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Selective and Tunable Galectin Binding of Glycopolymers Synthesized by a Generalizable Conjugation Method

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Abstract

Glycopolymers, conjugates of synthetic polymers with pendant carbohydrates, are becoming increasingly important to probe the role of carbohydrates in cellular processes and for applications like biosensors and drug delivery. A library of glycopolymers bearing different sugar moieties was synthesized by grafting amino-functionalized sugars to poly(acrylic acid) via DMTMM coupling. Primary amines were introduced at the anomeric (C-1) position to a number of unprotected mono- di- and tri-saccharides using ammonium carbamate, and conjugated to poly(acrylic acid) of different molecular weights, synthesized by reversible addition fragmentation chain transfer (RAFT) polymerization. This approach provides a simple and efficient route for the preparation of glycopolymers that differ only in the identity or degree of substitution of the sugar moiety on the polymer. The binding parameters (k_a, k_d, K_D) of these new glycopolymers to galectins-1 and -3 were quantified using surface plasmon resonance. The galectins selectively bound only to lactose-containing polymers, and the binding affinity was dependent on the galectin type, degree of sugar substitution and the molecular weight of polymer chains. Binding to both galectin-1 and -3 increased with a higher degree of sugar substitution, and higher molecular weight of the polymer backbone, reaching K_D values on the order of 10^{-11} M.

Introduction

Glycopolymers are an important class of synthetic carbohydrate-containing macromolecules that feature pendant sugars.[1-6] The presentation of multiple sugar moieties along a polymer backbone results in increased binding to the complementary carbohydrate-binding proteins (lectins) compared to the individual sugars, due to the multivalent carbohydrate-protein interactions, termed as "cluster glycoside effect".[7-13] They mimic the polyvalent display of oligosaccharides presented by a cell surface and can therefore serve as a valuable tool for probing carbohydrate-protein interactions in a number of important biological processes involving cell growth, cell differentiation, immune defense, pathogen invasion, inflammation, and cancer metastasis.[14, 15] In this capacity, glycopolymers have found a variety of biomedical and pharmaceutical applications such as matrices for cell culture and scaffolds for tissue repair[16, 17], anti-infective and anticancer therapeutics[18-20], and in vaccine design.[21]

Although studies of glycopolymers have attracted increasing interest in the fields of biochemistry, biomaterials, and biomedical science, their availability are limited due to the difficulties associated with their synthesis or production.[1, 3, 4] Currently, there are two general synthetic strategies for the generation of well-defined glycopolymers that bear sugars in their native closed cyclic forms through a glycosidic linkage. One strategy relies on the polymerization of sugar-containing monomers, while the other is based on the conjugation of functionalized sugar derivatives to polymer backbones. In the first strategy, a variety of glycopolymers have been prepared from different carbohydrate-substituted monomers utilizing various polymerization techniques including free radical polymerization[22, 231], atom transfer radical polymerization (ATRP),[24] reversible addition fragmentation chain transfer (RAFT) polymerization,[25] nitroxide mediated radical polymerization (NMP),[26] cationic polymerization,[27]

anionic polymerization,[28] ring-opening polymerization,[29] ring-opening metathesis polymerization (ROMP),[30, 31] and Suzuki coupling polymerization.[32] However, this approach involves rather complicated procedures for the synthesis of carbohydrate-containing monomers, and in some cases, requires the use of protected monomers and subsequent deprotection after polymerization to generate the desired glycopolymer, making the introduction of different oligosaccharides into glycopolymers synthetically challenging and time-consuming. In the second strategy, the direct attachment of oligosaccharides to an existing polymer backbone is achieved with a variety of different chemistries.[33-35] For example, Menzel et al. described the successful DMTMM coupling of D-glucosamine to polypeptides. However, the method relied on sugars with pre-existing amino functional groups, which significantly limited the synthesis of very diverse glycopolymer libraries.[36] Ladmiral et al. employed Cu(I)-catalyzed "click chemistry" to graft sugar-derived azides to alkyne functionalized polymers, which requires chemical elaboration on the sugar derivatives and prefunctionalization of the polymers.[37] Schlaad demonstrated the direct synthesis of glycopolypeptides by a radical thiol-ene reaction without the need for a protecting group, but the method can only be applied to those saccharides with a free thiol functional group.[38, 39] Godula et al. reported a general synthetic strategy for the synthesis of glycopolymers via ligation of reducing sugars to polymer backbones carrying hydrazide groups.[40] This approach eliminates the inconvenience and limitations of laborious carbohydrate prefunctionalization and offers rapid access to glycopolymer libraries with a broad scope of carbohydrate structures. However, it has the drawback of creating glycopolymers with mixed isomeric forms of the sugar, including α or β cyclic forms and open forms, which can significantly impact their interactions and binding specificity with receptor lectin proteins. Similarly, Deming et al. reported a general synthetic strategy for the synthesis of glycopolypeptides by coupling the

N-methylaminooxy-functionalized polypeptides with unmodified reducing saccharides. However, this method can yield glycoconjugate isomers that differ in type of anomer and ring size. It also required a large excess of sugar, therefore may not be practical for conjugation of expensive saccharides.[41]

Here, we describe a simple, efficient, and generalizable method for the preparation of glycopolymers with exclusively β conformation of the sugar via DMTMM coupling of amino functionalized sugars to poly(acrylic acid). The binding of the resulting glycoprotein conjugates to galectins-1 and -3 were systematically investigated by surface plasmon resonance (SPR), showing that lactose-containing polymers retained selective and high-affinity to the proteins.

