Article

pubs.acs.org/Biomac

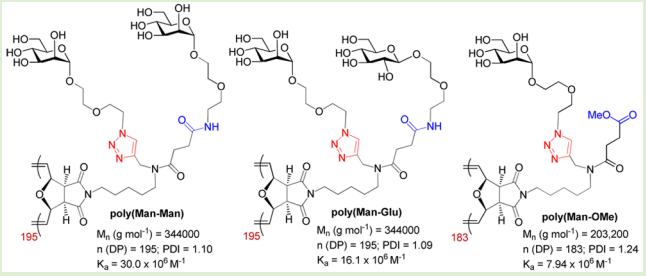
Studies of Highly-Ordered Heterodiantennary Mannose/Glucose-Functionalized Polymers and Concanavalin A Protein Interactions Using Isothermal Titration Calorimetry

Ravi S. Loka,[†] Matthew S. McConnell,[†] and Hien M. Nguyen*

Department of Chemistry, University of Iowa, Iowa 52242, United States

Supporting Information

ABSTRACT: Preparations of the highly ordered monoantennary, homofunctional diantennary, and heterofunctional diantennary neoglycopolymers of α-D-mannose and β-D-glucose residues were achieved via ring-opening metathesis polymerization. Isothermal titration calorimetry measurements of these synthetic neoglycopolymers with Concanavalin A (Con A), revealed that heterofunctional diantennary architectures bearing both α-mannose and nonbinding β-glucose units, poly(Man-Glc), binds to Con A (K_a = 16.1 × 10^6 M^{-1}) comparably to homofunctional diantennary neoglycopolymer (K_a = 30 × 10^6 M^{-1}) bearing only α-mannose unit, poly(Man-Man). In addition, poly(Man-Glc) neoglycopolymer shows a nearly 5-fold increasing in binding affinity compared to monoantennary neoglycopolymer, poly(Man). Although the exact mechanism for the high binding affinity of poly(Man-Glc) to Con A is unclear, we hypothesize that the α-mannose bound to Con A might facilitate interaction of β-glucose with the extended binding site of Con A due to the close proximity of β-glucose to α-mannose residues in the designed polymerizable scaffold.



INTRODUCTION

Glycans have a wide variety of roles in cellular processes that require specific recognition by glycan binding proteins (GBPs). Examples of these GBPs include plant,[1] viral lectins,[2] bacterial adhesins,[3] and sulfated glycosaminoglycan binding proteins.[4] In addition, glycan binding proteins can bind to the same glycan at different sites or multiple glycans in solution or on a cell surface.[5−8] Many of these proteins are functionally multivalent; in that they have multiple receptors for interacting with glycans.[9−12] In general, individual carbohydrate−protein receptor interactions are weak,[13] but the summation of these interactions results in an enhanced binding affinity which has been referred to as "avidity".[14]

In order to mimic glycan function, carbohydrates have been chemically displayed on a range of macromolecular architectures, such as polymers,[15−17] dendrimers,[18] quantum dots,[19−21] polypeptides,[22,23] and nanoparticles.[24−26] To date, neoglycopolymers (a polymer scaffold with incorporated carbohydrate residues) have illustrated great promise in studying GBP-governed processes. This is likely due to the ability to vary the length of the polymer chain, length and flexibility of the carbohydrate-polymerizable linker, and individual spacing of the pendant carbohydrate moieties.[27,28] For this work, neoglycopolymers can be classified as monoantennary **1**, homofunctional diantennary **2**, or heterofunctional diantennary **3** (Figure 1). Polymers **1**−**3** can be synthesized via a postpolymerization attachment of the glycan molecule[29] or polymerization of a monomer bearing a pendant carbohydrate unit.[30]

Concanavalin A (Con A), which is known to bind strongly to α-mannose residues, is widely utilized as a model protein to probe multivalent properties due to its low cost and well-established structure.[31,32] Isothermal titration calorimetry (ITC) is a very useful methodology of examining the binding affinities of GBP-glycan substrates. Many detailed ITC studies of multivalent α-mannose functionalized architectures with Con A protein have been reported. For instance, the Cloninger group has studied the thermodynamics of the interaction of α-mannose-functionalized dendrimers with Con A;[33] this study found that the fifth-generation dendrimer had the highest binding affinity to dimeric Con A. Wang and co-workers also used ITC to study the binding affinity of mannose-functionalized nanoparticles to Con A.[34] The Brewer group has thoroughly examined the binding of branched trisaccharides to Con A and concluded that Con A has an extended binding site that exhibits high affinity for the branched structure.[35] Gupta and co-workers have also illustrated that the nonhelical structure of glycopolypeptide, poly(α-manno-*O*-lys), has higher binding stoichiometry than the corresponding polypetide with α-helix by ITC studies.[23]

