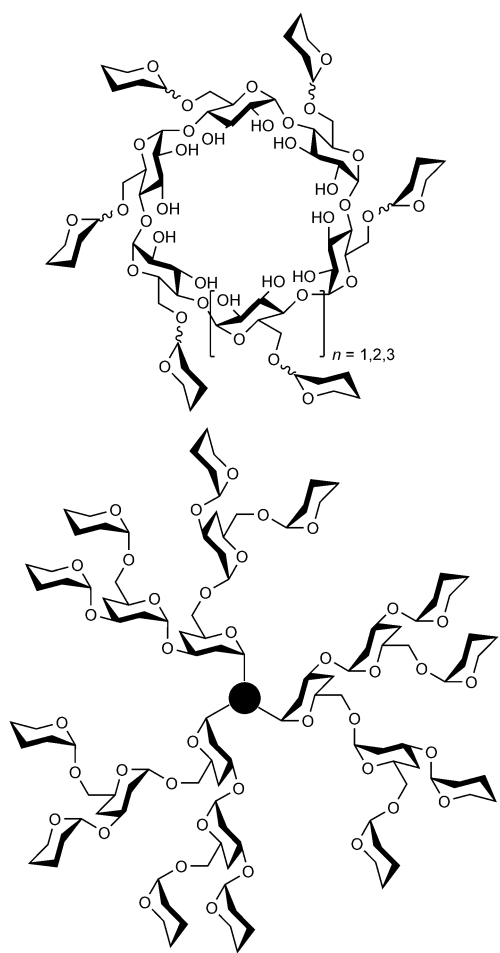
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Scheme 1. Schematic representations of carbohydrate-based supports for carbohydrate ligands.

Herein, we report a novel chemical method for the preparation of well-defined homogenous polysaccharide mimetics by grafting three different synthetic oligosaccharide ligands onto a biologically inert dextran backbone. In addition, we describe a preliminary NMR spectroscopy molecular recognition study of their interactions with the galactose-binding lectin galectin-3 (Gal-3). Gal-3 was selected as the model lectin because it is one of the most studied human galectins with an important role in several biological processes.^[10–12] It is widely known that Gal-3 interacts with galactose through the canonical binding site and with other polysaccharides through a noncanonical site on the opposite side^[13–15] of the lectin; this makes it an ideal protein for screening our novel polysaccharide-based molecular probes.