Experimental Section

Chemicals and Materials. All reagents were used as received unless otherwise noted. Acrylic acid (monomer), 4,4'-azobis(4-cyanopentanoic acid) (radical initiator), and 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid (chain transfer agent, CTA) were purchased from Sigma-Aldrich. D-Glucose, D-galactose, D-lactose monohydrate, D-maltose monohydrate, maltotriose, ammonium carbamate, ammonium hydroxide solution (28.0-30.0% NH_3), and tris(2-carboxyethyl) phosphine hydrochloride were purchased from Sigma-Aldrich. 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium Chloride (DMTMM) was purchased from TCI America. Octyl maleimide was purchased from Santa Cruz Biotechnology, Inc. HBS buffer (10×10^{-3} M 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 150×10^{-3} M NaCl, pH 7.2) was prepared with deionized (DI) water and filtered through a 0.2 μm PES membrane. Recombinant human galectin-1 and galectin-3 constructs were obtained from C. Bertozzi, recombinantly expressed in XL1-Blue competent *E. coli*, and purified using β-lactosyl

Sepharose affinity chromatography methods as previously reported.[42]

Characterization. ^{1}H NMR was performed on an Inova 600 MHz spectrometer with deuterium oxide (D_2O) as the solvent. Size exclusion chromatography (SEC) was performed using a Waters gel permeation chromatography system equipped with three Ultrahydrogel columns (Waters) in series (2000, 500 and 250 Å), a 1515 isocratic HPLC pump, and a 2414 refractive index detector. Temperature throughout the system was maintained at 30 °C. Phosphate buffer saline (pH = 7.4) was used as the eluting solvent at a rate of 0.8 mL/min. The system was calibrated using six individual poly(methacrylic acid), sodium salt standards with peak molecular weights ranging from 1670 to 110 000 Da and PDI from 1.02 to 1.11.

Nomenclature. Polymer conjugates are named according to the following rubric: polymer-*g*-sugar(polymer molecular weight- mol% sugar conjugation). For example, PAA-*g*-lactose(40-56%) reflects a Mn = 40,000 poly(acrylic acid) polymer backbone with 56 mol% substitution of lactose.

Synthesis of Poly(acrylic acid) (PAA) Homopolymers. PAA homopolymers with different molecular weights were synthesized by RAFT polymerizations followed by NaOH hydrolysis.[43] In a 100 mL round-bottom flask equipped with a magnetic stir bar, acrylic acid (10 mL, 146 mmol), 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid (50.9 mg, 0.182 mmol; in 5 mL of MeOH), 4,4'-azobis(4-cyanopentanoic acid) (12.8 mg, 0.046 mmol; in 5 mL of MeOH), and MeOH (28.5 mL) were added and sealed with a septum stopper. The solution was purged with high purity nitrogen for 20 min and then placed in a 60 °C oil bath under continuous stirring, which was stopped at 48 h by cooling the reaction mixture in an ice bath. The product was purified by dialysis using Spectra/Por regenerated cellulose dialysis tubing (1 kDa MWCO) against DI water for 3 d and recovered by lyophilization for 2 d to afford PAA with a

number-average molecular weight of ~40,000 (abbreviated PAA-CTA(40)). The other PAA-CTA homopolymer with the number-average molecular weights of ~80,000 (abbreviated PAA-CTA(80)) were made by using a higher monomer to CTA ratio in the reaction mixture.

The PAA-CTA homopolymers were treated with NaOH aqueous solution to remove methanol adducts as previously described[32] and the CTA end group. In a representative synthesis, PAA-CTA(40) (0.5 g) was dissolved in 15 mL of MeOH, purged with nitrogen for 5 min, and then mixed with NaOH (2M, 30 mL). The solution was stirred at room temperature under nitrogen for 12 h, neutralized with formic acid (88%), and purified by dialysis using Spectra/Por regenerated cellulose dialysis tubing (1 kDa MWCO) against DI water for 3 d. The product was recovered by lyophilization for 2 d to give PAA(40) homopolymer.

Synthesis of Amino-functionalized Sugars (Glycosylamines). Glycosylamines were synthesized by amination of unprotected mono- and oligosaccharides following the procedure reported by Likhosherstov.[44, 45] In a representative synthesis to amino-functionalized lactose (lactosylamine), the first step was to produce the salt form (lactosylammonium carbamate). D-lactose monohydrate (12.96 g, 36 mmol), ammonium carbamate (11.22 g, 144 mmol) and MeOH (150 mL) were added to a 500 mL round-bottom flask and heated to 40 °C for 20 min under stirring. Next, 70 mL of ammonium hydroxide solution (NH_4OH, 28.0-30.0% NH_3) were introduced to ensure completed dissolution of the D-lactose monohydrate. The reaction mixture was stirred at 40 °C for 24 h, after which 150 mL of MeOH was added. The reaction was then cooled and left at 0 °C overnight for complete precipitation of the product. The resulting precipitate was separated by filtration, washed with cold isopropanol and ether, and dried under vacuum to give the intermediate lactosylammonium carbamate (12.30 g). ^{1}H NMR (D_2O, 600 MHz): 4.74 (d, ~0.1H, J = 9.2 Hz, OC*H*NH), 4.46 (d, 1H, J = 7.8 Hz, OC*H*O), 4.13 (d, 1H, J =

8.8 Hz, OC*H*NH_2), 4.0-3.2 (m, 12H, C*H*O and CH_2OH). In the second step to generate the lactosylamine from its salt form, triethylamine (4 mL) was first mixed with MeOH (48 mL), and then slowly added to a solution of lactosylammonium carbamate (6.4g) in 8 mL of H_2O, during which a precipitate formed. EtOH (48 mL) was also added to ensure complete precipitation and the mixture was kept at 0 °C overnight. The resulting product was separated by filtration, washed with EtOH and ether, and dried under vacuum. The precipitation process was repeated one more time to afford the final product lactosylamine (5.0 g). ^{1}H NMR (D_2O, 600 MHz): 4.46 (d, 1H, J = 7.8 Hz, OC*H*O), 4.13 (d, 1H, J = 8.8 Hz, OC*H*NH_2), 4.0-3.2 (m, 12H, C*H*O and CH_2OH). Galactosylamine and maltotriosylmaine were prepared from D-galactose and maltotriose respectively, using the same synthetic protocol. Glucosylamine and maltosylamine were synthesized from D-glucose and D-maltose monohydrate, respectively, with the same procedure except that the addition of ammonium hydroxide in the first step was not needed.

