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Carbohydrate-Lectin Recognition of Sequence-Defined Heteromultivalent Glycooligomers

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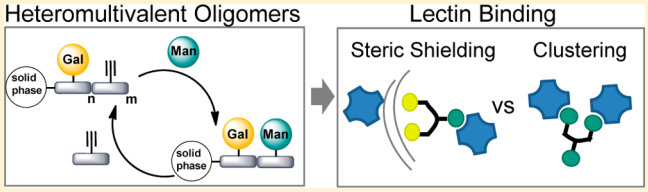
Supporting Information

ABSTRACT: Multivalency as a key principle in nature has been successfully adopted for the design and synthesis of artificial glycoligands by attaching multiple copies of monosaccharides to a synthetic scaffold. Besides their potential in various applied areas, e.g. as antiviral drugs, for the vaccine development and as novel biosensors, such glycomimetics also allow for a deeper understanding of the fundamental aspects of multivalent binding of both artificial and natural ligands. However, most glycomimetics so far neglect the purposeful arranged heterogeneity of their natural counterparts, thus limiting more detailed insights into the design and synthesis of novel glycomimetics. Therefore, this work presents the synthesis of monodisperse glycooligomers carrying different sugar ligands at well-defined positions along the backbone using for the first time sequential click chemistry and stepwise assembly of functional building blocks on solid support. This approach allows for straightforward access to sequence-defined, multivalent glycooligomers with full control over number, spacing, position, and type of sugar ligand. We demonstrate the synthesis of a set of heteromultivalent oligomers presenting mannose, galactose, and glucose residues. All heteromultivalent structures show surprisingly high affinities toward Concanavalin A lectin receptor in comparison to their homomultivalent analogues presenting the same number of binding ligands. Detailed studies of the ligand/receptor interaction using STD-NMR and 2fFCS indeed indicate a change in binding mechanism for trivalent glycooligomers presenting mannose or combinations of mannose and galactose residues. We find that galactose residues do not participate in the binding to the receptor, but they promote steric shielding of the heteromultivalent glycoligands and thus result in an overall increase in affinity. Furthermore, the introduction of nonbinding ligands seems to suppress receptor clustering of multivalent ligands. Overall these results support the importance of heteromultivalency specifically for the design of novel glycoligands and help to promote a fundamental understanding of multivalent binding modes.



INTRODUCTION

Multivalency has been recognized as an important feature of ligand−receptor interactions in biology. In nature, multivalent interactions are often generated by weak monovalent ligands that enhance their association to the binding partner due to their multiple presentation on a larger scaffold. An important example is the recognition and tight binding between protein receptors and oligosaccharide ligands.[1,2] Moreover multivalency has been recognized as a key concept for the design of more effective therapeutics and diagnostic substances.[3−5] However, apart from basic concepts, the molecular mechanisms that can lead to enhanced affinities of multivalent species, e.g. cooperative binding modes, steric receptor shielding, or the role of the ligand composition on the backbone are not well understood. To systematically investigate the underlying principles of multivalency, synthetic multivalent ligands have been used, as natural multivalent ligands are often obtained only in scarce amounts and as structurally heterogeneous mixtures.[6] Especially, synthetic oligosaccharide mimetics can overcome this limitation and therefore, have become an important tool to investigate multivalency.[7−9]Among other architectures, such as glycopeptides[10,11] and -dendrimers,[12−14] glycooligo- and polymers are one important class of such multivalent mimetic structures that have successfully been applied for several biomedical and biotechnological applications and have helped to identify underlying effects in multivalent binding.[1,2,15−17] Most of the glycoligands studied so far are homomultivalent, i.e. they contain only a single kind of sugar residue. Natural oligosaccharides, however, consist of a variety of sugars and use this kind of heterogeneity to tune affinity and selectivity toward a specific receptor. There are a few examples

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in the literature for the combination of different sugar ligands within an artificial scaffold, with major focus devoted to the combination of binding and nonbinding sugar ligands.[18] Some heteromultivalent systems with nonbinding sugars are able to enhance binding affinities,[19−21] whereas other systems exhibit only minor or no contribution from the nonbinding residues on the resulting affinity.[22−24] Overall, heteromultivalent interactions seem to strongly depend on the number as well as ligand density of the constructs and also on their mode of action, namely on secondary neighboring effects such as a promoted sliding of the heterocluster over the primary binding site.[17] However, systematic studies correlating the chemical structure of the ligand with its binding mode and the resulting receptor affinity are scarce. On the one hand, the synthesis of structurally defined heteromultivalent glycoligands still remains a challenging task and more flexible yet controlled synthetic strategies are required. On the other hand, detailed studies of the ligand/receptor complex formation looking beyond affinity studies will help to understand the influence of non- or less-binding ligands in more detail and potentially derive novel glycomimetics for therapeutically relevant receptors. Previously, we introduced a novel platform for the synthesis of monodisperse, sequence-defined glycooligomers based on the stepwise assembly on solid support of specifically designed building blocks.[25−27] This platform allows for the straightforward variation of the number, spacing, and position of sugar ligands along the scaffold and gives access to a large variety of structurally controlled glycoligands. However, so far only homomultivalent glycooligomers, i.e. structures presenting one kind of carbohydrate, were accessible, and we previously focused on the interaction of these structures with the well-characterized mannose specific lectin receptor Concanavalin A (Con A). Here, we expand on this approach and introduce for the first time a sequential functionalization protocol for the synthesis of heteromultivalent glycooligomers introducing high affinity mannose, lower affinity glucose, and nonbinding galactose ligands at specific positions along the oligomer backbone. While the combination of different sugar ligands has already been realized, for example by Kiessling et al., so far only statistical distributions of the different ligands along the polymer scaffold have been obtained via polymer-analogue modifications of polymer precursors or copolymerization of different sugar-functionalized monomers.[15] In contrast to these systems, the presented glycooligomers are monodisperse and sequence-controlled, thus allowing for the precise positioning of different sugar ligands along the scaffold. For comparison, all systems are characterized by means of competitive inhibition assays (IC_{50} values) with Con A. Furthermore, Saturation Transfer Difference Nuclear Magnetic Resonance (STD-NMR) and Dual-Focus Fluorescence Correlation Spectroscopy (2fFCS) measurements were performed to evaluate the role of the nonbinding sugar ligands and to infer possible binding modes.