Results and Discussion

Synthetic modification of dextran

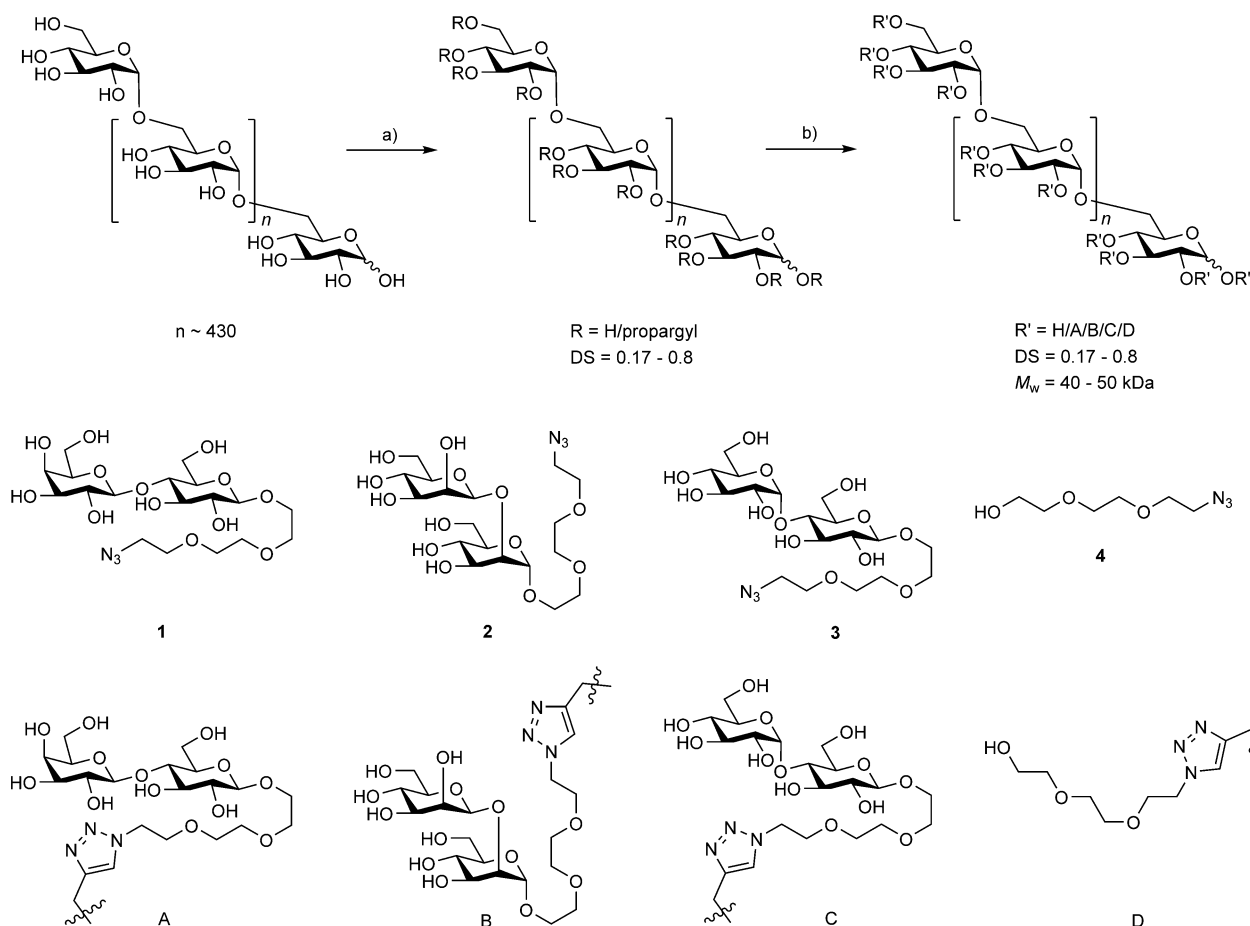
For construction of the molecular probes, dextran with an approximate molecular weight of 70 kDa was selected as the carrier material. This polysaccharide consists of α -(1 \rightarrow 6)-linked

glucose units with α -(1 \rightarrow 3) branches at approximately every 25 glucose residues and contains approximately 430 glucose units per molecule. It was additionally predicted that dextran would be a chemically inert material that adequately mimics natural polysaccharides but that, as such, does not interact with the galactose binding lectin Gal-3. The starting material was first modified by propargylation following a modified literature method (Scheme 2).^[11]

The degree of substitution (DS) of the propargylated dextran was varied by changing the amount of propargyl bromide (PgBr) used in the reaction. With this method, six different DS values were obtained ranging from 0.17 to 0.8 (**P1**: DS=0.17, **P2**: DS=0.25, **P3**: DS=0.41, **P4**: DS=0.51, **P5**: DS=0.60, **P6**: DS=0.80), with the DS referring to the number of propargyl groups per glucose unit.

The practical upper DS limit was shown to be approximately 0.8, which was obtained with 4 equivalents of propargyl bromide per sugar unit of the polysaccharide. A larger excess amount resulted in a material that was insoluble in common organic solvents. Higher DS values were therefore not pursued in this work, although, it should be noted that the use of dissolving ionic liquids may tolerate higher DS values.^[12,13]

In recent work, we showed that the general copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC) reaction conditions resulted in the controlled degradation of several polysaccharide backbones in a size-dependent manner.^[14] Therefore, in this study we decided to investigate further if this methodology could be applied to a one-pot bioconjugation–degradation sequence to give polysaccharide–disaccharide biomolecular probes within the same molecular range. To screen the outcome of the reaction, the bioconjugation reaction between propargylated dextran and synthetically prepared disaccharide azides **1–4** (Scheme 2) was conducted under typical CuAAC conditions, and the results are summarized in Table 1. Lactose (**1**) was selected for interaction with the canonical binding site, and mannobiose (**2**) and maltose (**3**) were selected for eventual interaction with the noncanonical binding site. Tri(ethylene glycol) linker **4** was grafted onto the dextran and was used as a negative control. The same phenomenon was observed here in the grafting reactions during the preparation of the polysaccharide probes. The CuAAC reactions resulted in natural-like biomolecular probes with molecular weights of 40–50 kDa (as determined by HPSEC/MALLS) instead of the expected 110–250 kDa (if simultaneous fragmentation would not have occurred). These results are significant for a number of reasons. First and foremost, these results prove that natural-like homogenous and well-defined polysaccharide–oligosaccharide molecular probes can be constructed by chemical reaction technologies. Moreover, the bioconjugation–fragmentation reaction can be conducted in a single synthetic step, thus removing the need for fractionation and other tedious purification protocols associated with the isolation of naturally occurring, biologically relevant polysaccharides. From a biological perspective, the main advantage of this approach is that the molecular probes are all in the same molecular-weight region, that is, the comparison of their biological interactions can only be related to the biologically active disaccharide fragments grafted onto the