Synthesis of Glycopolymers (GlycoPAA Graft Polymers). Glycopolymers were synthesized by conjugating the glycosylamines to the carboxy groups of PAA using the coupling agent DMTMM.[46] Using lactose-containing poly(acrylic acid) (PAA-*g*-lactose) graft polymers as a representative synthesis, lactosylamine (283.9 mg, 0.83 mmol) was introduced into a solution of PAA(40) (30 mg, 0.41 mmol of AA unit) in 8 mL of H_2O and stirred at room temperature for 5 min. A solution of DMTMM (230.4 mg, 0.83 mmol) in 2 mL of H_2O was added drop-wise and the reaction was stirred at room temperature for 24 h. The product was purified by dialysis using Spectra/Por regenerated cellulose dialysis tubing (2 kDa MWCO) against DI water for 3 d and recovered by lyophilization for 2 d to give PAA-*g*-lactose(40-56%). PAA-*g*-lactose graft polymers with different degrees of lactose substitution were obtained by controlling the initial ratio of [AA]:[lactosylamine]:[DMTMM] in the reaction mixture. Similarly, other

sugar-containing poly(acrylic acid)s were successfully prepared by the conjugation of corresponding glycosylamine to PAA homopolymers following the same procedure. Samples investigated in this work are listed in Table 1, Table 2 and Table 3.

Synthesis of Octyl End-caped PAA-*g*-lactose (PAA-*g*-lactose-C8) Graft Polymer. Reduction of disulfide bonds between PAA-g-lactose(40-56%) polymer chains and protection of the free thiols with octyl maleimide were performed following a procedure previously established by our group.[43] PAA-*g*-lactose(40-56%) (90 mg) was dissolved in 12 mL of HBS buffer, and purged with nitrogen for 10 min. Tris(2-carboxyethyl) phosphine hydrochloride (TCEP·HCl; 30 mg; in 3 mL HBS) was added, and purged with nitrogen for 5 min. The reaction was stirred at room temperature under nitrogen for 1 h. Octyl maleimide (43.8 mg; in 15 mL DMF) was added, purged with nitrogen for 5 min, and the reaction was stirred overnight at room temperature. The product was purified by dialysis using Spectra/Por regenerated cellulose dialysis tubing (2 kDa MWCO) against DI water for 3 d and recovered by lyophilization for 2 d to afford PAA-*g*-lactose-C8 (40-56%).

The product of each reaction step was confirmed by ^{1}H NMR spectroscopy and characterized by size exclusion chromatography (SEC) as described above.

Surface Plasmon Resonance (SPR). The interactions between glycopolymers and galectins (galectin-1 and galectin-3) were analyzed by SPR on a Biacore 3000 instrument (GE Healthcare). Stock solutions of galectin-1 and galectin-3 (1 mg/mL) in DI water were prepared from galectin samples (human recombinant, ~ 8 mg/mL in 8 mM DTT and 0.1 M lactose) using Zeba spin desalting columns (0.5 mL). The stock solutions were diluted to either 0.01 or 0.02 mg/mL using sodium acetate buffers (0.01 M, pH 5.0 or 5.5) for immobilization on carboxymethylated dextran-coated (CM5) sensor chips by an amine-coupling procedure. The

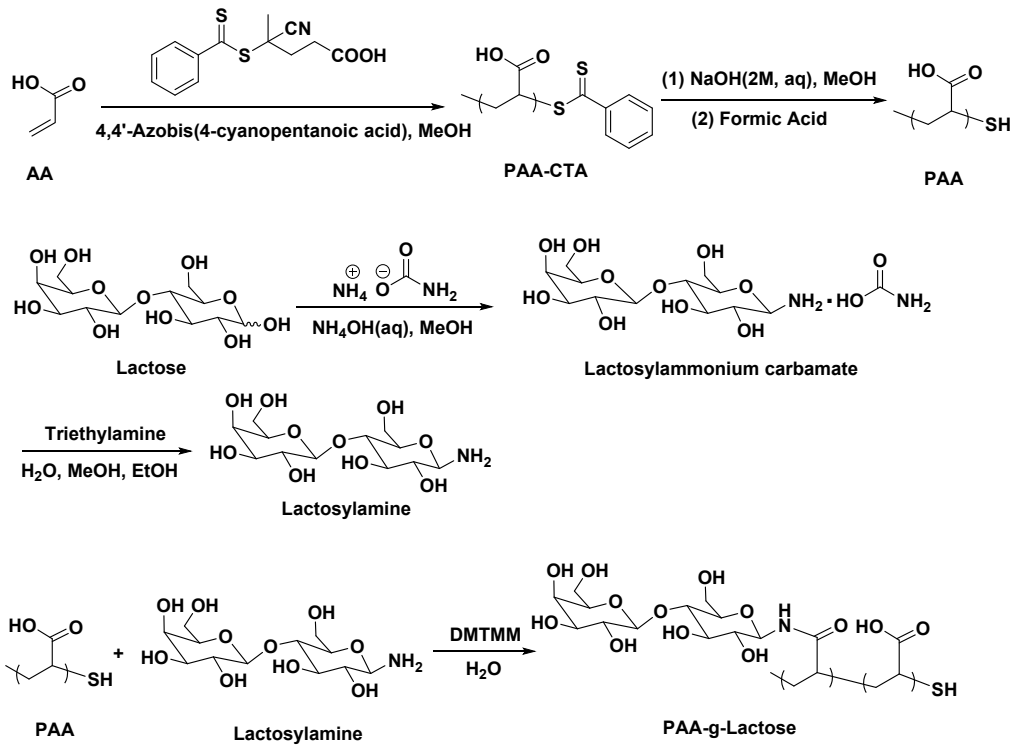
chip surface was activated with a freshly prepared aqueous solution of 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 0.1 M N-hydroxysuccinimide (NHS) (1:1, v/v), followed by surface coupling of galectins in sodium acetate buffer at a flow rate of 10 μL/min, then blocked by 1 M ethanolamine-HCl (pH 8.5). Each step was followed by HBS-EP buffer (0.01 M HEPES, 0.15 M NaCl, 0.03 M EDTA, and 0.0005% (v/v) surfactant P20, pH 7.4) rinses for a few minutes at a flow rate of 10 μL/min. The control flow cell was also activated and blocked by the same reagents (EDC/NHS and ethanolamine-HCl) but without the coupling of galectins. For the binding analysis, galectin-3 (0.02 mg/mL, pH 5.5) and galectin-1 (0.02 mg/mL, pH 5.0) with an injection time of 10 min were used to achieve ~6,000 and ~5000 resonance units, respectively. While for the kinetic experiments, galectin-3 (0.01 mg/mL, pH 5.5) and galectin-1 (0.01 mg/mL, pH 5.0) with an injection time of 5 min were employed to reach a level of ~1800 and ~2800 resonance units, respectively.