RESULTS AND DISCUSSION

Synthesis of Heteromultivalent Glycooligomers. The main objective of this work was the introduction of different sugar ligands, mannose (Man), glucose (Glc), and galactose (Gal), at well-defined positions within a monodisperse oligomer segment. This was achieved by a solid-phase supported synthesis route with functional building blocks.[26] The general concept is based on standard peptide synthesis and the stepwise addition of building blocks on solid support. Therefore, the building blocks are equipped with a free carboxyl and a Fmoc-protected amine group. After activation of the carboxyl group, the building block is coupled to an amine-functionalized resin followed by Fmoc-deprotection and release of the free amine group for addition of the next building block. Highly optimized activation chemistry and deprotection protocols were adapted to meet the requirements of the oligomer synthesis ensuring full conversion and avoiding side reactions. Thus, the synthetic route allows for the synthesis of monodisperse oligomers, exhibiting a precisely defined monomer sequence and the absence of both chemical and molecular weight distributions.[25,26]

In order to obtain an oligomer presenting sugar ligands, we applied the alkyne functionalized building block 1-(fluorenyl)-3,11-dioxo-7-(pent-4-ynoyl)-2-oxa-4,7,10-triazatetradecan-14-oic acid (TDS) allowing for the functionalization with sugar azides via Cu-catalyzed Azide−Alkyne Cycloaddition (CuAAC).[28,29] A second building block based on 2,2′(Ethylenedioxy)bis(ethylamine) (EDS) introduces a diethyleneglycol spacer in the main chain.[25]

For the introduction of different sugar ligands, such as mannose and galactose at specific positions within the oligomer segment, we applied a sequential coupling and functionalization protocol (Figure 1). The following section will describe an exemplary synthesis of a heteromultivalent glycooligomer: In the first step, a TDS building block (equipped with an alkyne moiety in the side chain) is attached to the resin followed by coupling of the desired sugar azide e.g. Gal via CuAAC resulting in the first attachment of a sugar ligand to the

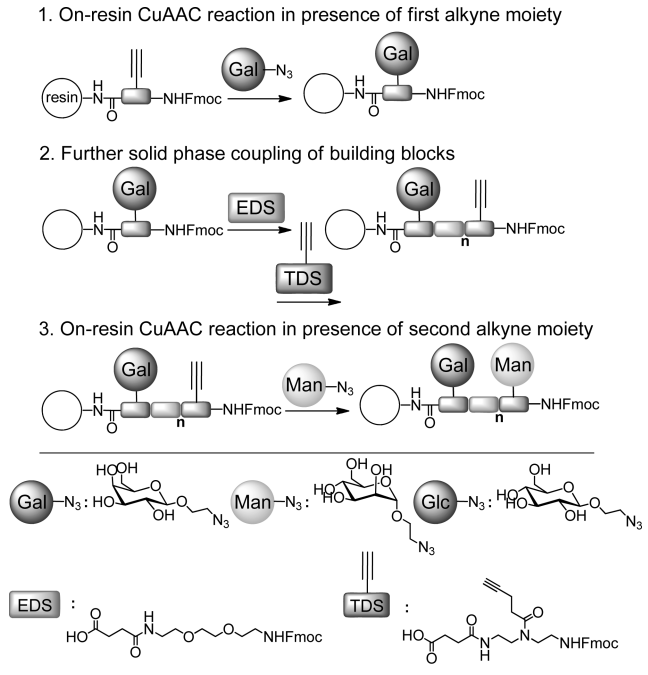

Figure 1. Schematic representation of the solid phase synthesis of sequence-defined, monodisperse, heteromultivalent glycomacromolecules: 1) At first, TDS is coupled on solid phase followed by conjugation of its alkyne moiety to, e.g. 2-azidoethyl-galactopyranoside, in the presence of the temporary Fmoc protecting group via CuAAC reaction. 2) After Fmoc cleavage, coupling of further building blocks proceeds according to solid phase coupling protocols. 3) A newly attached alkyne moiety is again clicked via CuAAC reaction to another sugar azide, e.g. 2-azidoethyl-mannopyranoside. This coupling sequence can be repeated further until the desired heteromultivalent sequence is obtained.

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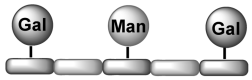
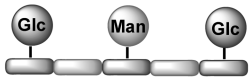
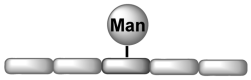
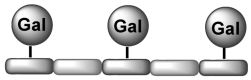
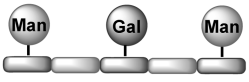
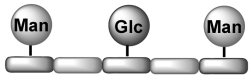
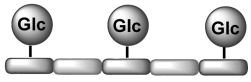
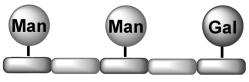
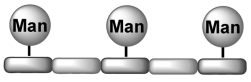
Table 1. List of Glycomacromolecules Used for Lectin Binding Studies: All Heteromultivalent Structures Were Synthesized As Described above, Homomultivalent Structures 6−11 Were Synthesized According to Previously Described Protocols[25]

Heteromultivalent				Homomultivalent			
1	 GalManGal(1,3,5)-5	4	 GlcManGlc(1,3,5)-5	6	 Man(3)-5	9	 Gal(1,3,5)-5
2	 ManGalMan(1,3,5)-5	5	 ManGlcMan(1,3,5)-5	7	 Man(1,5)-5	10	 Glc(1,3,5)-5
3	 ManManGal(1,3,5)-5			8	 Man(1,3,5)-5	11	 EDS-5

oligomer. After removal of the temporary Fmoc protecting group, the spacer building block EDS followed by the next TDS are coupled to the main chain. The latter allows for the introduction of a second sugar ligand, applying the standard protocol for CuAAC functionalization. However, here we can now choose a different sugar ligand, e.g. Man, resulting in the precise positioning of different sugars along the oligomer chain. Repeating these synthetic steps with sequential building block coupling followed by side chain functionalization now allows for the straightforward synthesis of heteromultivalent glycooligomers.