We report herein the simple and efficient synthesis of a highly ordered bifunctional neoglycopolymers **2** and **3** (Figure 1) from monomer consisting of both α-mannose and nonbinding β-glucose units. ITC studies[36] were used to

Received: October 13, 2015
Revised: November 16, 2015

DOI: 10.1021/acs.biomac.5b01380
Biomacromolecules XXXX, XXX, XXX−XXX

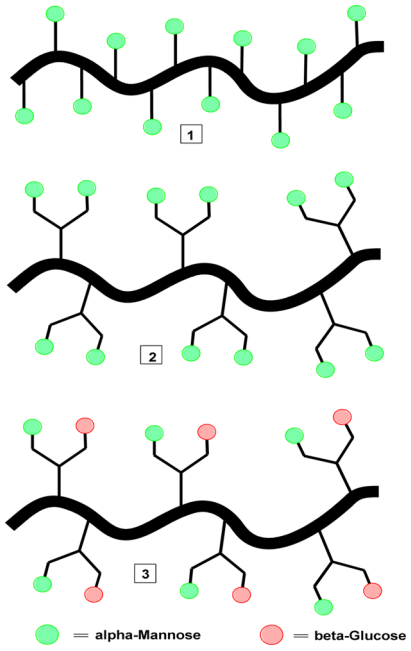


Figure 1. Neoglycopolymers of monoantennary **1**, homofunctional diantennary **2**, and heterofunctional diantennary **3**.

examine the avidity and thermodynamics of heterobifunctional neoglycopolymer **3** to Con A protein. These ITC results were compared to those of homofunctional neoglycopolymers **1** and **2** (Figure 1). Despite the presence of nonbinding β-glucose residues on the polymer chain, we observe that heteropolymer **3** can also effectively bind to Con A comparably to homopolymer **2** bearing only α-mannose units.

EXPERIMENTAL SECTION

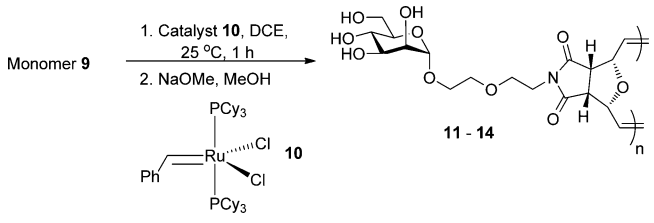
Methods and Reagents. All reactions were performed in oven-dried Schlenk flasks fitted with glass stoppers or round-bottom flasks under a positive pressure of nitrogen or argon. Organic solutions were concentrated by rotary evaporation below 40 °C at 25 Torr. Analytical thin-layer chromatography (TLC) was routinely used to monitor the progress of the reactions and performed using precoated glass plates with 230–400 mesh silica gel impregnated with a fluorescent indicator (250 nm). Visualization was achieved using UV light or ceric ammonium molybdate. Flash chromatography was performed and employed a 230–400 mesh silica gel. Milli Q water was used to make buffers. All other chemicals were obtained from commercial vendors and used without further purification.

Analysis. All proton (^{1}H) nuclear magnetic resonance spectra were recorded on 300, 400, and 500 MHz spectrometers. All carbon (^{13}C) nuclear magnetic resonance spectra were recorded on 100 and 125 MHz NMR spectrometer. Chemical shifts are expressed in parts per million (δ scale) downfield from tetramethylsilane and are referenced to the residual proton in the NMR solvent ($CDCl_3$: δ 7.27 ppm; D_2O: δ 4.79 ppm). Reference peaks for $CDCl_3$ in ^{13}C NMR spectra were set at 77.23 ppm. Data are presented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, and bs = broad singlet), integration, and coupling constant in hertz (Hz). Infrared (IR) spectra were reported in cm^{-1}. High resolution (ESI) mass spectrometry was performed to identify the purity of the compounds. Average polymer molecular weight (M_n) and polydispersity index (PDI) of the synthetic neoglycopolymers are determined by Gel Permeation Chromatography (GPC) using a Wyatt Dawn Heleos-II light scattering detector. Microcal iTC$_{200}$ from GE health care was used to perform ITC experiments.