To determine the affinitive binding between the glycopolymers and galectins (binding analysis), two different polymer concentrations (0.1 and 0.01 mg/mL) in HBS-EP buffer were introduced to the flow cells at a flow rate of 30 μL/min with an injection time of 3 min and a dissociation time of 3 min. To determine the kinetic parameters (association rate constant (k_a), dissociation rate constant (k_d), and equilibrium dissociation constant (K_D)) of the binding interaction between PAA-*g*-lactose graft polymers and galectins, a concentration series of PAA-*g*-lactose polymers in HBS-EP buffer were individually injected to the flow cells at a flow rate of 30 μL/min. For both binding and kinetic analysis, the chip surface was regenerated with a 30 s pulse of the regeneration buffer (0.2 M lactose in DI water) at a flow rate of 10 μL/min. All sensogram data were recorded at 25 °C and normalized by subtracting the data from the control flow cell to correct for non-specific binding. All samples were repeated at least twice to check

the reproducibility. In the kinetic analysis, the sensorgrams measured at different concentrations were fitted by BIAevaluation software using one-step biomolecular association reaction model (1:1 Langmuir binding), which gave the optimal mathematical fits with the lowest χ-values.

Results and Discussion

Scheme 1. Synthesis of PAA-g-lactose Graft Polymers



Scheme 1 shows the synthetic route for the glycopolymers using PAA-*g*-lactose as an example. PAA homopolymers were synthesized by reversible addition fragmentation chain transfer (RAFT) polymerization followed by NaOH treatment. The PAA-CTA polymers obtained by RAFT polymerization were treated with NaOH to remove the small amount of impurities including both the methyl ester of acrylic acid (δ = 3.7 ppm) and the dimer form of

acrylic acid (δ = 2.8, 4.4 ppm) (Figure S1). It should be noted that this treatment also removed the CTA end group to afford the free thiol in the polymer chain end, which can lead to disulfide bond formation to bridge two polymer chains as suggested by the appearance of high molecular weight shoulder in the SEC trace (Figure S2). Two PAA homopolymers with the number-average molecular weights of ~40,000 and ~80,000 were prepared and their molecular characteristics are summarized in Table 1.

Table 1. Synthesis of PAA Homopolymers

Sample[a]	Reaction condition [AA]:[CTA]:[Initiator][b]	N_{AA}[c]	$Đ$[d]
PAA-CTA(40)	800:1:0.25	555	1.19
PAA(40)			1.32
PAA-CTA(80)	1600:1:0.25	1110	1.35
PAA(80)			1.48

[a] The numbers in the parentheses represent the approximate number-average molecular weight of PAA homopolymers, in kg/mol. [b] [AA]:[CTA]:[Initiator] represents the initial concentration ratio of AA:CTA:Initiator in the reaction mixture. [c] Number average degree of polymerization was calculated from monomer conversion as determined by ^{1}H NMR spectroscopy. [d] The polydispersity ($Đ$) was measured by SEC with PBS as the eluting solvent.

Amino-functionalized carbohydrates (glycosylamine) were synthesized by introducing the amino functionality to the anomeric carbon (C-1) of the sugar. Using lactose as an example, lactosylamine was synthesized by introducing the amino function via a one-step amination of the unprotected sugar with ammonium carbamate. The use of ammonium carbamate as the amination reagent leads to the formation of lactosylamine in the salt form (lactosylammonium carbamate), which precipitates as a white solid from the reaction mixture (mixed methanol-water solution). The salt formation was confirmed by ^{1}H NMR analysis of the anomeric hydrogens of lactosylammonium carbamate (δ = 4.1 ppm) and the rearranged form ammonium lactosylcarbamate (δ = 4.7 ppm) in D_2O (Figure S3).[44] The lactosylamine was easily recovered

by a simple treatment of lactosylammonium carbamate with triethylamine. Due to the precipitation of the salt intermediate from the reaction conditions, this method can give lactosylamine in high yield and purity without having a prolonged and labor-consuming procedure. It is also worth noting that the ammonium carbamate protocol leads to the formation of lactosylamine with exclusively β conformation, which was confirmed by ^{1}H NMR analysis of the anomeric α-hydrogens (δ = 4.1 ppm) (Figure S3). The amination modification method is readily applied to other mono- and oligosaccharides. Here, galactose, glucose, maltose, and maltotirose were also successfully amino-functionalized to afford the corresponding glycosylamine for glycopolymer synthesis (Scheme S1).

Table 2. Synthesis of Glycopolymers

Sample[a]	Glycosylamine	Reaction condition [COOH]:[NH_2]:[DMTMM][b]	Conjugation (%)[c]
PAA-g-galactose(40-29%)	galactosylamine	1:2:2	29
PAA-g-glucose(40-55%)	glucosylamine	1:2:2	55
PAA-g-lactose(40-56%)	lactosylamine	1:2:2	56
PAA-g-maltose(40-70%)	maltosylamine	1:2:2	70
PAA-g-maltotriose(40-75%)	maltotriosylamine	1:2:2	75

[a] The numbers in the parentheses represent the number-average molecular weight of PAA, in kg/mol, and ratio of [NH_2]:[COOH] in the reaction mixture. [b] [COOH]/[NH_2]/[DMTMM] represents the initial ratio of [AA]:[Glycosylamine]:[DMTMM] in the reaction mixture. [c] Degree of sugar substitution as determined by ^{1}H NMR spectroscopy.