To the best of our knowledge, this is the first time such sequential coupling and click chemistry protocol has been presented for the introduction of different sugar ligands with precise positioning. So far, sequential click chemistry in terms of a sequential coupling followed by functionalization with different moieties has only been shown for heterogeneous pendant groups such as ferrocene, fluorophores, and estradiol on peptidomimetic structures.[30,31]

Following our approach, we synthesized five glycooligomers of a total length of five building blocks displaying different combinations of sugar ligands (Table 1): GalManGal(1,3,5)-5 (**1**), ManGalMan(1,3,5)-5 (**2**), ManManGal(1,3,5)-5 (**3**), GlcManGlc(1,3,5)-5 (**4**), and ManGlcMan(1,3,5)-5 (**5**). Homomultivalent ligands Gal(1,3,5)-5 (**9**), Glc(1,3,5)-5 (**10**), and EDS-5 (**11**) were synthesized by the previously introduced concept for the synthesis of homomultivalent glycooligomers.[25] Structures **6**−**8** have been presented previously. The nomenclature specifies the kind of monosaccharide (mannose (Man), galactose (Gal), and glucose (Glc)), its position on the backbone in parentheses, and the overall length of the backbone (5=pentameric).

After assembly of the final oligomer structures on solid support, the terminal amine group was capped with acetic anhydride to give N-acetyl terminated products which were cleaved from tentagel trityl resin by addition of 30% TFA in DCM. To our surprise, for Glc functionalized structures **4**, **5**, and **10**, treatment with acetic anhydride resulted in partially acetylated sugar ligands as determined by ESI-MS and RP-HPLC (see the Supporting Information). However, these acetyl groups could be completely removed via additional treatment of the resin with sodium methanolate in methanol prior to final cleavage. All structures were lyophilized after cleavage and analyzed by RP-HPLC, ESI-MS, and NMR. The structures were isolated in high purity of above 90% as crude products directly after cleavage from the resin as determined by integration of UV signal at 214 nm (see the Supporting Information).

Con A Competitive Inhibition Assay: Heteromultivalent Glycooligomers Show Higher Affinity than Comparable Homomultivalent Ligands. With these glycoligands in hand, we investigated the influence of different sugar ligands presented on the oligomer scaffold on the binding affinity toward sugar recognizing protein receptors. As a model system, the well-characterized lectin receptor Con A was employed. Con A is a plant lectin isolated from jack bean seeds (*Canavalia ensiformis*). It exists as tetrameric form at pH higher than 7, as dimer at pH lower than 6. Each monomer is equipped with one saccharide binding site as well as a transition metal ion site and a Ca^{2+} site.[32] Its binding pockets are 6.5 nm apart, as determined by X-ray crystal structures.[33] Con A binds specifically to Man and has a lower affinity to Glc and no affinity to Gal. Previously, we reported on the synthesis of a first set of glycooligomers presenting Man ligands: Man(3)-5 (**6**), Man(1,5)-5 (**7**), Man(1, 3, 5)-5 (**8**). The heteromultivalent systems represent analogues of the trivalent Man oligomer (**8**) with successive replacement of the Man ligands toward Gal or Glc ligands. Taking into account only the Man moieties of the heteromultivalent systems, these oligomers can be considered analogues of the monovalent or divalent ligands **6** and **7**, with Gal or Glc ligands now occupying the previously non-functionalized positions 1, 3, or 5 of the oligomer chain.

All glycooligomer segments have the same contour length of approximately 10 nm with a maximal distance between the sugar units of ∼10 nm for sugar ligands attached to positions 1 and 5 and ∼7 nm for sugar ligands attached to positions 1 and 3 on the fully extended oligomer scaffold (these distances were obtained by addition of bond lengths with the structures presenting an all-*trans* linear conformation and were calculated by Chem3D Pro).[25] The hydrodynamic radius of the glycooligomers in solution was determined by 2fFCS measurements and is 1.2 nm for all four glycoligands (see the

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Supporting Information). Therefore we assume a coiled conformation for all glycoligands in the unbound state but cannot exclude a potential stretching of the polymeric backbone upon binding to the receptor.

First, the affinities of all glycoligands **1**−**11** were determined via soft colloidal probe reflection interference contrast microscopy (SCP-RICM) assay.[35] This assay is based on the adhesion of ligand-functionalized soft colloidal probes (SCP) on receptor-functionalized glass surfaces resulting in a defined contact area. The latter can be detected via optical microscopy showing a specific interference pattern, which can be used to calculate adhesion energies.[34,35] We used a competitive inhibition assay to measure IC_{50} values. Therefore, a Man-functionalized SCP was measured against a Con A covered glass slide. Upon addition of the glycoligand in solution and in dependence of the concentration, binding between SCP and the Con A surface is inhibited as the glycoligands bind to the Con A surface. IC_{50} values are obtained by plotting the decrease in SCP contact area with increasing glycoligand concentration and fitting the resulting data points with the Hill equation (Figure 2).