General Procedure for Polymerization of Homofunctional Monomer. An oven-dried 10 mL Schlenk flask was charged with α-mannose monoantennary monomer (50 mg, 0.06 mmol, 1.0 equiv) and anhydrous degassed dichloroethane. Bis(tricyclohexylphosphine)-benzylidine ruthenium(IV) dichloride, (2.5 mg, 0.003 mmol, 0.05 equiv) was then added. The reaction mixture was stirred at room temperature for 1 h and monitored by TLC. The mixture was quenched with ethyl vinyl ether (0.1 mL) and concentrated *in vacuo* to a brown oil. The crude product was dissolved in a minimal amount of ethyl acetate and precipitated with an excess of petroleum ether. The solid was filtered and then redissolved in ethyl acetate. The precipitation process was repeated twice to produce the protected neoglycopolymer as a white solid (yields reported in Table 1). Purity and molecular weight (M_n) were characterized by ^{1}H NMR and GPC analyses. Degassing was done using the freeze–pump–thaw method.

General Procedure for Polymerization of Homofunctional Diantennary Monomer. An oven-dried 10 mL Schlenk flask was sequentially charged with homofunctional diantennary monomer bearing α-mannose moiety (50 mg, 0.017 mmol, 1.0 equiv) and anhydrous degassed dichloroethane

Table 1. ROMP of Monoantennary Monomer 9



entry	glycopolymers[a]	cat. **10** (mol %)	M_n[b] (g mol^{-1})	n (DP)[b]	PDI[b]	ROMP yield (%)[c]	hydrolysis yield (%)[c]
1	**11**	5	25 290	30	1.09	83	67
2	**12**	3	47 150	57	1.10	80	65
3	**13**	2	147 700	175	1.08	79	40
4	**14**	1	184 300	222	1.08	78	62

[a]Neoglycopolymers **11–14** correspond to the schematic monoantennary polymers **1** illustrated in Figure 1. [b]Number of average molecular weight (M_n), degree of polymerization (DP), and polydispersity index (PDI) of **11–14** were determined by GPC analysis. [c]Isolated yield.

DOI: 10.1021/acs.biomac.5b01380
Biomacromolecules XXXX, XXX, XXX–XXX

(500 μL). Dichloro[1,3-bis(2,4,6-trimethylphenyl)-2-imidazolidinylidene](benzylidene)-bis(3-bromo- pyridine) ruthenium (0.75 mg, 8.5×10^{-4} mmol, 0.03 equiv) was then added. The reaction mixture was stirred at room temperature for 1 h and monitored by TLC. The mixture was quenched with ethyl vinyl ether (0.1 mL) and concentrated *in vacuo* to a brown oil. The crude product was dissolved in a minimal amount of ethyl acetate and precipitated with an excess of petroleum ether. The solid was filtered and then redissolved in ethyl acetate. The precipitation process was repeated twice to produce the protected diantennary neoglycopolymer as a white solid (yields reported in Scheme 3). Purity and molecular weight were characterized by ^{1}H NMR and GPC. Degassing was done using the freeze−pump−thaw method.

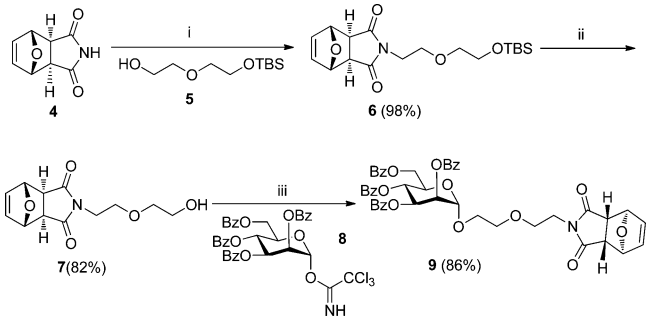
General Procedure for Removal of Benzoyl Groups of Neoglycopolymers. An oven-dried 10 mL Schlenk flask was charged with the protected neoglycopolymer (40 mg, 0.00158 mmol, 1.0 equiv), anhydrous dichloromethane (2 mL), anhydrous methanol (2 mL), and sodium methoxide (5 mg, 0.0926 mmol). The resulting mixture was stirred overnight at room temperature. The reaction mixture was poured off and the oily residue was washed with methanol (3 × 1 mL) and dichloromethane (3 × 1 mL). The residue was dissolved in water (5 mL) and brought to neutral pH with Amberlyst 15. The aqueous solution was filtered and lyophilized to produce the deprotected neoglycopolymer as a white solid. The deprotected neoglycopolymer was dialyzed using Slide-A-Lyzer dialysis cassette before ITC experiments were performed.