Glycopolymers were synthesized by grafting glycosylamines to PAA homopolymers using the coupling agent DMTMM. DMTMM successfully conjugates amino-containing organic compounds to PAA homopolymers in aqueous solutions.[47, 48] In this work, we found the conjugation efficiency was greatest when using DI water, as determined by ^{1}H NMR (Figure S4), and that conjugations in borate buffers (pH = 6.5, 7.5, 8.5) yielded degrees of substitution <5%. ^{1}H NMR analysis of the anomeric α-hydrogens (δ = 5.0 ppm) also confirmed the β conformation of sugar unit next to polymer backbone for all glycopolymers listed in Table 2. Interestingly, the

degree of conjugation is lowest for PAA-*g*-galactose, which could be due to steric hindrance resulting from the cyclic form of galactose next to the PAA backbone in the case of PAA-*g*-galactose, while it is glucose for all the other glycopolymers. The degree of substitution in glycopolymers can be controlled by changing the molar ratio of AA:glycosylamine:DMTMM. PAA-*g*-lactose graft polymers with five different lactose conjugation levels were achieved by controlling the initial ratio of [AA]:[lactosylamine]:[DMTMM] in the reaction mixture (Figure S5). Additionally, changing the molecular weight of PAA (while keeping the same reaction condition) does not change the degree of lactose substitution (Table 3).

Table 3. Synthesis of PAA-*g*-lactose Graft Polymers

Sample[a]	Reaction condition [COOH]/[NH_2]/[DMTMM][b]	Conjugation (%)[c]	$N_{lactose}$ [d]
PAA-*g*-lactose(40-4%)	1:0.2:0.2	4	22
PAA-*g*-lactose(40-8%)	1:0.5:0.5	8	44
PAA-*g*-lactose(40-14%)	1:0.75:0.75	14	78
PAA-*g*-lactose(40-24%)	1:1:1	24	133
PAA-*g*-lactose(40-56%)	1:2:2	56	311
PAA-*g*-lactose(80-55%)	1:2:2	55	611

[a] The numbers in the parentheses represent the number-average molecular weight of PAA, in kg/mol, and ratio of [NH_2]:[COOH] in the reaction mixture. [b] [COOH]/[NH_2]/[DMTMM] represents the initial ratio of [AA]:[lactosylamine]:[DMTMM] in the reaction mixture. [c] Degree of substitution of lactose as determined by ^{1}H NMR spectroscopy. [d] Number of lactose units per polymer chain.

The hydrodynamic sizes of the glycopolymers were characterized by SEC. PAA has the most extended solution conformation, likely from charge repulsion, and the hydrodynamic radii of the glycopolymers decreased, relative to PAA, with increasing degree of carbohydrate substitution (Figure 1, Figure S6). A shoulder is observed on each SEC trace which is from the formation of a disulfide bridge between two polymer chains. The disulfide bond can be reversed by exposing the polymers to a reducing agent (TCEP) followed by thiol-capping with a maleimide (Scheme S2). The successful reduction and thiol-capping of PAA-*g*-lactose(40-56%)

was confirmed by the appearance of the proton signal corresponding to the C8 alkane of the maleimide (δ = 0.9, 1.3, 2.6 ppm) in the ^{1}H NMR spectrum (Figure S7) and disappearance of high molecular weight shoulder in the SEC trace (Figure S8).

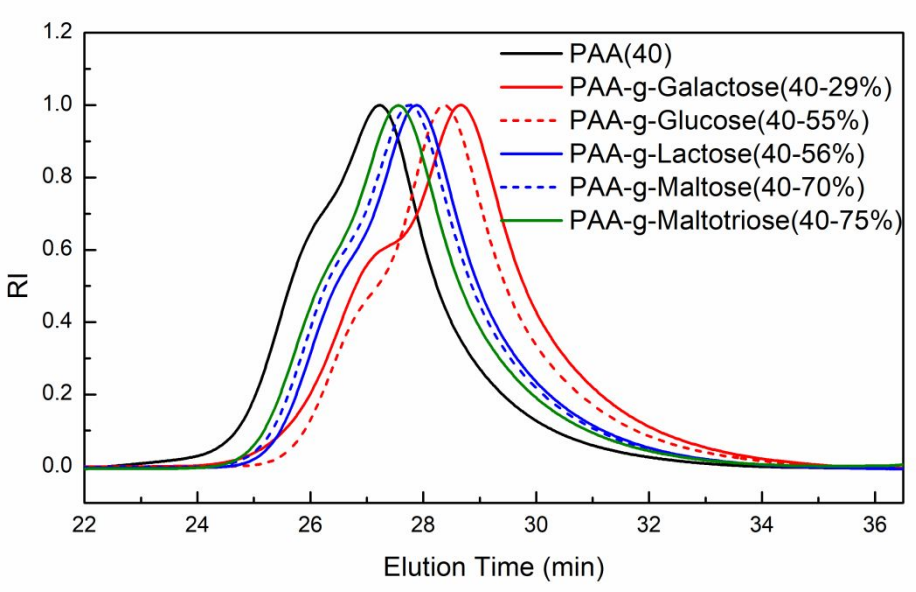


Figure 1. SEC trace of PAA (40), PAA-*g*-galactose(40-29%), PAA-*g*-glucose(40-55%), PAA-*g*-lactose(40-56%), PAA-*g*-maltose(40-70%), and PAA-*g*-maltotriose(40-75%).

Using DMTTMM, the amino-functionalized carbohydrates were coupled to poly(acrylic acid), creating a library of glycopolymers with different sugar identities and different degrees of substitution. Their binding to galectins-1 and -3 was quantified using SPR. SPR has emerged as a very good method to measure both the binding affinity and binding kinetics of glycopolymers with various lectins.[19, 49, 50] In this work, the galectins were immobilized on the surface of the sensor chip and the glycopolymers were flowed over the surface. The opposite SPR set-up (i.e., glycopolymer immobilization and galectin flowed over the surface) did not give stable signals in our hands. Binding events lead to changes in the surface plasmon resonance of the system, thereby allowing quantitation of binding kinetics. The galectin family of lectins have binding

specificity for β-galactoside sugars.[51] Two galectins were selected in this study. Galectin-1 possess a single carbohydrate recognition domain and can form homodimers through *N*-terminal interactions. Galectin-3 forms pentamers via the non-lectin domain, resulting in a pentavalent carbohydrate-binding molecule.[42] Biochemically, these galectins are functional in cancer metastasis, innate and adaptive immune regulation and inflammation among other processes.[52-54] Retention of the β-galactoside stereo-conformation of the sugars following functionalization with the amino group, as well as retention of the biorecognition by proteins following sugar conjugation to the PAA are both validated by the SPR studies.