Scheme 2. Grafting disaccharides onto dextran by propargylation followed by CuAAC. a) PgBr, KOH, H₂O; b) 1/2/3/4, CuSO₄, Na-ascorbate, H₂O, 55 °C.

Table 1. Structural features of the modified dextrans			
Compound	Dextran	Ligand	DS
Lac1	P1	A	0.17
Lac2	P2	A	0.25
Lac3	P3	A	0.41
Lac4	P4	A	0.51
Lac5	P5	A	0.60
Lac6	P6	A	0.80
Man	P6	B	0.80
LM1	P6	A/C = 1:3	0.80
LM2	P6	A/C = 3:1	0.80
T	P6	D	0.80

polysaccharide backbone and the frequency by which these occur (DS). This eliminates the aspect of comparing molecules of significantly different sizes that may have deviating biophysical properties.

Characterization of the materials

The propargylated dextrans were characterized by NMR spectroscopy to determine the DS as well as the substitution pattern. Owing to the close proximity of the water signal to the

anomeric signals and CH₂ signals from the propargyl groups, the DS was determined by quantitative ¹³C NMR spectroscopy. In the carbon spectrum, the anomeric region ($\delta \approx 93\text{--}102$ ppm) as well as the CH₂ signals from the propargyl side chains ($\delta \approx 57\text{--}62$ ppm) were found to be well separated from the other signals, making the integration reliable (Figure 1).

Although the chemical shift of a proton attached to the same carbon atom as a hydroxy group is generally not affected very much by ether substituents, the chemical shift of a carbon atom with a hydroxy substituent is significantly different ($\Delta\delta \approx 7\text{--}10$ ppm) from that of a carbon atom with an ether substituent. Furthermore, both the carbon and proton chemical shifts are sensitive to the substituent on the neighboring carbon atom, which makes 2D correlation experiments, such as HSQC and HMBC, powerful tools for determining the substitution pattern.

HMBC spectra were recorded as a band-selective modification for increased resolution along the F1 (¹³C) axis without folding artifacts or unreasonably long experiment times. Two spectra were recorded, $\delta = 54\text{--}64$ and $75\text{--}85$ ppm, that allowed for identification of relevant peaks in the HSQC spectra. From the HMBC spectra it could be seen that the signals at $\delta = 58.1$, 59.8 , and 60.1 ppm corresponded to propargyl groups on C2, C4, and C3, respectively. Integration of these peaks showed