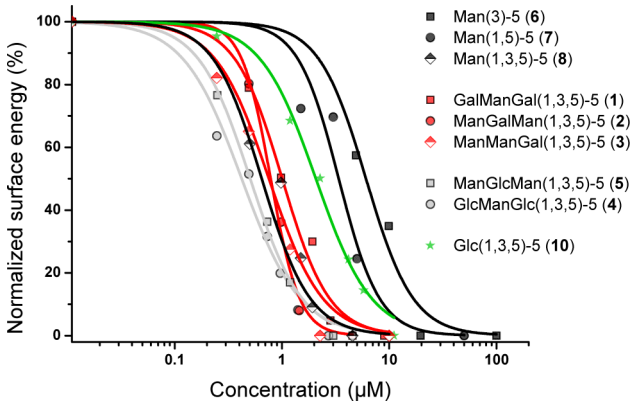

Figure 2. Measurement of inhibitory potential of glycomacromolecules by SCP-RICM. Increasing concentrations of compounds **1**−**9** resulted in a decrease of surface energy. The resulting IC_{50} values are reported in Table 2. Inhibition curves of compounds specified in Table 1, but not shown here are included in the Supporting Information. Error bars are smaller than the symbols and result from more than 20 independent measurements.

Figure 2 shows the obtained data for structures **1**−**9** with their respective fit curves. All structures showed inhibition in the low μM range. Three control structures, a trivalent Glc functionalized oligomer Glc(1,3,5)-5 (**10**), a trivalent Gal functionalized oligomer Gal(1,3,5)-5 (**9**), and an oligomer consisting only of EDS spacer moieties EDS-5 (**11**) were also included (see the Supporting Information). No inhibition was observed for oligomers **9** and **11**, thereby discarding nonspecific interactions of the scaffold or nonbinding sugars during the measurements.

First, we compare the IC_{50} values obtained for four trivalent structures with the same geometry but different ligand composition: GalManGal(1,3,5)-5, ManManGal(1,3,5)-5, ManGalMan(1,3,5)-5, and Man(1,3,5)-5. A decrease in inhibition would be expected by exchanging binding Man ligands toward nonbinding Gal ligands due to a decrease in statistical probability of one or more Man ligands binding to the Con A receptor. However, all four trivalent structures present very similar IC_{50} values of about 1 μM. This could indicate that all trivalent systems undergo monovalent binding via one Man ligand, while the other, binding or nonbinding, ligands promote steric stabilization of the ligand-protein complex. Steric shielding so far has been discussed mainly for homomultivalent glycopolymers where the nonbinding parts of the glycopolymer, both scaffold and ligands, shield the glycopolymer−protein complex against competition by other ligands or inhibitors.[36,37]

If we assume such a steric contribution for the presented heteromultivalent glycoligands, we would expect similar results also for the Man and Glc modified oligomers. However, quite surprisingly, the IC_{50} values for the trivalent GlcManGlc(1,3,5)-5, ManGlcMan(1,3,5)-5 are around 0.5 μM and thus lower than the IC_{50} of Man(1,3,5)-5 oligomers with 0.8 μM. As a control we synthesized the Glc trivalent oligomer Glc(1,3,5)-5 showing an increase in IC_{50} up to 2.1 μM. This can be readily explained by the lower affinity of Glc toward Con A compared to Man. The IC_{50} of Glc(1,3,5)-5 (2.1 μM) is roughly 2.6 times higher for the trivalent Glc oligomer than for the trivalent Man oligomer (0.8 μM). This is in accordance with previous reports highlighting that monovalent Glc structures has ca. four times lower affinity than mannose functionalized analogues (affinity(Man) = 4*affinity(Glc)[38]).

Table 2. Results of Con A Inhibition Studies Determined by SCP RICM

ligand	IC_{50} [μM]	relative activity[a]
(**1**) GalManGal(1,3,5)-5	1.0 ± 0.1	620
(**2**) ManGalMan(1,3,5)-5	0.8 ± 0.1	388
(**3**) ManManGal(1,3,5,)-5	0.7 ± 0.1	442
(**4**) GlcManGlc(1,3,5)-5	0.4 ± 0.1	1550
(**5**) ManGlcMan(1,3,5)-5	0.5 ± 0.1	1240
(**6**) Man(3)-5	6.1 ± 0.7	102
(**7**) Man(1,5)-5	3.4 ± 0.5	91
(**8**) Man(1,3,5)-5	0.8 ± 0.1	258
(**9**) Glc(1,3,5)-5	2.1 ± 0.1	-
(**10**) Gal(1,3,5)-5	nb[b]	-
(**11**) EDS-5	nb[b]	-
methyl α-D-mannopyranoside	620 ± 20	1

[a]IC_{50} value of methyl α-D-mannopyranoside divided by normalized IC_{50} (normalized to number of mannoses). [b]nb = not binding

If we assume a monovalent binding mode of the presented trivalent ligands mediated by a single Man ligand only and consider steric shielding contributions from additional ligands on the same scaffold, the Glc presenting oligomers would have the same IC_{50} values as the Gal or all-Man presenting structures. Indeed, this has been observed in the literature for tri- and tetravalent heterogeneous dendron structures where the exchange of Man units to Glc units resulted in similar IC_{50} values as for the all-Man structures in binding to Con A.[24] In contrast, we observe a clear difference between the Glc and Gal modified oligomers. Alternatively, statistical effects can be taken into account, where the overall chance of a Man or Glc ligand binding to a Con A receptor molecule is increased with the overall number of binding ligands. Then again, the trivalent Glc and Man heteromultivalent oligomers should have at maximum the same inhibitory potency as the all-Man system; however, 'mixtures' of Glc and Man on the oligomer exhibit a lower IC_{50} value and thus increased inhibitory potency. A similar finding was reported by García Fernandez et al., who presented a ß-cyclodextrin scaffold displaying different combinations of Glc