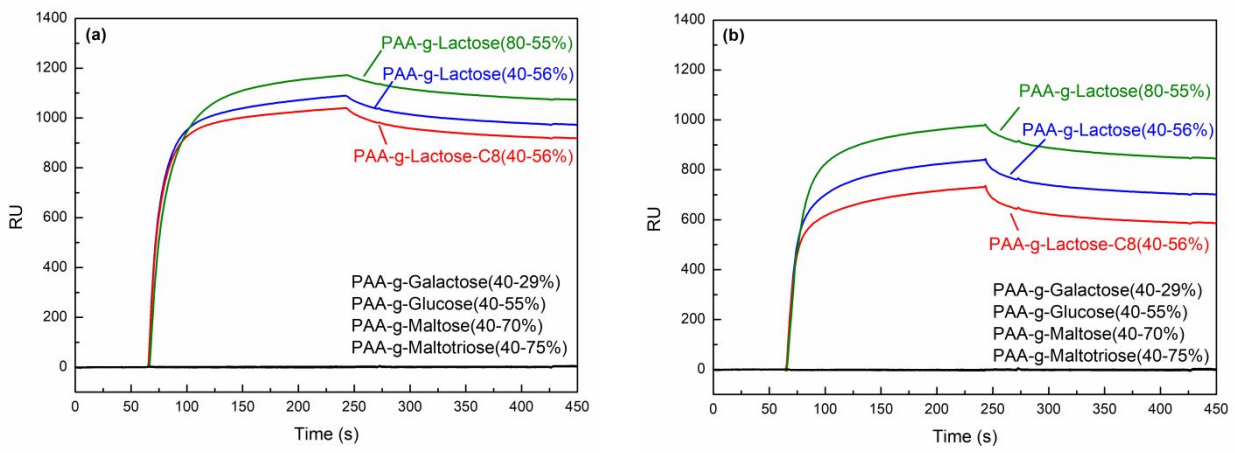


Figure 2. SPR sensorgrams for the binding interactions between glycopolymers and (a) galectin-3 and (b) galectin-1 obtained by flowing 0.01 mg/mL glycopolymers over the galectin-modified sensor chip surface.

Of all the sugar moieties investigated in this work, the only β-galactoside sugar is lactose. To measure the binding parameters of each glycopolymer, galectin-3 and galectin-1 were separately immobilized on the surface of a sensor chip at the level of ~6000 and ~5000 response units, respectively. Solutions of glycopolymers at the concentration of 0.01 mg/mL were introduced to the chip surface to initiate the binding analysis. Sensorgrams for the binding of glycopolymers to galectin-3 and galectin-1 are shown in Figure 2. Only lactose-containing

glycopolymers are able to interact with galectin-3 and galectin-1, confirming the retention of the pendant β-galactoside structure on the polymers. The higher molecular weight PAA-*g*-lactose(80-55%) had stronger binding affinities to the galectins than the smaller PAA-*g*-lactose(40-56%), likely the result of increased cluster valency (Table 3). Along the same lines, PAA-*g*-lactose-C8(40-56%), wherein the thio-end group is protected, exhibited weaker binding to the galectins than PAA-*g*-lactose(40-56%), the population of which includes polymer dimers created by disulfide formation. It is worth noting that increasing the glycopolymer concentration that is flowed in the system to 0.1 mg/ml gave almost identical results in Figure 2 for both galectin-3 and galectin-1 (See Figure S9).

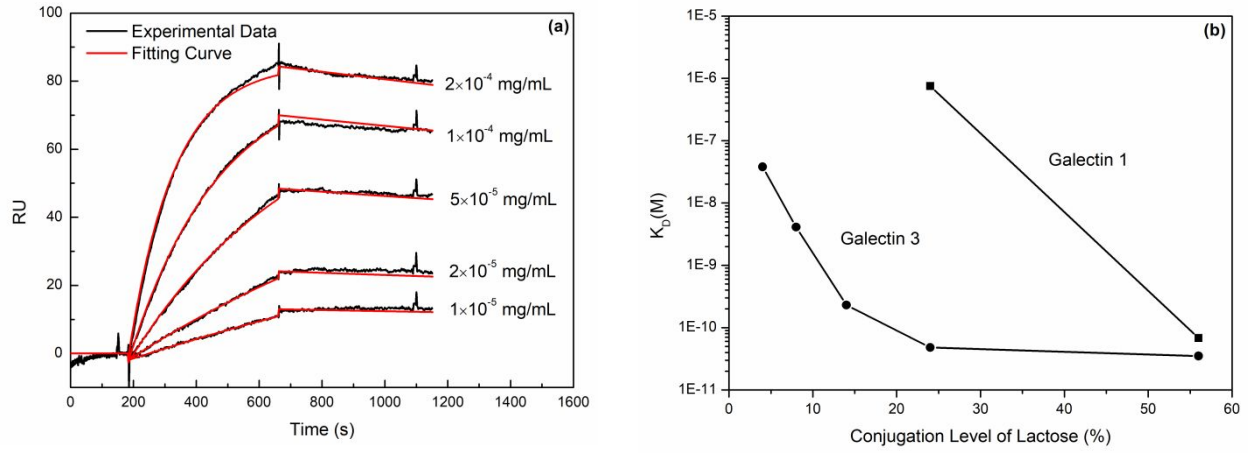


Figure 3. (a) SPR sensorgrams and curve fits for the binding interactions between galectin-3 and PAA-*g*-lactose(40-24%). The black curves represent experimental data, while the red curves represent model fits. (b) Equilibrium dissociation constants (K_D) of binding interactions between galectins and PAA-*g*-lactose as a function of lactose conjugation level in PAA-*g*-lactose graft polymers.

