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Block-Sequence-Specific Glycopolypeptides with Selective Lectin Binding Properties

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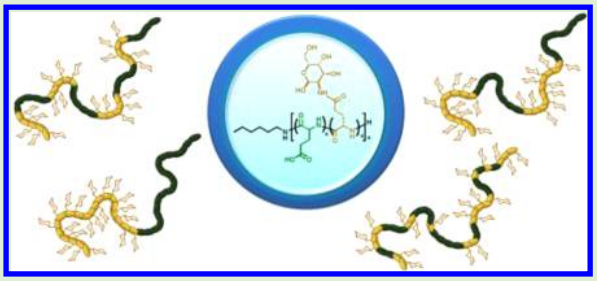
Supporting Information

ABSTRACT: Glycopolypeptides with defined block sequences were prepared by sequential addition of two different *N*-carboxyanhydrides (NCAs), followed by selective deprotection and functionalization of predefined positions within the polypeptide backbone. The sequential arrangement of the galactose units and the block-sequence length have been systematically varied. All the glycopolypeptides have been obtained with a similar overall composition and comparable molecular weights. Circular dichroism measurements revealed some dependence of the secondary structure on the primary composition of the glycopolypeptides at physiological pH. While statistical, diblock, and tetrablock glycopolypeptides adopted a random coil conformation, the octablock glycopolypeptide was mostly α-helical. The ability to selectively bind to lectins was investigated by turbidity measurements as well as surface plasmon resonance (SPR) studies. It was found that the extent of binding was dependent on the position of the galactose units and thus the primary glycopolypeptide structure. The octablock glycopolypeptide favored interaction with lectin RCA_{120} while the tetrablock glycopolypeptide demonstrated the strongest binding activity to Galectin-3. The results suggest that different lectins are very sensitive to glyco coding and that precise control of carbohydrate units in synthetic polymeric glycopeptides will remain important.



INTRODUCTION

Carbohydrates (glycans) play an important role in almost every biological recognition process, i.e. cell communication, cell−cell recognition, and fertilization, as well as in significant diseases, such as cancer, inflammation, and microbial infection.[1,2] In many cases the recognition events are based on specific carbohydrate−protein (lectin) interactions that occur on the surface of the cells.[3] The pharmacological exploitation of glycoscience has already begun, and glycosylated drugs (e.g., vaccines) have seen enormous growth rates.[4,5] However, development of applicable glycomaterials is highly complicated due to the structural complexity of biological glycans; for example, glycans can have different branching patterns and different linkages between the sugars.[6] They can also be linked to proteins in many different ways. Lectins bind specifically, but weakly, to carbohydrates. This binding can be enhanced by combining several carbohydrates in the same molecule or aggregate, allowing for multiple binding events to occur at the same time (avidity, or the cluster glycoside effect).[7] Polymer chemists have thus proposed a strategy whereby the structural complexity of glycomaterials is reduced while keeping biological activity high by the design of glycan conjugated polymers (glycopolymers).[8] Synthetic glycopolymers are convenient multivalent ligands, exhibiting various carbohydrate moieties along a polymer backbone.[9] Many synthetic glycopolymers have been produced by free or controlled radical polymerization of vinyl monomers, and by ring-opening metathesis polymerization of norbonenes.[10−14] In these cases, all polymer backbones are carbon chains, with no other function than connecting the carbohydrate moieties and acting as spacer units. The question remains whether these simplistic systems are "too simple" and their broader biological activity compromised? Researchers are now beginning to respond to this question by designing the next generation of glycopolymers with the goal to understand the effect of glycan positioning along the polymer chain on the biological activity.

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We and others have specifically focused on glycosylated synthetic polypeptides derived from the ring-opening polymerization of *N*-carboxyanhydrides (NCA). Unlike acrylate derived polymer backbones, polypeptides are biodegradable and more closely mimic the peptidic backbone of natural glycoproteins.[15] For example, synthetic glycopolypeptides have the ability to fold into secondary, tertiary, and higher order structures due to multiple noncovalent interactions between amino acid units.[16,17] Special attention has also been paid to synthetic glycopolypeptides that can respond to external stimuli by conformational changes, such as to pH, temperature, or oxidation, as well as their self-assembly into nanostructures.[18−24] Some initial studies have reported on the influence of different degrees of glycosylation on the conformation and biological properties of synthetic glycopolypeptides,[16,25] while there is no systematic study on the effect with regard to the position of the glyco units in the polypeptide.

In the present work, we devise a method to obtain block-sequence-specific polypeptides, in such a way that certain units within the polypeptide backbone can be selectively functionalized, to afford glycopolypeptides with galactose units located in predefined positions. The sequential arrangement of galactose units and block-sequence length is systematically varied while keeping the overall composition constant. Detailed lectin binding studies reveal for the first time an influence of the glycopolypeptide primary structure on the lectin binding properties, providing fundamental guidance for the design of more efficient glycopolypeptides.

EXPERIMENTAL SECTION

Materials. All chemicals were purchased from Sigma-Aldrich and used as received unless noted. Chloroform-*d* (99.8% atom-*d*), trifluoroacetic acid-*d* (99.5% atom-*d*), dimethyl sulfoxide-*d* (99.9% atom-*d*), and deuterium oxide (99.9% atom-*d*) were purchased from Cambridge Isotope Laboratories. Galectin-3 protein (>90%) was obtained from R&D Systems Company.

General Methods. Nuclear magnetic resonance (NMR) spectra were recorded on a Mercury 400 spectrometer (400 MHz for ^{1}H NMR). Samples were dissolved in DMSO-*d*, in D_2O, or in a mixture of $CDCl_3$/TFA-*d* (9:1). All chemical shifts are reported in parts per million (ppm) with tetramethylsilane (TMS) as an internal reference.

Size exclusion chromatography (SEC) was performed on a system equipped with a Waters 1515 Isocratic HPLC pump, a Waters 2414 refractive index detector (35 °C), a Waters 2707 auto sampler, and a PSS PFG guard column followed by two PFG-linear-XL (7 μm, 8 × 300 mm) columns in series at 40 °C. Hexafluoroisopropanol (HFIP) with potassium trifluoroacetate (3 g·L^{-1}) was used as eluent at a flow rate of 0.8 mL·min^{-1}. The molecular weights were calculated against poly(methyl methacrylate) standards (Polymer Laboratories, M_p = 580 Da up to M_p = 7.1 × 10^6 Da).