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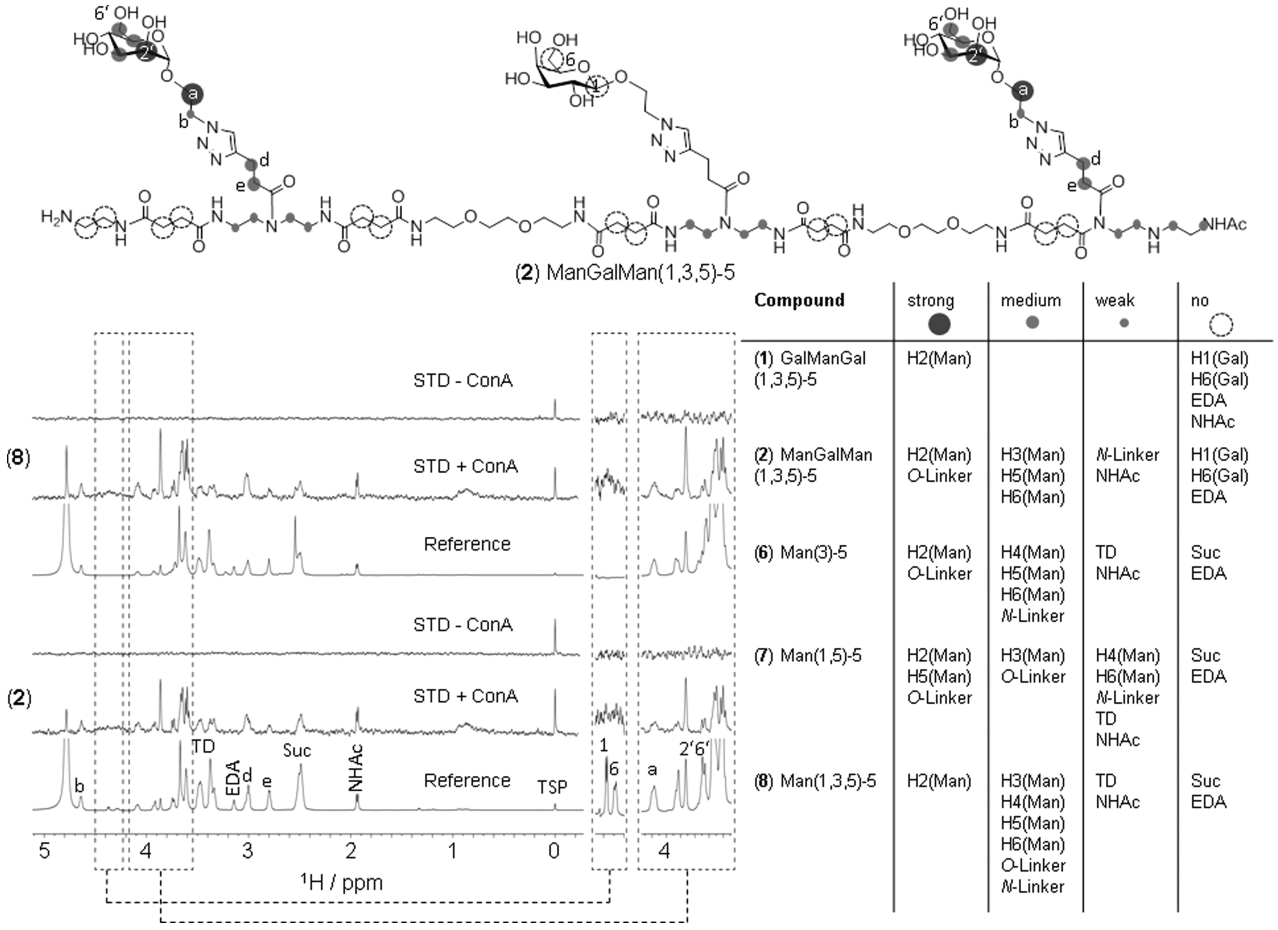

Figure 3. Binding epitope of ManGalMan(1,3,5)-5. The relative degrees of saturation of the individual protons are depicted on the structure of **2** by differently sized circles proportional to the degree of saturation and categorized into strong, medium, low, and no saturation. Protons that are not categorized could not be resolved directly due to chemical shift overlap. Binding epitopes were derived from STD build-up curves (see the Supporting Information). The table summarizes these findings for **1**, **2**, **6**, **7**, and **8**.

and Man ligands that showed an enhanced Con A binding determined by enzyme-linked lectin assay (ELLA) in comparison to the all-Man system of the same valence. For these systems, an increased affinity due to a more dynamic binding and release or so-called sliding of the heteromultivalent systems was suggested. The associated gain in entropy leading to enhanced binding was then experimentally determined by ITC.[23] The dynamic structure of Man-Glc combining glycoligands is also supported by a recently published study by Haddleton et al.[39] who showed that polymers with mixtures of Man and Glc side chains have about the same IC_{50} value as the all-Man polymer but are more easily inhibited with monovalent Me-Man.

At this point, to further elucidate the potential role of the non- or less binding ligands of the glycooligomer constructs and gain insight into their molecular interactions, additional measurements were required to study the ligand-protein complex in more detail. Hence, we applied STD-NMR and Dual-Focus Fluorescence Correlation Spectroscopy (2fFCS) to identify substructures of the glycoligands interacting with the Con A and how many glycoligand and protein molecules are involved in complex formation. We focus exclusively on the Man and Gal combining glycoligands. This should allow us to differentiate clearly between binding and nonbinding parts of the molecule, as we can exclude any unspecific interaction of the Gal residues and possibly identify secondary binding effects through the combination of Man and Gal ligands.