Isothermal Titration Calorimetry. Microcal iTC$_{200}$ from GE health care was used to perform ITC experiments. Concanavalin A (Con A) was dialyzed using Slide-A-Lyzer dialysis cassette with 10 000 MW cut off against 100 mM acetate buffer, pH 4.6 in the presence of 1 mM $MnCl_2$, 1 mM $CaCl_2$, and 30 mM NaCl. Concentrations of Con A were determined by spectrophotometry at 280 nm using $A^{1\%,1\ cm}$ = 12.4 (expressed in terms of the monomer, MW = 25 600 g mol^{-1}) and glycopolymers by colorimetry. Concentrations of Con A ranged from 0.42−0.06 mM, neo- glycopolymers from 0.338−0.017 mM, and glycomonomers from 7.55−8.03 mM. Titrations were performed at 25 °C with stirring speed of 1000 rpm. Injections of 1 μL of neoglycopolymer in the same buffer were added into the sample solution of Con A (cell volume = 200 μL) from a computer controlled 40 μL syringe at an interval of 2 s. Injections of 2 μL of glycomonomer in the same buffer were added into the sample solution of Con A (cell volume =200 μL) from a computer controlled 40 μL syringe at an interval of 4 s. The experimental data were fitted to a theoretical titration curve using software supplied by Microcal. A standard one-site model was used with ΔH (enthalpy change in kcal mol^{-1}), K_a (association constant in M^{-1}), and n (number of binding sites per monomer) as variable parameters.

RESULTS AND DISCUSSION

A. Synthesis of Homofunctional Neoglycopolymers. The work began with the synthesis of monomer **9** (Scheme 1) starting from Diels−Alder *exo*-adduct **4**.[37] We chose this *exo*-norbornene **4** because it has been reported that **4** undergoes ring-opening metathesis polymerization (ROMP) much faster than its *endo*-counterpart,[38] and allows for multivalent display of the ligands at defined, chemically controlled intervals to promote multivalent binding.[39] Furthermore, this polymerizable scaffold **4** increases the structural rigidity of the neoglycopolymers and allows efficient access to the corresponding copolymers between α-mannose and β-glucose units.[39] Accordingly, the Mitsunobu coupling of **4** with alcohol linker **5**[40] provided **6** in 98% yield (Scheme 1). Removal of the *tert*-butyl silyl (TBS) ether group afforded 82% yield of primary alcohol **7**, which served as a glycosyl acceptor in the glycosylation with trichloroacetimidate donor **8** mediated by 20 mol % of trimethylsilyl triflate (TMSOTf). The desired glycoside **9** was obtained in 86% yield.

Scheme 1. Synthesis of α-Mannose Monoantennary Monomer[a]



[a]Conditions: (i) Ph_3P, DEAD, DMF, 12 h. (ii) TBAF, THF, 0 °C, 1 h. (iii) 20 mol % TMSOTf, CH_2Cl_2, - 40 °C, 2 h.

ROMP of **9** was then investigated using Grubbs I catalyst **10** (Table 1). In our design, the benzoyl (Bz) protecting groups were chosen for the mannose hydroxyl groups in **9** because aromatic neoglycopolymers tend to have a high refractive index increment (dn/dc).[41] A high dn/dc would allow accurate number-average molecular weight (M_n) and polydispersity (PDI) determination via gel permeation chromatography (GPC) analysis of polymers.[41,42] In addition, the benzoyl protecting groups increase the solubility of **9** in noncoordinating, aprotic solvents, which are generally used for ROMP. Compound **9** dissolved in dichloroethane and underwent polymerization with 5 mol % of Grubbs catalyst **10** to the corresponding neoglycopolymer within 1 h (Table 1, entry 1, DP = 30, PDI = 1.09, and M_n = 25,290). By decreasing the amount of Grubbs I catalyst **10** from 5% to 1% (entry 2−4), we were able to achieve a series of the desired neoglycopolymers with increasingly larger M_n and degrees of polymerization (DP). Although lowering catalyst **10** increased molecular weights of polymers, the polydispersities remained relatively narrow (PDI = 1.08−1.10), indicating a high uniformity of molecular weight (M_n) in each neoglycopolymer. These polymers were also analyzed by ^{1}H NMR spectroscopy and were obtained in good yields (Table 1, 78−83%). Finally, the benzoyl groups were removed to afford neoglycopolymers **11**−**14** (Table 1) in 40−67% yield. (*Neoglycopolymers **11**−**14** correspond to the schematic monoantennary structure **1** shown in Figure 1.*)