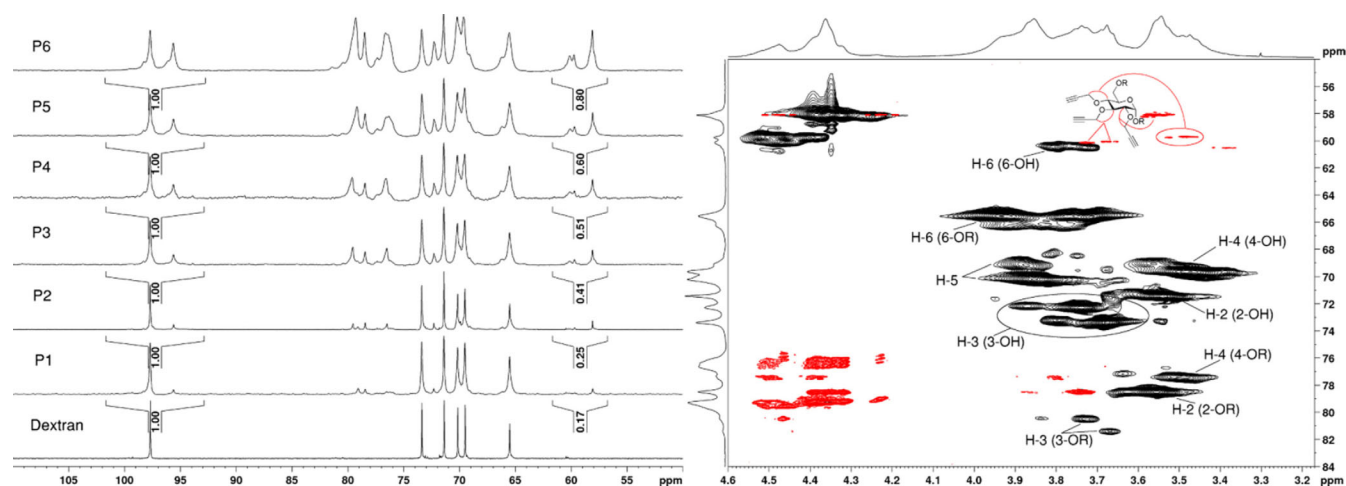


Figure 1. Left: Quantitative ^{13}C NMR spectra of the propargylated dextrans showing the different degrees of substitution. Right: HSQC (black) and HMBC (red) spectra of P6.

that approximately 57% of the substituents were on C2 and the remaining were on C3 and C4 in a ratio close to 1:1.

Upon treating the propargylated compounds with azido-functionalized compounds 1–4, the carbohydrate regions of the NMR spectra become visually rather complex. Some clear differences could, however, be seen in both the ^1H NMR and ^{13}C NMR spectra, and once again, these differences were most evident in the HSQC spectra of the compounds. The signals from the CH_2 group next to the azide appeared at approximately $\delta = 3.52/50.1$ ppm in the free azides, and after the reaction, once the triazole had formed, the signal shifted to a significantly lower field ($\delta = 4.67/50.1$ ppm) in the ^1H NMR spectrum. Similarly, the CH_2 groups of the propargyl chains shifted downfield by approximately $\Delta\delta = 0.5$ and 5 ppm in the ^1H NMR and ^{13}C NMR spectra, respectively (Figure 2). The fact that no signals from propargyl or azide chains could be seen confirmed that the reactions went to completion. This was further supported by quantitative ^{13}C NMR spectroscopy, which showed that the ratio between the anomeric signals of the dextran backbone and the added disaccharides was the same

as the ratio of anomeric signals to propargyl groups in the starting material.

The final size of the polysaccharides was determined by using HPSEC combined with both MALLS and refractive index detectors by using dextran standards with molecular weights ranging from 50 to 270 kDa for calibration.

Preliminary binding studies

A simple application of these molecules was explored. Thus, the novel bioconjugates were employed to monitor interactions by using the carbohydrate recognition domain (CRD) of Gal-3 as a model receptor with the use of standard $^{15}\text{N}, ^1\text{H}$ HSQC NMR methods, as typically employed to monitor carbohydrate–protein interactions.^[15] To simplify the interpretation of the results by reducing the complexity of the spectra, only the carbohydrate recognition domain of the protein was used, as different reports have indicated that the long and nonpolar N terminus of the full-length lectin may establish ill-defined interactions with different natural polysaccharides.^[16–18]