Fourier transform infrared spectroscopy (FTIR) was performed on a Varian Excalibur 3100 FTIR spectrometer, equipped with a diamond Specac Golden Gate attenuated total reflection (ATR) setup, and an Eurotherm 2416 temperature controller. Measurements were performed over a spectral range of 4000 to 650 cm^{-1} with a resolution of 2 cm^{-1}. 50 scans were signal-averaged, and the resulting spectra were analyzed using Varian Resolutions Pro software.

Circular dichroism (CD) measurements were performed on a Jasco J-815 spectropolarimeter (sensitivity: standard; response: 4 s; bandwidth: 1 nm; data pitch: 0.5 nm; scanning speed: 50 nm/min; accumulation: 2). Samples were prepared by dissolving the polypeptides in Milli-Q water or buffer. Typical CD measurements were performed at amide concentrations of 50 μM and using an optical pathway of 1 cm. Mean residue ellipticities were calculated from the CD spectra following a literature procedure[26] using the equation $[\theta] = (\theta \cdot \mathrm{MRW})/(10lc)$, where θ is experimental ellipticity in mdeg; MRW is mean residue weight (g·mol^{-1}); l is cuvette path length (cm); and c is glycopolypeptide concentration (g·mL^{-1}).

UV/vis measurements were performed with a Jasco V-650 spectrophotometer with a Peltier-type temperature controller. The lectin binding assays were performed by analyzing the change in absorbance at 450 nm, at 25 °C. First, the quartz cuvette was filled with 500 μL of RCA_{120} (2 mg/mL in pH 7.2 buffer), and the absorbance was set to zero. The lectin binding experiments were carried out by adding 50 μL of glycopolypeptide (1 mg/mL in pH 7.2 buffer) into the RCA solution, following the absorbance at 450 nm over time, corrected against 50 μL of glycopolypeptide solution (1 mg/mL in pH 7.2 buffer) diluted into 500 μL of pH 7.2 buffer. As control experiments, absorbance at 450 nm was followed over time when 50 μL of glycopolypeptide (1 mg/mL in pH 7.2 buffer) was added into 500 μL of Concanavalin A (ConA) solution (2 mg/mL in pH 7.2 buffer), also corrected as above.

Surface plasmon resonance (SPR) was used for interaction analysis using lectins RCA_{120} and Galectin-3 on a BIAcore 2000 system (GE Healthcare). RCA_{120} and Galectin-3 (0.05 mg/mL) were immobilized via a standard amine coupling protocol onto a CM5 sensor chip following pH scouting to determine the most favorable coupling conditions. Surfaces were activated by a 1:1 mixture of 0.1 M *N*-hydroxysuccinimide and 0.1 M *N*-ethyl-*N*′-(dimethylaminopropyl)-carbodiimide (5 min at 25 °C at a flow rate of 5 μL/min). After coupling, all activated channels were blocked by ethanolamine (1 M pH 8.5 for 10 min at 5 μL/min), followed by equilibration with filtered HEPES buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 0.01% P20 surfactant). Glycopolypeptide solutions were prepared at varying concentrations (10−0.62 μM) in the same HEPES buffer for binding and kinetic experiments. Sensorgrams were recorded with a 300 s injection of polymer solution (association) followed by 180 s of buffer alone (dissociation). Regeneration of sensor chip surfaces was performed using 100 mM NaOH solution. Kinetic data was evaluated using a 1:1 Langmuir binding model using BIAevalulation 3.1 software.

Glycopolypeptide Synthesis. Polypeptides containing both benzyl-L-glutamate and *tert*-butyl-L-glutamate units in different block sequences were synthesized following a literature procedure.[27] Selective deprotection of the side chain *tert*-butyl-ester groups to afford COOH groups, while maintaining intact benzyl-L-glutamate (BG) groups, was carried out by treatment with CH_2Cl_2:TFA (1:1, v:v). After stirring at room temperature for 3 h, the volatiles were removed under reduced pressure in the rotary evaporator. Successive coevaporation with small additional volumes of CH_2Cl_2 was carried out to facilitate TFA removal, and the selectively deprotected polypeptides were further dried in a vacuum oven at room temperature. Afterward, the side chain COOH groups of the selectively deprotected polypeptides were made to react further with galactosamine hydrochloride to afford glycopolypeptides: In a round-bottom flask, 500 mg of polypeptide containing BG and COOH groups, 610 mg of galactosamine hydrochloride (2.8 mmol), and 15 mL of Milli-Q water were added. The suspension was stirred at room temperature under N_2. After 15 min, a solution of 785 mg of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl morpholinium chloride (DMT-MM, 2.8 mmol) in 8 mL of Milli-Q water was added dropwise. After stirring for 24 h at room temperature, the pH was raised up to 7 by addition of NaOH 0.5 M, and the reaction mixture was further stirred for 3 h. The mixture was dialyzed against Milli-Q water using a Spectra/Por dialysis membrane (MWCO 1 kDa) for 72 h at room temperature. The resulting glycopolypeptide containing BG and galactosyl-L-glutamate units (GalG) was lyophilized. Yield glycosylation: $[(BG)_5\text{-}(GalG)_5]_4$, 667 mg, 91%; $[(BG)_{10}\text{-}(GalG)_{10}]_2$, 659 mg, 90%; $(BG)_{20}$-*b*-$(GalG)_{20}$, 677 mg, 93%; $(BG)_{20}$-*st*-$(GalG)_{20}$, 670 mg, 91%.