STD-NMR: Direct Binding of Heteromultivalent Glycooligomers to Con A Is Mediated by Mannose Exclusively. In STD-NMR, spin diffusion transfers saturation selectively introduced into the protein hydrogen network to a low molecular weight interaction partner. From this so-called on-resonance spectrum, a reference spectrum is subtracted in which the saturation does not perturb any resonances of the interaction partners. The resulting difference spectrum comprises only signals from the ligand that were in close proximity to the receptor interface. The build-up of the intensity of these resonances in the difference spectrum as a function of saturation time is related to the distance of the respective proton to the receptor which is an indicator for binding.[40,41] STD-NMR measurements were carried out for heteromultivalent glycoligands **1** and **2** and mono- to trivalent Man ligands **6**−**8** for comparison. Ligands in the absence of protein as well as the alkyne precursor backbone lacking mannose moieties served as negative controls. No signals were observed in the respective STD-NMR experiments. Then, binding epitopes of **1**, **2**, **6**, **7**, and **8** in the presence of 20 μM Con A were determined using STD build-up curves with saturation times ranging between 0.5 and 8 s (Figure 3).[40] All mannoses experience significant saturation transfer at H2(Man) (3.86 ppm). The anomeric proton of Man could not be

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examined due to interference with the solvent suppression scheme applied during the STD-NMR experiment. All other Man protons H3(Man) (3.63 ppm) − H6(Man) (3.73 ppm) show high to moderate saturation. Interestingly, the linker protons next to the anomeric center (3.92 and 4.07 ppm) also receive a high degree of saturation of above 70% in all structures (see Figure 3 and the Supporting Information). Hence, a possible direct contribution of this particular stretch of the linker cannot be excluded.

Most importantly, results from STD-NMR highlight lack of saturation of H1(Gal) (4.35 ppm) and H6(Gal) (4.27 ppm). Other protons from the Gal moiety could not be resolved directly due to overlap of chemical shifts, but the chemical shift pattern of the resulting STD-NMR spectra indicates complete absence of saturation of this sugar (for discussion see the Supporting Information). Hence, this subunit is not in close proximity to the receptor and not binding to ConA. Moreover, the backbone of the TDS building block (TD) (3.31, 3.35, 3.45 ppm) receives low saturation transfer, and the succinyl protons of both building blocks (Suc) (2.42, 2.43 ppm) experience no saturation at all. Protons from individual EDS building blocks (3.36, 3.59, 3.65 ppm) are not distinguishable as their chemical shifts overlap with H3 (3.63 ppm) and H4 (3.59 ppm) of the unresolved sugar protons. In conclusion, only Man interacts with the receptor upon complex formation, and no direct binding is observed for Gal residues in any of the ligands.

2fFCS: Heteromultivalent Glycooligomers Show Intermolecular Binding and Steric Shielding in Dependence of the Ligand Sequence. We hypothesized that the nonbinding sugars contribute by steric shielding as they spatially turn outward when the Man ligands are engaged in the binding pocket. This might prevent additional ligands from binding to a Con A molecule. To investigate the oligomer-protein complex further and elucidate potential steric effects, we performed experiments using 2fFCS,[42] a single-molecule based technique using fluorescence emission time correlations to determine hydrodynamic radii.[43,44] Additionally, it is possible to determine binding affinities which can be compared with the IC_{50} values obtained by SCP-RICM inhibition assay. Fluorescence correlation spectroscopy (FCS) involves the detection of fluorescently labeled molecules diffusing in and out of the detection volume of a confocal microscope setup.[45] Their size can be precisely determined from the transit times through the volume. 2fFCS is a variant of the FCS method.[42] It involves the use of two orthogonally polarized lasers of the same wavelength and a DIC prism to generate two confocal volumes laterally shifted in the sample. The distance between the confocal volumes only depends on the optical setup and constitutes an external ruler. Because this distance is defined by the DIC prism, there is no need to measure a reference sample, as is usually the case with FCS. By using two alternately pulsed lasers and a time-correlated single photon counting module, the autocorrelation of the fluorescence can be calculated for each confocal volume as well as the cross-correlation between both volumes. All three correlation curves are fitted simultaneously, resulting in a very robust method to obtain the diffusion coefficient of the diffusing emitters.[44]

Glycoligands GalManGal(1,3,5)-5 (**1**), ManGalMan(1,3,5)-5 (**2**), ManManGal(1,3,5)-5 (**3**), and Man(1,3,5)-5 (**8**) were labeled with Atto647N, a fluorescence marker with a peak excitation wavelength of 644 nm, which was coupled as an NHS ester to the primary amine at the C-terminus of the oligomer backbones (see the Supporting Information). For the measurement, glycoligands at a concentration of 0.5 nM were incubated for 18 min with Con A solutions at different concentrations ranging from 0.5 to 50 μM. Hydrodynamic radii were calculated from experimentally obtained diffusion coefficients via the Stokes−Einstein relation (see the Supporting Information). An important requirement is that the glycoligands do not self-aggregate at the measured concentrations. Therefore, we carried out 2fFCS measurements of Man(1,3,5)-5 with concentrations between 0.5 nM and 50 μM. The hydrodynamic radius remained constant at 1.2 nm at all concentrations, indicating that neither oligomerization nor aggregation was present (see the Supporting Information). NMR does also support this hypothesis (data not shown).

Hydrodynamic radii (R_H) of the different oligomers are plotted in Figure 4 as a function of Con A concentration. The

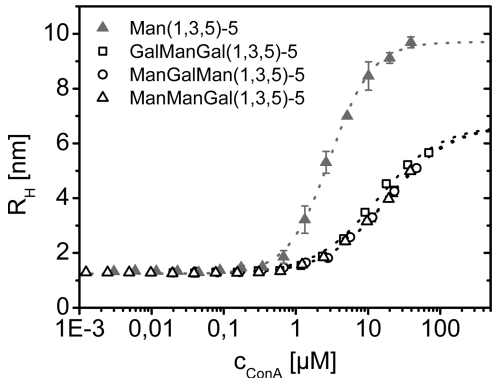

Figure 4. Hydrodynamic radius of examined glycomacromolecules Man(1,3,5)-5, ManGalMan(1,3,5)-5, GalManGal(1,3,5)-5, and ManManGal(1,3,5)-5 as a function of Con A concentration. The dotted lines are Hill fits of the measurements.