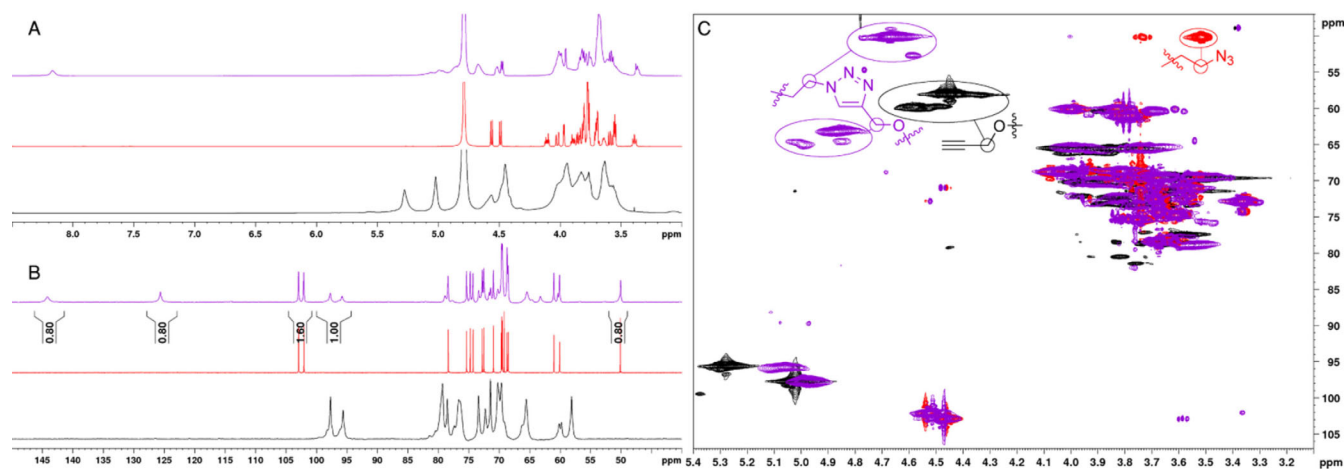


Figure 2. A) ^1H NMR, B) ^{13}C NMR, and C) HSQC NMR spectra of compounds P6 (black), 1 (red), and Lac6 (purple).

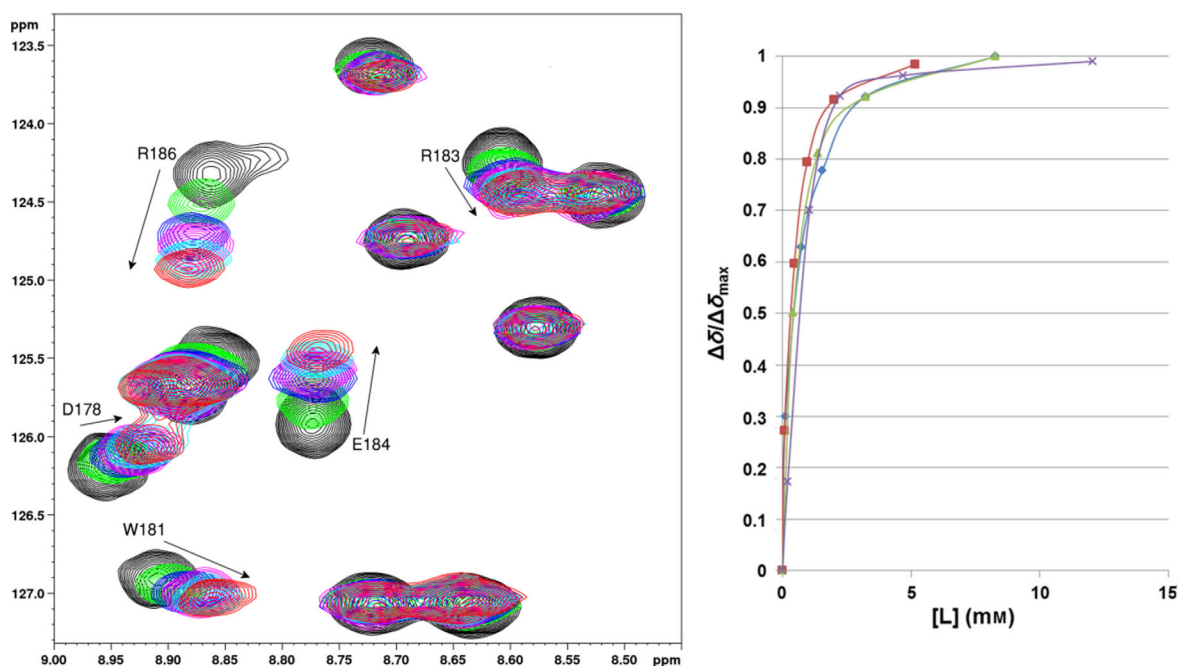


Figure 3. Left: Chemical-shift perturbation of the signals in a selected region of Gal-3 upon titration with lactose-functionalized dextran. Right: binding curves of dextrans functionalized with Lac1 (●), Lac3 (■), Lac4 (▲), and Lac6 (×).