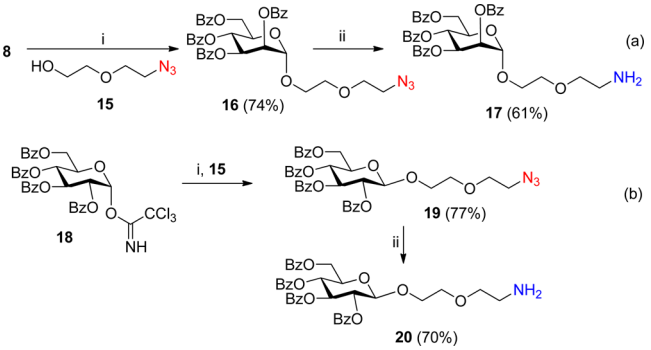
B. Synthesis of Homofunctional and Heterofunctional Diantennary Polymers. We next explored the synthesis and polymerization to produce the diantennary neoglycopolymers. There are two important criteria needed to be considered for effective synthesis of heterobifunctional neoglycopolymer. The first is to establish orthogonal reactivity with the α-mannoside and β-glucoside moiety that we intend to attach to the bifunctional, polymerizable scaffold **21** (Scheme 3).[43] Toward this end, we sought to functionalize both carbohydrates with amine and azido functional groups. While the amine group facilitates an amide coupling to the carboxylic acid of the

DOI: 10.1021/acs.biomac.5b01380
Biomacromolecules XXXX, XXX, XXX−XXX

scaffold, the azide group can react with the terminal alkyne of **21**. In addition, an ethylene glycol spacer was utilized in the sugars to increase flexibility and solubility of glycopolymers generated from ROMP.

Accordingly, use of 5 mol % of mild and commercially available $Pd(CH_3CN)_4(BF_4)_2$ catalyst, previously reported in our lab,[44,45] provided **16** in 74% yield (Scheme 2). Subsequent

Scheme 2. Preparation of α-Mannoside and β-Glucoside Bearing Azide and Amine Functionality[a]



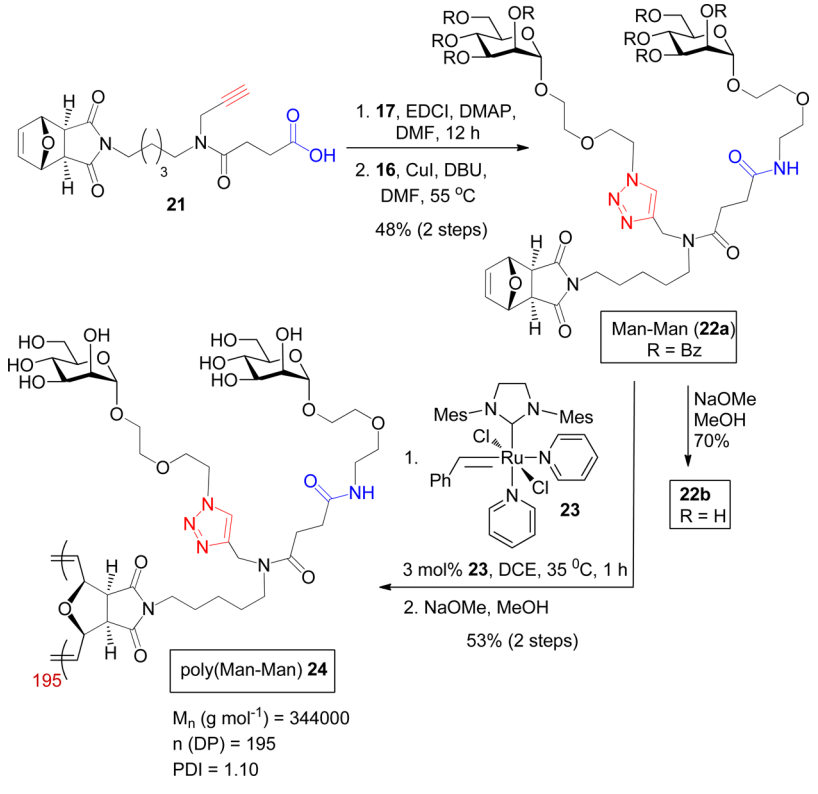
[a]Conditions: (i) 5 mol % $Pd(CH_3CN)_4(BF_4)_2$, CH_2Cl_2, 25 °C, 3 h. (ii) Ph_3P, THF/H_2O, 55 °C, 12 h.

subjection of **16** to a Staudinger reduction of the azide functionality provided the primary amine **17** in 61% yield. A similar route was used to make β-glucose azide **19** and amine **20** (Scheme 2).