To quantify the kinetic parameters for the binding between galectins and PAA-*g*-lactose with varying degree of lactose substitution, galectin-3 and galectin-1 were immobilized at a lower density of ~1800 and ~2800 response units, respectively to prevent mass transfer-limited kinetics. Solutions of different PAA-*g*-lactose graft polymers ranging in concentration from

1×10^{-5} mg/mL to 0.1 mg/mL (~7×10^{-11} M to ~7×10^{-7} M) were analyzed by SPR for their binding to galectins. The five lowest consecutive concentrations with detectable SPR signals were chosen from those analyzed and fit with BIAevaluation software using the one-step biomolecular association reaction model (1:1 Langmuir binding) to extract the kinetic rate constants. A representative example for the SPR sensorgrams and curve fits for the binding between galectin-3 and PAA-*g*-lactose(40-24%) is shown in Figure 3a (See Figure S10 and Figure S11 for the comprehensive SPR sensorgrams and curve fits) and rate constants (k_a and k_d) for these polymers to both galectin-3 and galectin-1 are presented in Table 4. All SPR experiments were repeated at least twice, and reproducible sensorgrams were obtained in both binding analysis and kinetic analysis (Figure S12).

Table 4. Kinetic Parameters of Binding between PAA-*g*-lactose and Galectins

PAA-*g*-lactose [a]	Galectin	k_a(1/Ms) [b]	k_d(1/s) [b]	K_D(M) [c]
PAA-*g*-lactose(40-4%)	galectin 3	8.8×10^{2}	3.3×10^{-5}	3.8×10^{-8}
PAA-*g*-lactose(40-8%)		7.6×10^{4}	3.1×10^{-4}	4.1×10^{-9}
PAA-*g*-lactose(40-14%)		1.1×10^{6}	2.5×10^{-4}	2.3×10^{-10}
PAA-*g*-lactose(40-24%)		2.8×10^{6}	1.4×10^{-4}	4.8×10^{-11}
PAA-*g*-lactose(40-56%)		5.4×10^{6}	1.9×10^{-4}	3.5×10^{-11}
PAA-*g*-lactose(80-55%)		5.8×10^{6}	1.4×10^{-4}	2.4×10^{-11}
PAA-*g*-lactose(40-4%)	galectin 1		NA [d]	
PAA-*g*-lactose(40-8%)			NA [d]	
PAA-*g*-lactose(40-14%)			NA [d]	
PAA-*g*-lactose(40-24%)		1.7×10^{2}	1.3×10^{-4}	7.5×10^{-7}
PAA-*g*-lactose(40-56%)		4.6×10^{6}	3.1×10^{-4}	6.8×10^{-11}
PAA-*g*-lactose(80-55%)		4.1×10^{6}	1.5×10^{-4}	3.7×10^{-11}

[a] The numbers in the parentheses represent the number-average molecular weight of PAA, in kg/mol, and the ratio of $[NH_2]$:[COOH] in the reaction mixture. [b] Association rate constants (k_a) and dissociation rate constants (k_d) were determined by fitting the sensorgrams of SPR with BIAevaluation software using the 1:1 Langmuir binding model. [c] Equilibrium dissociation constants (K_D) were calculated from k_a and k_d ($K_D = k_d / k_a$). [d] Kinetic parameters cannot be determined due to the very low SPR signals.

Increasing the polymer backbone length from PAA-*g*-lactose(40-56%) to PAA-*g*-lactose(80-55%) lowers the equilibrium dissociation constants (K_D) for both galectin-3

and galectin-1, consistent with the previous binding analysis in which increasing the polymer backbone molecular weight increases the binding affinities. The K_D increases as the degree of lactose substitution decreases mainly due to the contribution of the association rate constants (k_a). The trend is shown in Figure 3b, wherein K_D is plotted as a function of lactose degree of substitution. Figure 3b shows an abrupt increase in K_D at lactose degree of substitution between 14% and 24% for galectin-3. The K_D for the polymer-lactose conjugates is smaller for galectin-3 than that for galectin-1 at all lactose conjugation levels. For the SPR studies with galectin-1, the K_D was not measurable for three of the PAA-*g*-lactose conjugates with low degrees of substitution due to the very low SPR signal, but data for the conjugates that did give adequate signals are included in Figure 3b for the reader's reference and for completeness.

A model that represents the SPR results, primarily the higher avidity of the PAA-*g*-lactose conjugates to galactin-3 relative to galectin-1, is represented in Figure 4. The carboxymethylated dextrans coated on the gold chip surface are functionalized with a high density of galectins, placing the proteins in close proximity and allowing multivalent binding with the lactose residues within one PAA-*g*-lactose polymer chain. Additionally, galectin-3 is pentameric, bearing five carbohydrate recognition domains to interact with up to five lactose residues, whereas galectin-1 forms dimers, which can bind to up to two lactose moieties (Figure 4). The multivalent-multivalent interactions with galectin-3 is stronger than multivalent-divalent interactions for galectin-1, which lead to the lower K_D and lower critical lactose conjugation value for the abrupt increase of K_D for galectin-3 determined by the kinetic analysis (Figure 3).