results show that all four glycoligands have the same R_H of 1.2 nm in the absence of protein. This also indicates a similar conformation in solution for all glycoligands independent of their glycosylation pattern. With increasing concentration of Con A in the solution, R_H increases as a result of complex formation. In the transition region, R_H is an average over bare glycoligands and protein-bound glycoligands. The three heteromultivalent structures show a similar increase in hydrodynamic radius by ∼5 nm to a maximum value of 6.6 nm (see Table 3). We note that complete saturation could not be reached because of the solubility limit of Con A of ∼50 μM. Given that a Con A molecule has an approximate R_H of 4.3 nm,[46] we conclude that one Con A molecule binds to one heteromultivalent ligand, resulting in the observed increase in R_H. This result was expected for GalManGal(1,3,5)-5 (**1**), which presents only one binding ligand. The size increase by (5.4 ± 0.1) nm observed for all three heteromultivalent structures is a bit larger than what is expected for the protein alone (R_H of 4.3 nm). However, the R_H of the complex depends on the detailed structure, and the glycoligand may assume a different conformation in the complex with the protein than alone. The results of fitting with a Hill equation (see the Supporting Information) are shown in Figure 4 as dotted lines; the fit parameters are summarized in Table 3. Because all three heteromultivalent structures apparently bind only one Con A molecule, the hydrodynamic radius of the glycoligand-protein complex, i.e. the radius at saturation, R_{max}, was taken as a global parameter in the simultaneous fit of all three curves. For

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Table 3. Results of the Hill Fits Performed on the Hydrodynamic Radii of Ligands (1), (2), (3), and (8) Measured As a Function of Con A Concentration[a]

ligand	R_{max} [nm]	K_D' [μM]	IC_{50} [μM][b]	n
GalManGal(1,3,5)-5	6.6 ± 0.1	14 ± 1	1.0 ± 0.1	1
ManGalMan(1,3,5)-5	6.6 ± 0.1	19 ± 1	0.8 ± 0.1	0.99 ± 0.04
ManManGal(1,3,5)-5	6.6 ± 0.1	18 ± 1	0.7 ± 0.1	1.02 ± 0.04
Man(1,3,5)-5	9.7 ± 0.1	3.0. ± 0.1	0.8 ± 0.1	1.48 ± 0.04

[a] R_{max} is the hydrodynamic radius of the ligand when all its available binding sites are occupied by Con A molecules. K_D' is the free Con A concentration at half coverage, and n is the Hill coefficient. [b] For comparison the IC_{50} obtained by SCP-RICM are also shown.

GalManGal(1,3,5)-5 (**1**), which has only one Con A binding site, the Hill coefficient was fixed to 1. The Hill coefficients obtained from the fits for ManGalMan(1,3,5)-5 (**2**) and ManManGal(1,3,5)-5 (**3**) are very close to 1, corroborating our view that the heteromultivalent ligands bind only one Con A molecule. The concentration of free Con A at the transition midpoint is given by K_D'. In general, the obtained K_D' for all heteromultivalent structures is in the same range. For GalManGal(1,3,5)-5, a K_D' of (14 ± 1) μM was obtained. For structures **2** and **3** the dissociation coefficients, K_D', of ∼18 μM are identical within the error.

K_D' is comparable to the IC_{50} value obtained by the inhibition SCP-RICM experiment, as both values describe the ligand receptor interaction at 50% binding. Also for SCP-RICM the values of the three heteromultivalent glycoligands are very similar. The practically identical behavior of the three heteromultivalent structures is surprising and supports the hypothesis drawn from the results of the SCP-RICM studies: All three structures bind with only one Man unit, and the increase in binding affinity of the heteromultivalent structures is driven mostly by steric shielding of the other nonbinding sugar ligands.

For Man(1,3,5)-5 (**8**), a much greater size increase of (8.5 ± 0.1) nm was observed. This result clearly indicates that, unlike for the heteromultivalent ligands, we are not dealing with a 1:1 complex. Thus, we examined if multiple Man(1,3,5)-5 are involved in the complex by measuring the brightness of the molecules diffusing through the detection volume (see the Supporting Information). If glycoligands were to aggregate or multiple ligands were to bind to one Con A molecule, an increase in brightness would be expected because the glycoligands are fluorescently labeled. However, the brightness of the diffusing structures does not, at any Con A concentration, exceed the values obtained for the brightness of the single glycoligands in the absence of Con A. Therefore, we can conclude that a single Man(1,3,5)-5 molecule interacts with several Con A molecules to generate the additional size increase of 3.1 nm with respect to the 1:1 complex. The fact that ManGalMan(1,3,5)-5 and ManManGal(1,3,5)-5 apparently bind only one Con A molecule suggests that Man(1,3,5)-5 binds probably two Con A molecules, which appears entirely reasonable in view of the overall R_H of the complex. The obtained Hill coefficient of (1.48 ± 0.04) also clearly indicates cooperative binding. The K_D' of (3.0 ± 0.1) μM is significantly lower than for the heteromultivalent glycoligands, indicating an overall increased affinity of Con A for the homomultivalent ligand, which is caused by the simultaneous binding of more than one Con A molecule. This effect of the formation of soluble complexes has been observed before and is called intermolecular aggregation.[47] Additionally, this effect is observed at high concentration of Con A, where intermolecular binding leads to the formation of insoluble, three-dimensional clusters. This intermolecular aggregation was studied by a precipitation assay (see the Supporting Information) for the heteromultivalent samples **1** and **2** and Man(1,3,5)-5 (**8**). We obtained a comparable trend as observed by 2fFCS, where the homomultivalent Man(1,3,5)-5 structure is able to cross-link different Con A molecules, leading to intermolecular aggregation and precipitation at high Con A concentrations, whereas the heteromultivalent ligand shows no aggregation or precipitation.