The second important criteria involves the spacing between the two carbohydrate units. Recently, our group synthesized a similar heterobifunctional monomer with the calculated spacing between anomeric centers of mannose units at about 13 Å using solid state 3D-modeling software.[46] Based on this calculation, we hypothesize that the proximity between two sugar units would facilitate enhanced binding to the extended binding site of Con A in a similar manner as observed by Brewer with branched trisaccharides.[47] With this approach in mind, each glycan (Scheme 2) with its respective orthogonal functionality was sequentially coupled to the polymerizable scaffold **21** (Schemes 3–5) bearing the terminal alkyne and carboxylic acid functional groups.

The synthesis of homofunctional diantennary neoglycopolymer **24**, poly(Man-Man), was first explored (Scheme 3). Accordingly, EDCI-mediated amide coupling of linker **21** with α-mannosyl amine **17**, followed by Sharpless-Huisgen cycloaddition of azide **16**, produced homofunctional diantennary monomer, Man-Man (**22a**), in 48% yield over two steps. Removal of the benzoyl groups gave the fully deprotected monomer **22b** in 70% yield. Unfortunately, Man-Man (**22a**) monomer did not undergo polymerization with Grubbs I catalyst **10**. To address this problem, we investigated ROMP of **22a** with the more reactive Grubbs III catalyst **23**, and the desired neo-homopolymer (DP = 195 and PDI = 1.10) was obtained. Hydrolysis of the benzoyl groups produced the homobifunctional neoglycopolymer, poly(Man,Man) **24** (Scheme 3), in 53% over two steps. (*The homobifunctional polymer **24** corresponds to the schematic structure **2** shown in Figure 1.*) While higher and lower catalyst loadings were also employed to make larger and smaller homofunctional diantennary polymers (e.g., use of 5 mol % of catalyst **23** provided **24** with DP = 97 and PDI = 1.08), ITC studies of neoglycopolymers **11**–**14** illustrated the optimal valency for binding to be 175 repeating units (*vide infra*, Table 2).

Scheme 3. Synthesis and Polymerization of Homofunctional Diantennary Glycomonomer 22a (Man-Man)



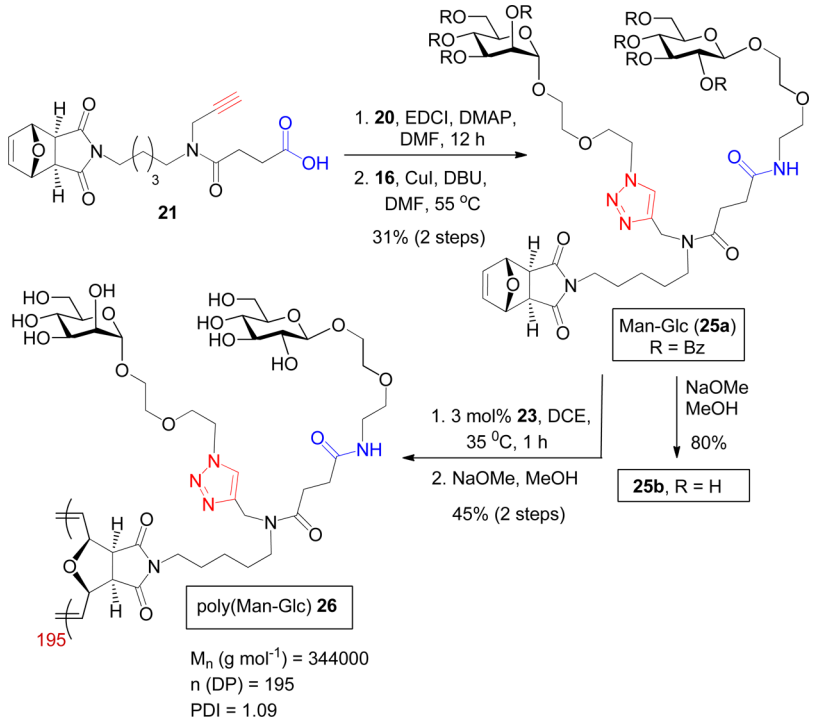
DOI: 10.1021/acs.biomac.5b01380
Biomacromolecules XXXX, XXX, XXX−XXX