Samples of the ^{15}N -labeled protein were titrated with the polysaccharide mimics as described in the Experimental Section, and the chemical-shift perturbations of the amide NH crosspeaks were monitored (Figure 3) and employed to deduce binding affinities. As a comparison, Gal-3 was also titrated with mannans extracted from the cell wall of *Candida albicans* [both native and a hydrolyzed fraction, enriched with β -(1 \rightarrow 2)-linked mannans].^[19] The negative control (T) showed no interactions with Gal-3.

Lactose-containing dextrans

The estimated binding affinities (from the HSQC spectra, Figure 3) were very similar among all lactose-containing polysaccharides. The equilibrium dissociation constant (K_D) values are rather moderate, ranging from 0.25 to 0.45 mM, and correspond to the range of binding affinities reported for free lactose^[20,21] (Table S1 in the Supporting Information). The perturbed HSQC crosspeaks correspond to the amino acids that form the canonical galactose binding site of the protein,^[21] providing CH- π and hydrogen-bond interactions (Figure S2A).^[22] The data suggest that the multiple interactions at different sites of the dextran do not cooperatively provide added value to the single interaction site, probably as a result of unfavorable entropic effects.

β -(1 \rightarrow 2) linked manno- biose

In contrast, upon titrating Gal-3 with the manno-
biose-functionalized dextran (Man), no significant chemical-shift perturbations were observed, but a different phenomenon was evidenced. Approximately 15 NMR signals showed a marked de-

crease in intensity over the course of the titration (Figure S1); this suggests that the Man polysaccharide does indeed interact with the lectin. The disappearance of these NMR crosspeaks suggests the existence of a dynamic process on a particular timescale, within a defined lectin region.^[23] This phenomenon indicates different, yet specific, binding than that observed for the lactose-functionalized dextrans. A feasible explanation is that the signal intensity loss is due to broadening caused by interconversion of an ensemble of different complexes.^[24] Thus, the Man ligand binds to the lectin in a specific region defined by the affected residues but adopts multiple orientations, in a way that could be described as nonspecific binding from the perspective of the ligand (see the Supporting Information).^[24] Interestingly, natural galactomannans as well as α - and β -mannan polysaccharides were previously reported to interact with Gal-3 in this same region, as evidenced by the same resonance broadening effect as that observed with the manno-
biose bioconjugate.^[21]

Titration of Gal-3 with the mannans extracted from the cell wall of *C. albicans*, however, showed no interaction, which could be due to less than optimal availability of the β -(1 \rightarrow 2) epitopes caused by the complex nature of the polysaccharide or the helical structure^[25,26] adopted by larger β -(1 \rightarrow 2)-linked mannosides.

Binding of maltose

A different construct displaying a mixture of maltose and lactose units was also prepared. Interestingly, in this particular case, the superimposition of both events took place. In addition to the expected perturbations at the canonical lactose binding site, a phenomenon similar to that for the dextran

functionalized with β -(1 \rightarrow 2)-linked mannobiose was simultaneously observed. A more detailed description of the noncanonical binding of dextrans functionalized with β -(1 \rightarrow 2)-linked mannobiose or maltose can be found in the Supporting Information.

Thus, these initial binding studies show that these lactose-containing dextrans are able to bind the CRD of Gal-3 in the same binding mode as lactose. However, the multiple presentation of lactose on the dextran backbone does not provide a stronger interaction with respect to monomeric lactose, as could be expected from a multivalent effect. Still, it is notable that the observation of multivalent effects is very much dependent on the experimental technique, as previously observed for a rigid tetravalent lactose dendrimer interacting with Gal-3.^[27] Indeed, the lectin presentation (surface vs. solution) is known to have a strong impact on the observed affinities.^[28] For the Man β -(1 \rightarrow 2)Man-linked functionalized dextran, a different binding effect was observed. In this case, the observed fading of the receptor NMR signals upon ligand binding was also described for the interaction of other lectins with natural polysaccharides.^[21]