So far, these results support our hypothesis that the introduction of nonbinding Gal ligands leads to a steric stabilization of the ligand−receptor complex, and, thus, no aggregation could be observed. The all-Man ligand, on the other hand, binds several Con A molecules even at micromolar protein concentrations. From a structural point-of-view, it is not clear why the trivalent Man ligand is able to cross-link two Con A receptor molecules. Most likely, two Man ligands are involved in the cross-linking. However, both possible divalent presentations (adjacent (**3**) and distant (**2**)) did not show any receptor clustering. We could assume that the trivalent ligand not only clusters the receptor molecules but also promotes chelate binding. However, potential chelate binding of the glycooligomers is not supported by the results for the homo- and heteromultivalent divalent systems interchanging the position of the Man ligands. Thus, comparing the divalent and trivalent ligands, we should take into account a statistical effect where the trivalent ligand has a higher probability for binding and thus receptor clustering.

The trimannosyl moiety 3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyrose which is located on the outer arm of natural oligomannose-type carbohydrates has been demonstrated to be the major epitope recognized by Con A.[38] A significant enhancement when displaying three mannose ligands compared to di- or even tetravalent systems was also reported by Toone et al.[47] and Lehn et al.[48] It seems that the trimannosyl motif is a beneficial structural attribute. For the presented glycooligomers, this finding additionally correlates with the trivalent all-Man ligand as the minimal structure for receptor clustering in solution.

Interestingly, the K_D' values obtained by 2fFCS show a clear difference in affinity for the homo- and heteromultivalent systems while the competitive inhibition assay gave similar IC_{50} values for all trivalent systems. It is known that different affinity assays can lead to different results depending on the setup.[48] For the results obtained in this work we can differentiate between the assay performed at the interface (SCP-RICM) and the assay performed in solution (2fFCS). In contrast to complex formation in solution, during SCP-RICM measurements, first a protein receptor-functionalized surface is incubated with the glycooligomer and then tested against a sugar-functionalized SCP. Due to the packing of receptors and ligands on the surfaces, no differences in the competition of the

ligand−receptor surface for the homo- or heteromultivalent glycooligomer with the ligand-SCP can be observed (Figure 5),

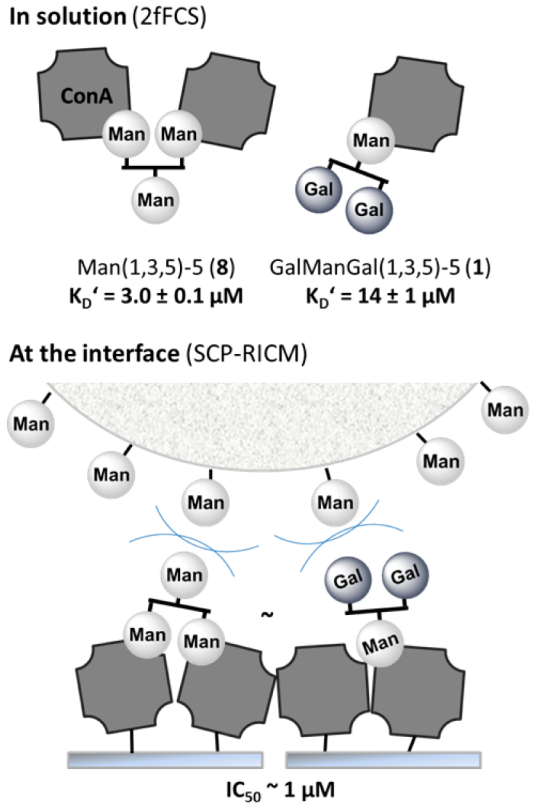

Figure 5. Schematic drawing of ligand−receptor binding in the 2fFCS and SCP-RICM measurements determining K_D' and IC_{50} values, respectively.

thus leading to similar IC_{50} values for both systems. While this clearly exemplifies the dependency of possible conclusions on binding mechanisms on the assay applied, this can also be highly relevant in the context of potential applications of multivalent glycoligands. Both assays, in solution or at the interface, have biological relevance, e.g. glycooligomers inhibiting pathogen/cell interactions.

In conclusion, the presented results show that the introduction of nonbinding ligands leads to the suppression of receptor clustering in solution and promotes steric shielding of the ligand/receptor complexes for divalent Man ligands. Thus, steric shielding effects alter the binding affinity properties of heteromultivalent glycooligomers and, depending on the targeted application, should be taken into consideration for the rational design of the next generation of glycoligands.

CONCLUSION AND OUTLOOK

In summary, the synthesis of a series of defined homo- and heteromultivalent glycooligomers by means of solid phase synthesis applying functional building blocks and sequential click chemistry has been reported. Due to its flexibility and efficiency, the reported methodology is a highly versatile and simple tool for accessing well-defined and tailor-made homo- and heteromultivalent glycomacromolecules. A series of heteromultivalent glycooligomers was synthesized presenting different combinations of Man, Glc, and Gal residues with controlled number and position of ligands. All glycooligomers were evaluated for their binding behavior toward Con A as a model lectin, and their binding mechanisms were further studied via STD-NMR and 2fFCS. Most interestingly, we observed a change in binding mechanism for trivalent glycooligomers presenting high affinity ligand Man or combinations of Man and nonbinding Gal residues. Via STD-NMR we could show that the Gal residues do not directly participate during the recognition of the receptor. 2fFCS measurements then revealed that the homomultivalent ligands can bind to several receptor molecules, while the heteromultivalent ligands interact only with one Man residue and seem to benefit from additional steric shielding. This effect seems to be particularly prominent for receptors bound to or clustered on a surface leading to similar affinities for both homo- and heteromultivalent glycooligomers with decreasing number of binding ligands as measured via SCP-RICM.

The presented studies will help to further understand the binding of synthetic multivalent glycoligands as well as their rational design. Ongoing work focuses on the design of homo- and heteromultivalent glycomacromolecules introducing also nonsugar residues, both hydrophilic and hydrophobic, investigating in detail the effects of hydration/dehydration on the effective binding affinity. Furthermore, heteromultivalent systems for targeting different lectin receptors and potential biomedical applications are currently explored.

ASSOCIATED CONTENT

Supporting Information

Experimental section, materials and instrumentation; more detailed experimental data and spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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