Table 2. ITC Studies of Neoglycopolymers and ConA Binding Affinity

entry	neoglycopolymers[a]	DP[b]	K_a ($M^{-1} \times 10^3$)[c]	ΔH (kcal mol^{-1})[c]	ΔH per sugar (kcal mol^{-1})	ΔG (kcal mol^{-1})	$T\Delta S$/kcal mol^{-1}	n[c]	N[d]
1	methyl α-mannose	-	7.6	−7.30	−7.30	−5.2	−2.1	1.120	1
2	**22b**	-	17.3	−7.30	−7.42	−5.7	−9.1	0.390	2
3	**25b**	-	8.56	−7.30	−3.79	−5.4	−2.2	0.813	1
4	**11**	30	537	−41.40	−1.38	−7.8	−33.6	0.171	6
5	**12**	57	3200	−92.80	−1.63	−9.3	−83.5	0.060	17
6	**13**	175	3880	−196.8	−1.12	−9.6	−187.2	0.060	17
7	**14**	222	2860	−456.4	−2.06	−10.7	−445.7	0.0184	54
8	poly(Man-Man) **24**	195	30000	−786.0	−2.01	−84.0	−701.8	0.0139	72
9	poly(Man-Glc) **26**	195	16100	−751.1	−1.93	−69.5	−682.6	0.0140	72
10	poly(Glc-Glc) **28**	137	NO BINDING						
11	poly(Man-OMe) **31**	183	7940	−414.2	−2.26	−8.72	−405.5	0.0148	67
12	poly(Man-OMe)-(Glc-OMe)	173	2340	−214.0	−1.24	−8.0	−206.0	0.0331	30

[a]According to schematic structures **1**−**3** in Figure 1, while neoglycopolymers **11**−**14** and **31** corresponds to monoantennary **1**, neoglycopolymers **24** and **28** correspond to homofunctional diantennary **2**, and neoglycopolymer **26** corresponds to hetereofunctional diantennary **3**. [b]DP = degree of polymerization determined by GPC. [c]Errors for K_a, n, and ΔH are 2−15%, 1−4%, and 1−12%, respectively. [d]Number of binding sites: $N = 1/n$ (n: number of lectin binding sites)

Scheme 4. Synthesis and Polymerization of Heterofunctional Diantennary Glycomonomer 25a (Man-Glc)



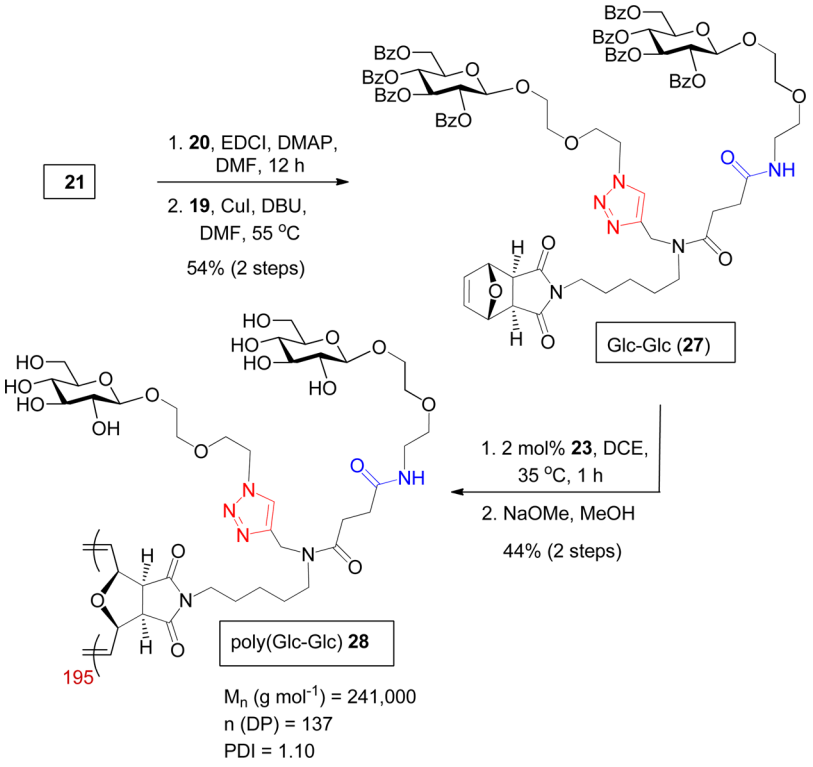
The heterodiantennary neoglycopolymer **26**, poly(Man-Glc), was prepared similarly to the route used for poly(Man-Man) **24** with the exception of utilizing amine-functionalized β-glucoside **20** for the amide-bond forming step (Scheme 4). While slightly less catalyst **23** was used {2.5 mol % vs 3 mol % for poly(Man-Man)}, a similar high valency was accomplished (DP = 195) with a narrow polydispersity (PDI = 1.09). The poly(Man-Glc) **26** (Scheme 4) was formed in 45% yield over two steps. (*The heterobifunctional polymer* ***26*** *corresponds to the schematic structure* ***3*** *shown in Figure 1*).