Deprotection of Side Chain Groups of the Glycopolypeptides. Glycopolypeptide (500 mg) containing BG and GalG units was dissolved in 20 mL of anhydrous DMF, and the solution was introduced into a PARR reactor. The reactor was purged with N_2 for 15 min. Subsequently, 50 mg of Pd/C (10 wt %) was added, and reactor was purged again with N_2 for 15 min. Afterward, the reactor was charged with H_2, and the hydrogenolysis reaction was carried out

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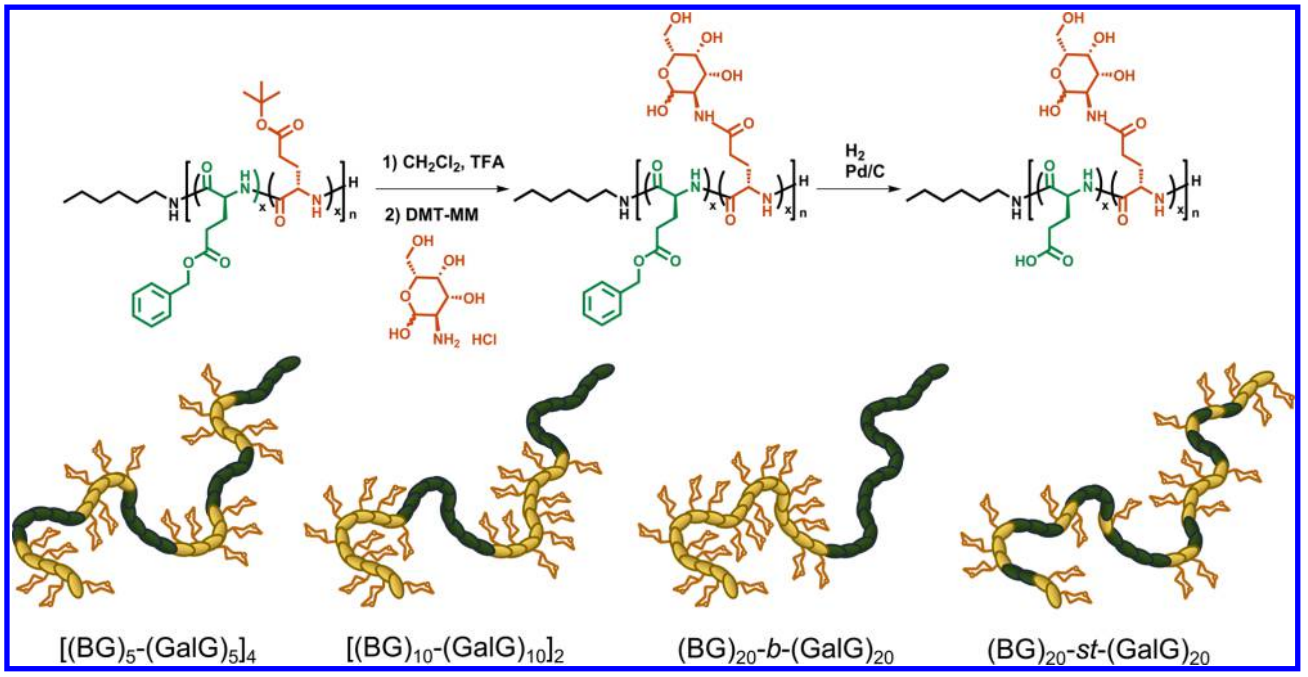
Scheme 1. Synthesis of Glycopolypeptides and Set of Glycopolypeptides with Identical Overall Composition and Systematically Varied Defined Primary Structure



at 5 bar, at 25 °C under stirring. The pressure was monitored, and samples were taken for ^{1}H NMR analysis to determine the conversion of the reaction. After 40−48 h, the pressure was decreased to atmospheric value, and the reactor was purged with N_2. The reaction mixture was filtered to remove the Pd/C catalyst, and it was washed with small volumes of DMF. Afterward, the glycopolypeptide solution was dialyzed against Milli-Q water using a Spectra/Por dialysis membrane (MWCO 1 kDa) for 72 h at room temperature, and lyophilized. The final conversion efficiency of hydrogenolysis (^{1}H NMR) and yields: $[(GA)_5\text{-}(GalG)_5]_4$, 71%, 430 mg; $[(GA)_{10}\text{-}(GalG)_{10}]_2$, 74%, 420 mg; $(GA)_{20}$-*b*-$(GalG)_{20}$, 79%, 412 mg; $(GA)_{20}$-*st*-$(GalG)_{20}$, 73%, 417 mg.

RESULTS AND DISCUSSION

Synthesis of Glycopolypeptides with Different Block Sequences. Glycopolypeptides with different block sequences were synthesized by functionalization of preselected positions of the polypeptide main chains. First, a set of copolypeptides having the same overall monomer composition and overall number of units (40), but different primary structures, were prepared by sequential addition of *tert*-butyl-L-glutamate and benzyl L-glutamate NCA monomers (ratio 1:1).[27] The structures comprised alternating octablock, tetrablock, and diblock copolymers as well as a statistical copolymer (Scheme 1). Since the *tert*-butyl ester and benzyl ester groups have different stabilities, selective and quantitative deprotection of the *tert*-butyl ester groups could be achieved while leaving the benzyl ester groups intact.[27] The polymers thus comprised of about 50% glutamic acid with COOH groups and the rest with protected benzyl ester protected glutamate.[27] The COOH groups were then reacted with excess galactosamine hydrochloride, using 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl morpholinium chloride (DMT-MM) as coupling agent (Scheme 1).[28,29] ^{1}H NMR confirmed that the yields of the glycosylations were more than 90% for the four reactions, leading to overall degrees of glycosylation around 45−50% in all glycopolypeptides with respect to the total number of amino acid units (Figures S1−S4). The molecular weights as well as the composition of the polymers after incorporation of the galactose units are gathered in Table 1, and the SEC traces are shown in Figures S5−S8.