Conclusions

A novel method for the synthesis of natural-like polysaccharide–disaccharide biomolecular probes was presented, and it features a one-pot CuAAC bioconjugation–fragmentation sequence resulting in homogenous and well-defined polysaccharide mimetics. The methodology was proven to be versatile and permitted the decoration of the dextran backbone with a variety of ligands with various DS values in a straightforward manner. The molecular probes were carefully characterized by NMR spectroscopy, HPSEC, and MALLS to provide information on the substitution patterns as well as the molecular weights of the final compounds. In addition, the binding of the functionalized dextrans to the model lectin Gal-3 was investigated by ¹⁵N,¹H HSQC NMR experiments, which allowed for estimation of the different binding affinities. The results provide a preliminary basis to refine further the synthetic bioconjugate approach, which is expected to find multiple applications in the biomaterials, chemical, and biological fields. More rigid linkers generally seem to give higher affinities, and that is the focus of our future work.^[29,30]

Experimental Section

General remarks: All reagents for synthetic work were purchased from Sigma–Aldrich and were used as such without further purification. Dry CH₂Cl₂ was distilled over CaH₂ under an argon atmosphere. TLC was performed on aluminium sheets precoated with silica gel 60F₂₅₄ (Merck), and the spots were visualized by treatment with H₂SO₄ in MeOH (20%, v/v) followed by heating. Column chromatography was performed by using silica gel 60 (0.040–0.060 mm, Merck) for normal phase and silica gel 100 C₁₈ (0.015–0.035 mm, Fluka) for reversed phase. HRMS data were recorded with a Bruker MicroToF-Q instrument with electrospray ionization (ESI) operating in the positive mode.

HPSEC/MALLS analysis was performed with an Agilent 1260 series (G1311B) instrument equipped with a refractive index detector (Shimadzu RID-10A) and MALLS detector (Wyatt Technology mini-DAWN Tristar). The setup consisted of a guard column (Waters, Ultrahydrogel 6 mm \times 40 mm) and two columns (2 \times Ultrahydrogel linear 7.8 mm \times 300 mm) connected in series. Eluent: 100 mM NaNO₃; flow rate: 0.5 mL min⁻¹; injection volume: 200 μ L.

NMR spectra for characterization of the compounds were recorded with a Bruker Avance III spectrometer operating at 500.20 MHz (¹H) and 125.78 MHz (¹³C) equipped with a Prodigy BBO CryoProbe or a Bruker AVANCE III spectrometer operating at 600.20 MHz (¹H) and 150.92 MHz (¹³C) equipped with a Prodigy TCI inverted CryoProbe optimized for proton detection. ¹⁵N,¹H HSQC experiments were performed with a Bruker AVANCE II with a standard triple-channel probe operating at 600.13 MHz (¹H) and 60.8 MHz (¹⁵N). Characterization of small molecular compounds was performed by using a standard set of experiments, ¹H NMR, ¹³C NMR, DQF-COSY, HSQC, and HMBC. The complexity of the spectra was reduced by using 1D-TOCSY. A band-selective modification of HMBC was used for increased resolution in the F1 axis for determining substitution patterns of the propargylated dextrans. Quantitative ¹³C NMR spectra were measured by using a recycle delay of 10 s and an acquisition time of 2.4 s with inverse-gated decoupling to remove NOE effects. These parameters were confirmed to give good quantitative spectra by using the unmodified dextran and fully propargylated methylglucoside as model compounds.

Binding studies: The observed chemical-shift perturbations were used to determine the binding site of the protein. Moreover, provided that the binding process is in the fast exchange regime, the binding affinity can be estimated by fitting the data to the following function [Eq. (1)]:

$$\Delta\delta/\Delta\delta_{\max} = \frac{[L]}{K_D + [L]} \quad (1)$$

in which $\Delta\delta/\Delta\delta_{\max}$ is the saturation, K_D is the dissociation constant, and $[L]$ is the concentration of the free ligand.^[22,29] The DS of the modified dextrans was converted into μ mol lactose per mg polysaccharide, which simplified comparison of the different spectra if the total amount of lactose at each point in the titration was known in all samples. The K_D values of the interactions between Gal-3 and the modified dextrans were estimated by plotting $\Delta\delta/\Delta\delta_{\max}$ versus $[L]$ and fitting a function $\Delta\delta/\Delta\delta_{\max} = [L]/(K_D + [L])$ to the plot. The $\Delta\delta$ values were measured as weighted average values for ¹H and ¹⁵N shifts by using $\alpha = 0.2$ as weighting factor for the ¹⁵N shifts according to convention.