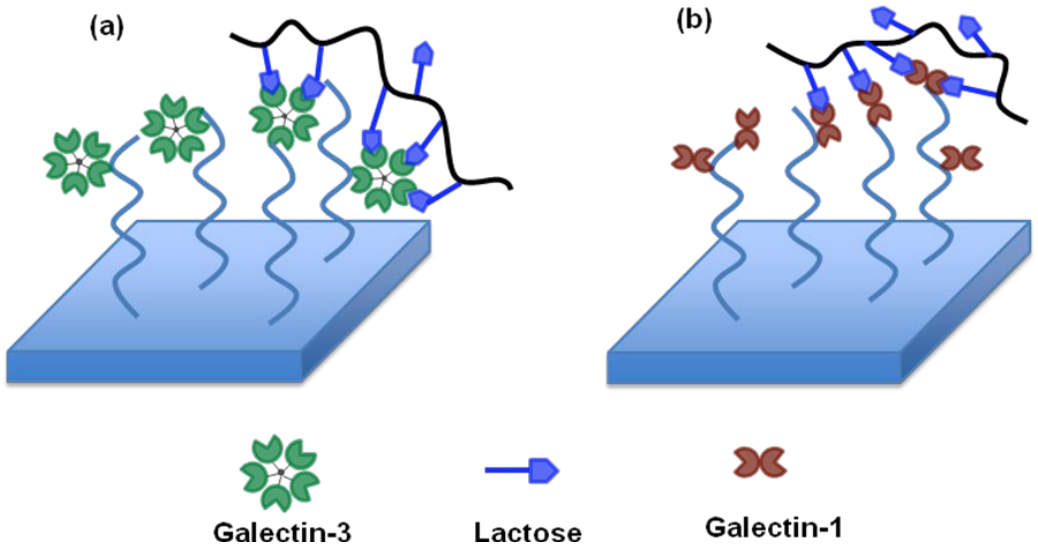


Figure 4. Schematic illustration of interactions between PAA-*g*-lactose graft polymers and (a) galectin-3 and (b) galectin-1.

The K_D for the glycopolymers with high lactose content is on the order of 10^{-11} M. This value is very low in comparison with other carbohydrate-lectin interactions reported in the literature by SPR, indicating the very high affinity for the glycopolymer-galectin interaction. To put these results into context, monovalent saccharide-protein interaction is typically in the range of 10^{-3}-10^{-4} M.[25, 30] Mannose-containing glycopolymers with low mannose content showed binding affinities to concanavalin A (Con A) in the range of 10^{-5}-10^{-6} M.[55] Galactose-containing glycopolymers with 100% galactose substitution showed avidities to *Ricinus communis* agglutinin 120 (RCA_{120}) in the range of 10^{-9}-10^{-10} M.[25] The trends in the literature (higher degree of substitution leads to lower K_D) support the trends observed in these PAA-*g*-lactose studies. Direct comparison among different studies is always a challenge, but it is clear that the PAA-*g*-lactose glycopolymers reported here do have very high affinity for galactins and this binding can be tuned by changing the molecular composition of the polymers.

Conclusions

We report a general approach for the preparation of glycopolymers via DMTMM coupling of amino functionalized sugars to poly(acrylic acid). The post polymerization modification offers a rapid and robust synthetic method, used herein to synthesize a glycopolymer library, by a simple sugar modification without any requirement for polymer pre-functionalization. The method enables good control of the glycopolymer structure, in particular retention of the sugar's β conformation, and control over the desired grafting density of the sugar on the polymer backbone. The method worked successfully with each sugar attempted. Given the commercial availability of many sugars, the method can serve as a very valuable tool for the preparation of very diverse glycopolymer libraries, which can be used as multivalent ligands to probe a large range of carbohydrate-binding protein interactions. Using this conjugation approach, a small library of glycopolymers with different sugar moieties and different degrees of sugar substitution were successfully synthesized and their binding interactions with galectins were quantified by SPR, and we found that galectins-1 and -3 are specific, high-affinity binding partners for these lactose-containing glycopolymers.

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discussions and expertise.

Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org. Contents of Supplemental Information includes: Synthetic Scheme for Glycosylamines and PAA-g-Lactose-C8 Graft Polymers (Page S3); ^{1}H NMR spectra and SEC for Table 1 Entries (Page S4); ^{1}H NMR spectra for Lactosylamine (Page S5); ^{1}H NMR spectra for Table 2 Entries (Page S6); ^{1}H NMR spectra and SEC for Table 3 Entries (Page S7); ^{1}H NMR spectra and SEC for PAA-g-Lactose-C8 Graft Polymers (Page S8); Molecular weights of polymers measured by SEC relative to pMAA (Page S9); SPR sensorgrams for interactions between glycopolymers and galectins (Page S10).

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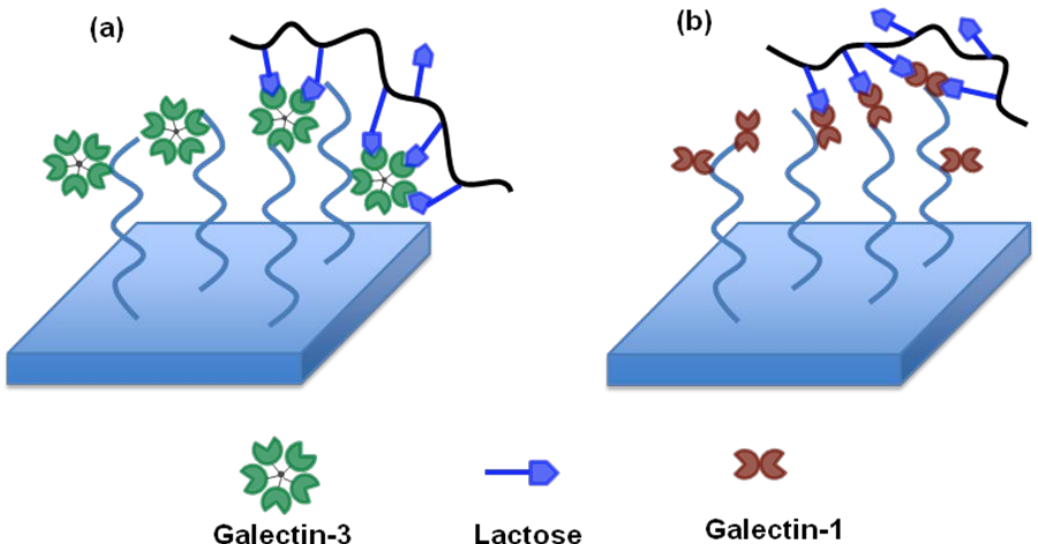
Table of Contents Graphic for

Selective and Tunable Galectin Binding of Glycopolymers Synthesized by a Generalizable Conjugation Method

Can Zhou,[1] Heidi L. Reesink[2] and David Putnam*,[1,3]



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