Table 1. Molecular Weights and Composition of the Glycopolypeptides

Glycopolypeptide	M_n^{th}[a] (g/mol)	M_n^{SEC}[b] (g/mol)	$Đ^{SEC}$[b]	Molar ratio[c] BG	GalG	GA
$[(BG)_5\text{-}(GalG)_5]_4$	10300	12400	1.17	0.53	0.45	0.02
$[(BG)_{10}\text{-}(GalG)_{10}]_2$	10300	11500	1.18	0.52	0.46	0.02
$(BG)_{20}$-*b*-$(GalG)_{20}$	10300	11900	1.20	0.51	0.45	0.04
$(BG)_{20}$-*st*-$(GalG)_{20}$	10300	12100	1.22	0.51	0.47	0.02
$[(GA)_5\text{-}(GalG)_5]_4$	8500	9300	1.23	0.16	0.45	0.39
$[(GA)_{10}\text{-}(GalG)_{10}]_2$	8500	8800	1.25	0.15	0.46	0.39
$(GA)_{20}$-*b*-$(GalG)_{20}$	8500	9400	1.24	0.12	0.45	0.43
$(GA)_{20}$-*st*-$(GalG)_{20}$	8500	9000	1.25	0.14	0.47	0.39

[a]Theoretical molecular weight calculated assuming quantitative monomer conversion during polymerization, complete removal of *tert*-butyl ester groups, and complete functionalization of COOH with galactosamine hydrochloride (BG-GalG glycopolypeptides), and assuming complete removal of BG groups (GA-GalG glycopolypeptides). [b]Determined by SEC in HFIP against PMMA standards. [c]Molar ratio determined by ^{1}H NMR of benzyl-L-glutamate (BG), galactosyl-L-glutamate (GalG), and L-glutamic acid (GA) in the glycopolypeptide backbone, by signals f-j and l-s (SI file).

The presence of galactose units was confirmed by FTIR, which showed an increase of the intensity of the C−O stretching band around 1000−1100 cm^{-1} as well as a broad O−H stretching band between 3200 and 3500 cm^{-1}. Qualitative secondary structure analysis by FTIR on solid samples (Figures S9−S10) confirmed that all of them formed mostly α-helices (Table S1). However, neither of the glycopolypeptides, containing both galactose and benzyl ester side chains, was water-soluble. To increase their hydrophilicity, deprotection of the benzyl ester groups was carried out. Several techniques have been reported in the literature for the deprotection of benzyl

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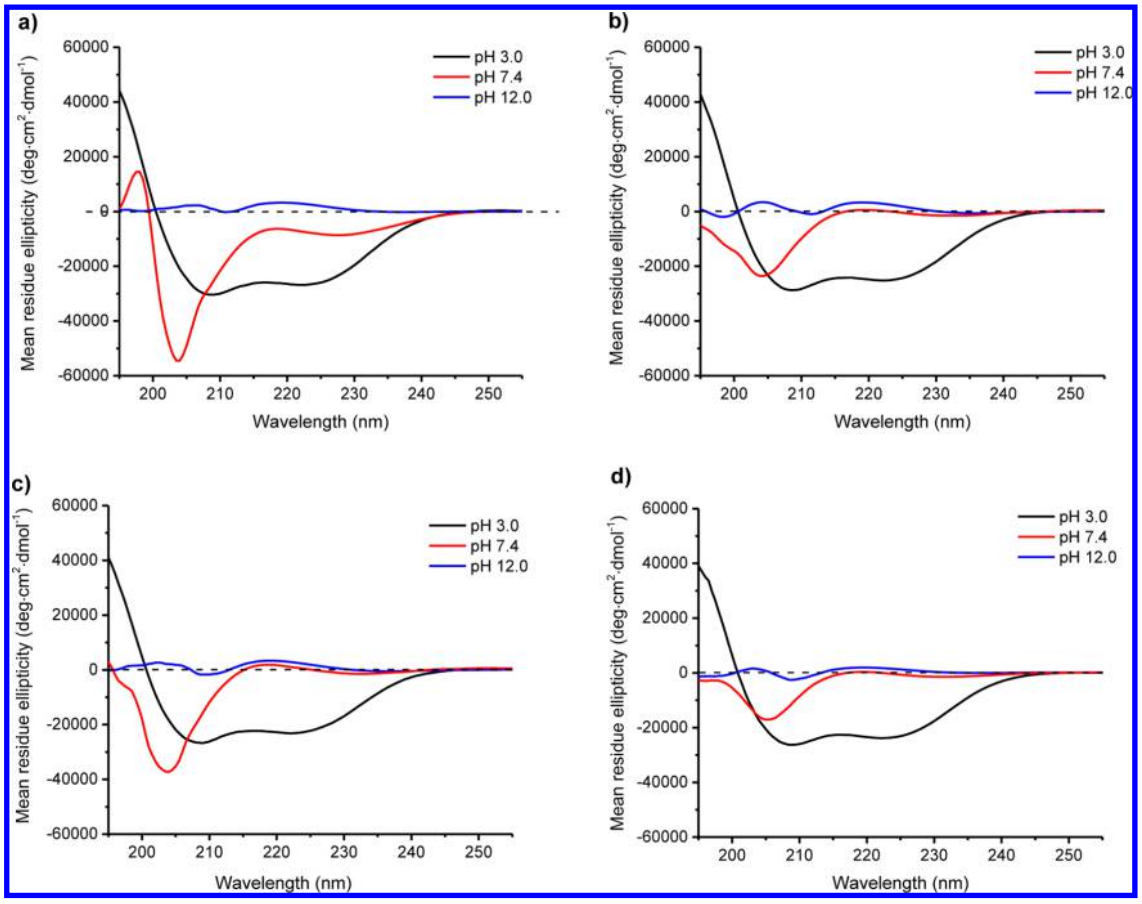

Figure 1. CD spectra in water at different pH, at 25 °C. (a) $[(GA)_5\text{-}(GalG)_5]_4$; (b) $[(GA)_{10}\text{-}(GalG)_{10}]_2$; (c) $(GA)_{20}$-*b*-$(GalG)_{20}$; (d) $(GA)_{20}$-*st*-$(GalG)_{20}$.

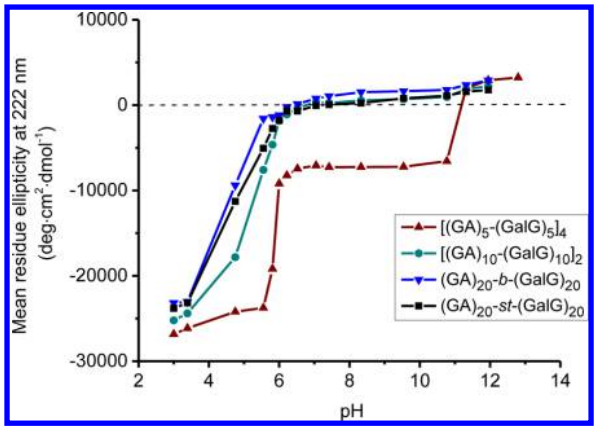

Figure 2. Mean residue ellipticity at 222 nm vs pH of block-sequence-defined glycopolypeptides.