Protein expression and purification: The gene encoding the carbohydrate recognition domain (CRD) of human galectin-3 (hGal3-CRD) (amino acid residues 114–250) inserted into the pET21a expression vector was a gift of Dr. Filipa Marcelo (Universidade Nova Lisboa). A single colony of BL21(D3) *Escherichia coli* cells harboring the expression construct was inoculated into lysogeny broth (5 mL) medium containing 100 μ g mL⁻¹ ampicillin and was allowed to grow overnight at 37 °C with shaking. The overnight culture was then added to 1 L of M9 media containing ampicillin and ¹⁵N-NH₄Cl (1 g) as the sole nitrogen source and was grown at 37 °C until the OD₆₀₀ reached 0.7–1.2. The cells were induced with isopropyl β -D-1-thio-galactopyranoside (IPTG; 1 mM) and growth was continued for 3 h at 37 °C. The induced culture was harvested by centrifugation at 5500 rpm for 20 min in a JLA 8:1000 rotor. The pellet was suspended in lysis buffer [10 mL, 22 mM Tris-HCl pH 7.5, 5 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM dithio-

threitol (DTT)], and the cell suspension was lysed by sonication (8 × 30 s, with 1 min intervals between each burst). The crude extract was clarified by centrifugation at 35 000 rpm for 30 min. The soluble fraction was loaded onto α-lactose-agarose resin (5 mL; Sigma-Aldrich) equilibrated with Tris-HCl (50 mM, pH 7.2) containing NaCl (150 mM). The column was washed with 50 mL of buffer equilibration. The recombinant hGal3-CRD was eluted with approximately 7 mL α-lactose (150 mM) in 1 × PBS (pH 7.2), and the protein purity was checked by 4–12% SDS-PAGE as one clear band, the position of which corresponded well to the calculated molecular weight of 15 kDa. The identification of the protein was further confirmed by LC-MS. Prior to the NMR spectroscopy experiments, recombinant hGal3-CRD was dialyzed against 1 × PBS (pH 7.2) containing NaCl (150 mM).

Propargylation of dextran: KOH (4.85 g, 86 mmol, 14 equiv) was dissolved in H₂O (8 mL) and cooled on an ice bath, after which 70 kDa dextran (1 g, 6 mmol glucose units, 1 equiv) was added, and the mixture was stirred until everything dissolved. Propargyl bromide (80% in toluene, 0.33–2.67 mL, 3–24 mmol, 0.5–4 equiv) was added, and the mixture was stirred on an ice bath for 1 h and at room temperature for 19 h. The mixture was then neutralized by adding acetic acid followed by precipitation into EtOH (100 mL). The residue was dissolved in H₂O (10 mL) and precipitated into EtOH (100 mL) two more times. A small amount of KOAc remained, but the amount was deemed small enough (a few mass% at most) to have no significance for further reactions.

Click reactions: Propargylated dextran (1 equiv propargyl groups) was dissolved in H₂O (3 mL), after which one of the azido-functionalized disaccharides (2 equiv/propargyl group), CuSO₄ (0.3 equiv), and sodium ascorbate (0.6 equiv) were added. The mixture was stirred at 55 °C for 19 h, after which the mixture was evaporated down to a few drops. MeOH (5 mL) was added, and the precipitate was collected and washed with additional MeOH (5 mL). The residue was then passed through a PD-10 G-25 desalting column according to the manufacturer's instructions. Any remaining copper residues were removed by QuadraPure TU thiourea-based metal-ion chelator.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: carbohydrates · glycomimetics · molecular recognition · NMR spectroscopy · polysaccharides

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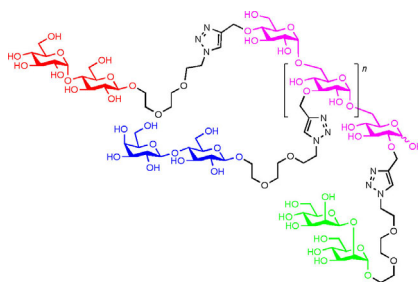
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Novel Dextran-Supported Biological Probes Decorated with Disaccharide Entities for Investigating the Carbohydrate–Protein Interactions of Gal-3



Carbohydrates squared: A new method to mimic complex natural polysaccharides by attaching disaccharide ligands to a polysaccharide backbone is presented. The approach reduces the heterogeneity commonly associated with natural polysaccharides, thereby simplifying the analysis of data.