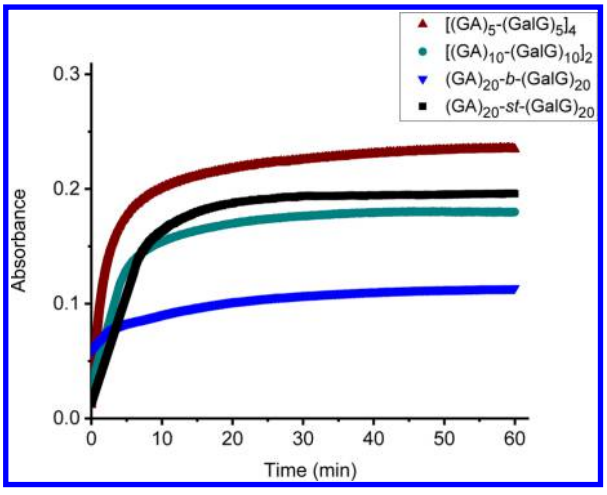

Figure 3. Absorbance vs time after adding 50 μL of glycopolypeptide (1 mg/mL) into 500 μL of RCA_{120} (2 mg/mL).

Table 2. Helicity of Glycopolypeptides in Aqueous Solution at Different pH

Glycopolypeptide	Helicity[a] (%)					
	pH 3.0	pH 4.7	pH 6.0	pH 7.4	pH 9.5	pH 12.0
$[(GA)_5\text{-}(GalG)_5]_4$	76.5	69.8	31.3	26.3	26.2	0
$[(GA)_{10}\text{-}(GalG)_{10}]_2$	72.3	53.3	12.4	0	0	0
$(GA)_{20}$-*b*-$(GalG)_{20}$	67.1	31.8	10.7	0	0	0
$(GA)_{20}$-*st*-$(GalG)_{20}$	68.7	36.6	12.3	0	0	0

[a]Helix content calculated as $(-[\theta_{222}]+3000)/39000$ from CD spectra.

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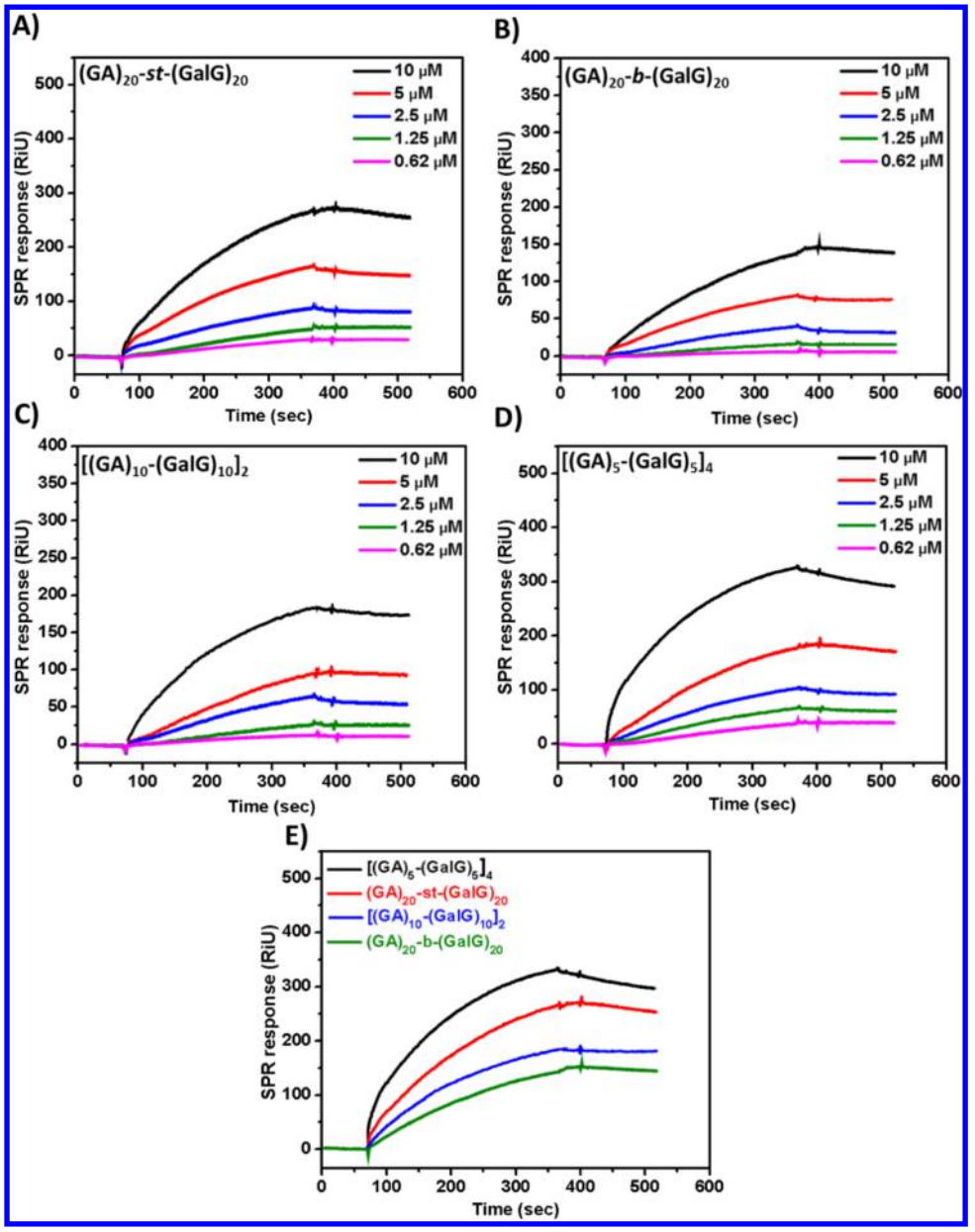

Figure 4. SPR sensorgrams showing concentration-dependent interactions between glycopeptides and RCA_{120}: (A) $(GA)_{20}$-*st*-$(GalG)_{20}$; (B) $(GA)_{20}$-*b*-$(GalG)_{20}$; (C) $[(GA)_{10}$-$(GalG)_{10}]_2$; (D) $[(GA)_5$-$(GalG)_5]_4$; (E) comparison of the binding of all glycopolypeptides to RCA_{120} at the same concentration (10 μM).

ester groups, such as basic deprotection, acidic deprotection, and deprotection through hydrogenolysis. The final glycopolypeptide materials must be obtained using a deprotection technique that ensures the removal of the benzyl ester groups without racemization, an issue which is often not considered carefully and could induce important changes in the immunological properties and the degradation profile in vivo.[30] Basic deprotection by using sodium methoxide in methanol solution has been reported as a fast method to remove the benzyl ester groups. However, this strong deprotection method has a high chance of leading to racemization of the polypeptide backbone. On the other hand, acidic deprotection using, e.g., HBr in CH_3COOH is a widely used technique without any racemization reaction. In our case, however, the use of these reagents could lead to undesired reactions in the sensitive sugar units. Therefore, hydrogenolysis under high pressure was chosen for deprotection. Dong et al. previously employed this method to successfully deprotect the benzyl ester units in triblock copolymers containing both glutamate and lactoside units.[31] DMF was selected as the solvent, since it could solubilize the multiblock glycopolypeptides and did not interfere with the hydrogenolysis reaction. Reaction conversion was followed by ^{1}H NMR, and the reactions were stopped when no further changes in conversion were monitored within a reasonable period. Afterward, the glycopolypeptides were dialyzed against water to remove the remaining DMF, and the samples lyophilized. ^{1}H NMR revealed that deprotections were in the range 70−80% in all cases (Figures S11−14). Furthermore, the molecular weights of the glycopolypeptides decreased after the hydrogenolysis reaction, as expected (Table 1). Although full deprotection could not be achieved by this milder method, which could also be due to the decreasing solubility of the

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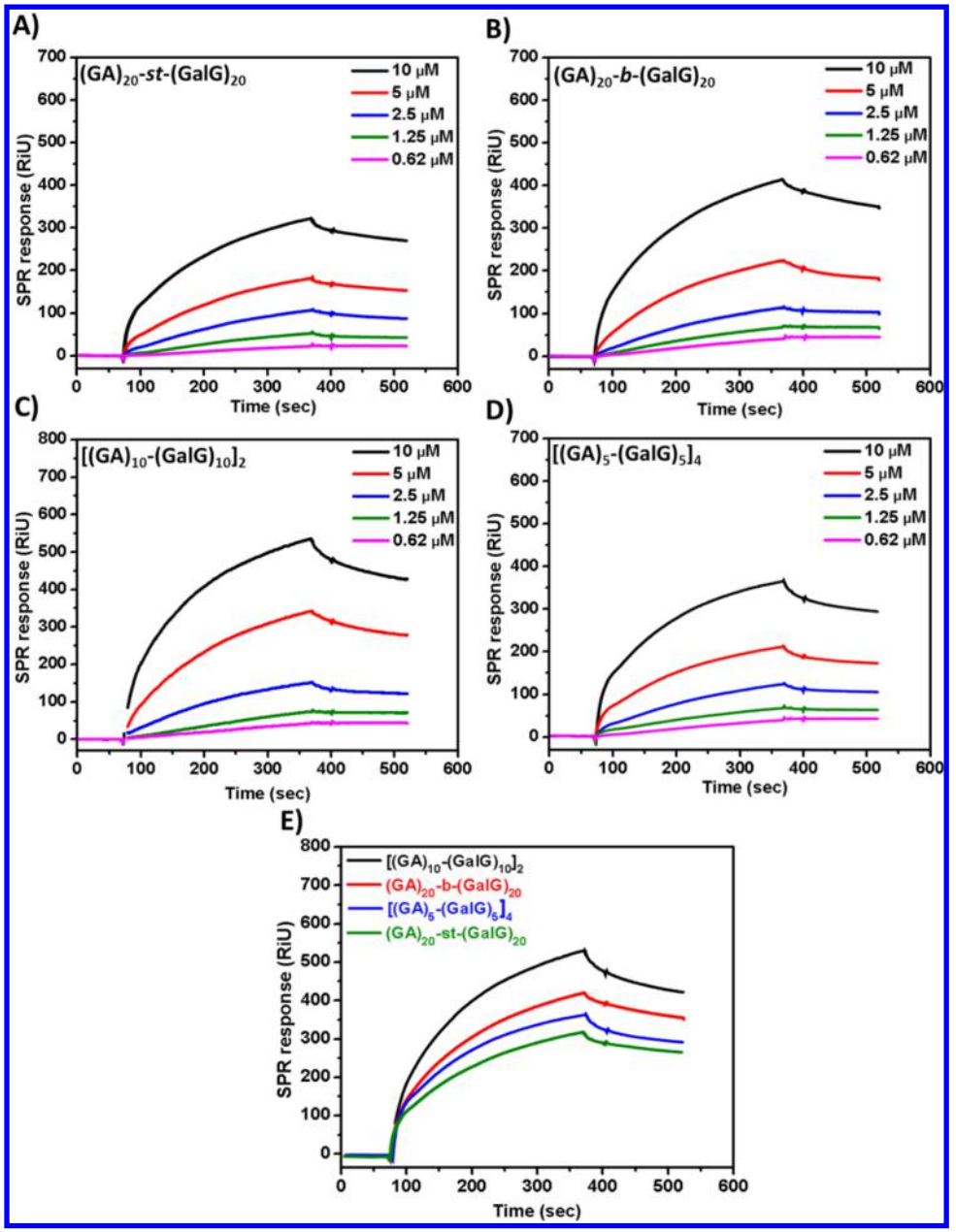

Figure 5. SPR sensorgrams showing concentration-dependent interactions between glycopolypeptides and Galectin-3: (A) $(GA)_{20}$-*st*-$(GalG)_{20}$; (B) $(GA)_{20}$-*b*-$(GalG)_{20}$; (C) $[(GA)_{10}$-$(GalG)_{10}]_2$; (D) $[(GA)_5$-$(GalG)_5]_4$; (E) Comparison of the binding of all glycopolypeptides to Galectin-3 at the same concentration (10 μM).

Table 3. Kinetic Binding Data of Glycopeptides and Lectins as Calculated by SPR[a]

	RCA$_{120}$				Galectin-3			
Ligand	k_a (1/Ms)	k_d (1/s)	K_a (1/M)	R_{max} (RU)	k_a (1/Ms)	k_d (1/s)	K_a (1/M)	R_{max} (RU)
$(GA)_{20}$-*st*-$(GalG)_{20}$	174	2.12×10^{-5}	8.20×10^{6}	260	236	5.66×10^{-4}	4.17×10^{5}	310
$(GA)_{20}$-*b*-$(GalG)_{20}$	87	1.63×10^{-5}	5.34×10^{6}	135	364	6.54×10^{-4}	5.56×10^{5}	420
$[(GA)_{10}$-$(GalG)_{10}]_2$	112	1.87×10^{-5}	5.99×10^{6}	175	406	5.96×10^{-4}	6.81×10^{5}	515
$[(GA)_5$-$(GalG)_5]_4$	208	2.36×10^{-5}	8.81×10^{6}	300	302	5.11×10^{-4}	5.91×10^{5}	360

[a]A 1:1 Langmuir binding model was used in biaevaluation software.

glycopolypeptide samples in DMF with increasing reaction times, the overall chemical composition of the four different block-sequenced glycopolypeptides with mostly COOH and galactose groups was comparable as summarized in Table 1.

Secondary Structure of Glycopolypeptides with Different Block Sequences. After deprotection, the influence of the sequential positioning of the galactose units on the secondary structure in aqueous solution was investigated by circular dichroism (CD) spectroscopy, along with pH-dependent conformations of the glycopolypeptide chains. At the concentration used (0.03 mg/mL), all aqueous solutions were transparent in the pH range studied (pH 3−12).

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At pH 12 the CD spectra are characteristic of a random-coil conformation for all four glycopolypeptides, with a maximum ellipticity at λ= 218 nm (Figure 1). At this pH, the glutamate carboxyl groups are ionized ($pK_a \sim 4.3$).[18,32] At pH 3, the glycopolypeptide chains adopt an α-helical conformation, as indicated by the two ellipticity minima at λ= 208 and 222 nm. At physiological pH, the diblock glycopolypeptide $(GA)_{20}$-*b*-$(GalG)_{20}$, the tetrablock glycopolypeptide $[(GA)_{10}$-$(GalG)_{10}]_2$, and the statistic glycopolypeptide $(GA)_{20}$-*st*-$(GalG)_{20}$ adopt a random-coil conformation, while the octablock glycopolypeptide $[(GA)_5$-$(GalG)_5]_4$ adopts a partially helical conformation.

The mean residue ellipticity at λ = 222 nm, $[\theta]_{222}$, can be used to estimate the helicity of the glycopolypeptide chains. The helical contents of the four glycopolypeptides at different pH (Figure 2) are summarized in Table 2. The helical contents at pH 3 are within the range 67−77%, with the helicity being slightly higher for the octablock $[(GA)_5$-$(GalG)_5]_4$ and the tetrablock $[(GA)_{10}$-$(GalG)_{10}]_2$ than for the diblock $(GA)_{20}$-*b*-$(GalG)_{20}$ and the statistical $(GA)_{20}$-*st*-$(GalG)_{20}$ glycopolypeptides. When pH is increased, there is a strong rise of $[\theta]_{222}$ for the diblock, the statistic, and the tetrablock glycopolypeptides, which invariantly adopt a random-coil conformation for pH values above 6. Conversely, the octablock glycopolypeptide shows only a small variation of $[\theta]_{222}$ until pH 6, and at this pH the $[\theta]_{222}$ rises suddenly, to reach a constant value of −7000 deg·cm^2·dmol^{-1} (corresponding to a helicity of ∼25%) up to pH 11. This unusual behavior, leading to a partially helical conformation at physiological pH, even at mildly basic pH, reveals that the α-helix conformation is strongly promoted in this glycopolypeptide, where a few units of galactosyl-L-glutamate are alternated with blocks of a few glutamic acid units. Surprisingly, the statistical glycopolypeptide behaves similarly to the diblock glycopolypeptide, which could suggest different reactivity ratios of the original NCA monomers leading to a tapered (block-like) chain structure rather than a random arrangement.[27,33,34] Recently, Mildner and Menzel described the use of hydrophobic spacers to enhance the helicity of pH-responsive glycopolypeptides.[35] From the different hydrophobic spacers studied, the use of an aromatic hydrophobic spacer resulted in enhanced helicity and a partial helical conformation at neutral pH, although their study did not include the behavior of the polypeptides under basic pH. In our case, however, we observe a strong enhancement of the helicity of the glycopolypeptides not by changing their chemical composition, but by the position of the sugar units in the peptidic backbone. As in natural peptides, the sequence of the units within the primary structure appears to determine the secondary structure of the biomacromolecules.

Lectin Binding of Glycopolypeptides with Different Block Sequences. Carbohydrates play a major role in biological recognition events, often mediated by specific carbohydrate−lectin interactions,[36,37] making the binding of glycopolypeptides to lectins a very effective method for ascertaining the bioactivity of these materials. Glycopolypeptides with pendant sugar moieties are capable of multivalent binding to some lectins in a process known as the "cluster−glycoside effect".[7] Preliminary lectin binding studies were performed using turbidimetric assays, in order to assess the ability of these block-sequenced glycopolypeptides to interact with biological systems. Typically, these tests are conducted by mixing the glycopolypeptide with a lectin that is selective for the sugar conjugated to the polymer. Successful galactose−lectin binding is denoted by the appearance of a precipitate due to aggregation of the lectins. Since single sugar units bind only weakly to the lectin receptors, only multivalent binding leads to clustering and precipitation.[38,39]

The lectin chosen was RCA_{120} (*Ricinus communis* agglutinin), a galactose-specific lectin which is present in the seeds of *Ricinus communis*, the castor bean plant. RCA_{120} is a 120 kDa tetramer which has two identical and independent sugar binding sites.[40] The lectin binding experiments were carried out by adding 50 μL of glycopolypeptide (1 mg/mL) into 500 μL of RCA_{120} (2 mg/mL), both dissolved in pH 7.2 phosphate buffer. Although RCA_{120} showed affinity for the four glycopolypeptides, the response over time was dependent on the sequence of the galactose units in the glycopolypeptide chain (Figure 3). The octablock glycopolypeptide $[(GA)_5$-$(GalG)_5]_4$ lead to stronger formation of clusters, the diblock glycopolypeptide $(GA)_{20}$-*b*-$(GalG)_{20}$ lead to the weakest interaction, whereas the tetrablock $[(GA)_{10}$-$(GalG)_{10}]_2$ and the statistical $(GA)_{20}$-*st*-$(GalG)_{20}$ glycopolypeptides presented intermediate behaviors. These results suggest that the particular disposition of the galactose units in the octablock polypeptide favors their interaction with lectins. When the same experiments were carried out with Concanavalin A lectin (ConA), which is selective for glucose and mannose, but unable to bind galactose residues,[41] no change in absorbance was measured (Table S2). These experiments confirm that the glycopolypeptides are available for biorecognition to selective lectins and that this recognition is dependent on the block-sequence composition of the glycopolypeptides.

For further insights to the binding activities of glycopeptides, surface plasmon resonance (SPR) was employed.[42] In addition to RCA_{120}, Galectin-3, a human galactose-specific lectin, was included. Galectin-3 contains carbohydrate recognition domains linked to nonlectin domains of about 130 amino acids.[43] Galectin-3 was selected because it plays a crucial role in human physiological responses to different types of pathology, such as cancer, inflammation, and autoimmunity.[44] In particular, it has attracted significant attention for the development of diagnostic marker systems for different cancers. Therefore, the synthesized glycopeptides are of interest for the development of multivalent biological systems for effective therapeutic applications.[45] It has to be noted, though, that galectin-3 has a much deeper recognition domain than RCA_{120} and efficient binding would require a disaccharide.[46]

As depicted (Figure 4 and Figure 5), the glycopolypeptides bound to both galactose binding lectins, and the strength of the interactions varied with the display of galactose units along the backbone. The results obtained from the SPR measurements are in good agreement with RCA_{120} turbidimetric assays. For instance, the octablock glycopolypeptide $[(GA)_5$-$(GalG)_5]_4$ showed the highest turbidity and SPR binding ability with RCA_{120} whereas the diblock $(GA)_{20}$-*b*-$(GalG)_{20}$ represented the weakest recognition (Figure 4E). However, when the binding properties of RCA_{120} are compared to those of Galectin-3, a different set of interaction behaviors is seen (Figure 5E). For example, the tetrablock glycopolypeptide $[(GA)_{10}$-$(GalG)_{10}]_2$ showed the strongest binding with Galectin-3, the statistical glycopeptide $(GA)_{20}$-*st*-$(GalG)_{20}$ showed the weakest binding, while the diblock $(GA)_{20}$-*b*-$(GalG)_{20}$ and the octablock $[(GA)_5$-$(GalG)_5]_4$ glycopolypeptides demonstrated intermediate interaction profiles. In order to understand these differences better, we evaluated the kinetic binding parameters of all glycopolypeptides using SPR (Table 3).

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There is little difference in association rate constants (k_a) or dissociation rate constants (k_d) for the various galactopolypeptides against each lectin, and hence for the affinities (K_a) in each case. However, the lectin RCA_{120} was seen to have more than a 10-fold higher affinity compared to Galectin-3, essentially because of the lower disassociation rate constant. The increase in affinity was greatest for $(GA)_{20}$-*st*-$(GalG)_{20}$ (approximately 20-fold).

The SPR binding isotherms do not represent classical 1:1 Langmuir kinetics, but this was anticipated given that we are presenting polyvalent ligands. It is most likely that our SPR data show the result of multivalent interactions, sometimes referred to as high avidity reactions. In many sensorgrams (Figures 4 and 5), an initial rapid binding event is seen, but the rate then appears to slow. In multivalent interactions the first binding events will proceed at a single-site k_a, but then these ligands may interact with more sites locally, creating a polyvalent binding complex. Given that binding of a galactopeptide at a second, third, or higher order site will not add any mass to the SPR chip surface, and that they will be favored due to the local high concentration of galactose units at the chip surface, the association rate curve slows. Dissociation is also complex, and the very slow dissociation rates seen with all the polymers are also consistent with multivalent interactions. For each single dissociation event, the adjacent galactose units remain bound, hence promoting rebinding rather than dissociation of the complex. Similar multivalent complexes with galactose polymers have been observed before.[47] No doubt, these multivalent kinetics mask some of the differences between binding specificities, but even so, the data illustrate that the sequence and composition of sugar moieties have significant influences on the details of recognition.

CONCLUSIONS

Several glycopolypeptides with the same overall composition and number of galactose units, but having these distributed in different block sequences, were successfully synthesized by a block-sequence-controlled ring-opening polymerization approach followed by selective functionalization of preselected positions within the polypeptide chain. Circular dichroism measurements revealed some dependence of the secondary structure on the primary composition of the glycopolypeptides at physiological pH. While statistical, diblock, and tetrablock glycopolypeptides adopted a random coil conformation, the octablock glycopolypeptide was mostly α-helical. All galactopeptides were biologically active and bound to lectins. However, the extent of binding was shown to be dependent on the position of the galactose units and, thus, the primary glycopolypeptide structure. Combined with the higher tendency to adopt a α-helical conformation, the octablock glycopolypeptide favored interaction with lectin RCA_{120}. With Galectin-3 the tetrablock glycopolypeptide $[(GA)_{10}$-$(GalG)_{10}]_2$ demonstrated the strongest binding activity, although in all cases calculated affinity values differed little. This lack of kinetic differentiation is probably due to multivalent binding interactions which invariably lead to long dissociation times. Such long dissociation times may be beneficial in diagnostic and therapeutic applications, and we believe that precise control over the glyco-polymer/peptide architecture will provide a much desired selectivity toward specific lectins. Most importantly, the results suggest that different lectins are very sensitive to glyco coding and that precise control of carbohydrate units in synthetic polymeric glycopeptides will remain important if we are to realize the value of these reagents for therapy and diagnosis in medicine.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biomac.7b00356.

Additional tables, NMR, ATR-FTIR, CD spectra, and SEC traces (PDF)

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Notes

The authors declare no competing financial interest.

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