In order to investigate whether polymeric β-glucoside can bind to Con A protein, we also synthesized a homofunctional diantennary neoglycopolymer poly(Glc-Glc) **28** (Scheme 5) in a similar fashion to that for the poly(Man-Man) **24** (Scheme 3) and poly(Man-Glc) **26** (Scheme 4). The valency (DP = 137) of poly(Glc-Glc) **28** is somewhat lower than both poly(Man-Man) **24** and poly(Man-Glu) **26** despite the polymerization being performed with a lower loading of catalyst **23** (Scheme 5). Nevertheless, **28** still has a narrow PDI of 1.10. (*The homobifunctional polymer* ***28*** *corresponds to the schematic structure* ***2*** *shown in Figure 1*).

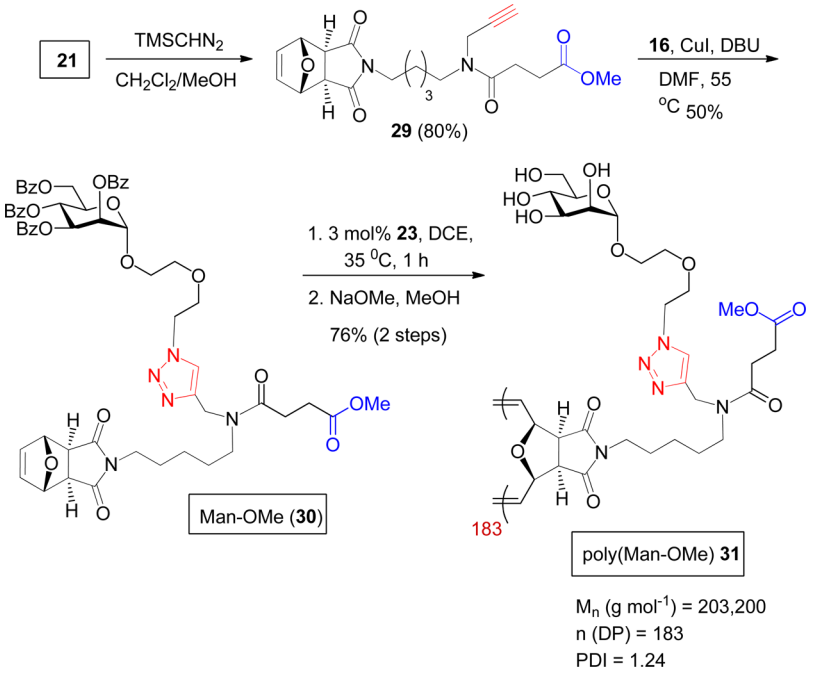
In order to accurately compare the binding affinity of monoantennary neoglycopolymers to Con A with diantennary neoglycopolymers, the change in polymerizable linker must be taken into consideration. A neoglycopolymer **31** (Scheme 6) using scaffold **21** as the building block was made as a control experiment for this purpose. The monomer **30** was polymerized using catalyst **23** to produce the corresponding poly(Man-OMe) **31** (Scheme 6) with similar valency (DP = 183) and higher polydispersity (PDI = 1.23) to the poly(Man-

DOI: 10.1021/acs.biomac.5b01380
Biomacromolecules XXXX, XXX, XXX−XXX

Scheme 5. Synthesis and Polymerization of Homofunctional Diantennary Glycomonomer 27 (Glc-Glc)



Scheme 6. Synthesis and Polymerization of Monoantennary Glycomonomer 30 (Man-OMe)



Man) **24** (Scheme 3) and the homofunctional glycopolymer **13** (Table 1, entry 3). (*The neoglycopolymer **31** corresponds to the schematic monoantennary **1** shown in Figure 1*).

We also sought to investigate whether the highly ordered nature of the heterofunctional diantennary neoglycopolymer poly(Man-Glc) **26** (Scheme 4) would enhance binding affinity to Con A. To achieve this goal, a control experiment was setup in which a random copolymer of Man-OMe **30** and Glc-OMe (prepared using the similar route for **30**) was synthesized in a 1:1 ratio. The copoly(Man-OMe)-(Glc-OMe) (see the Supporting Information) was obtained with a high valency (DP = 173 or about 87 mannose residues per polymer) and relatively narrow polydispersities (PDI = 1.05). Given the structural similarity between mannose and glucose unit, we postulated that there is no real difference in reactivity to the Grubb's catalyst **23** between Man-OMe and Glc-OMe monomers. As a result, they are randomly oriented along the polymer. Overall, this operationally simple synthetic approach

DOI: 10.1021/acs.biomac.5b01380
Biomacromolecules XXXX, XXX, XXX−XXX

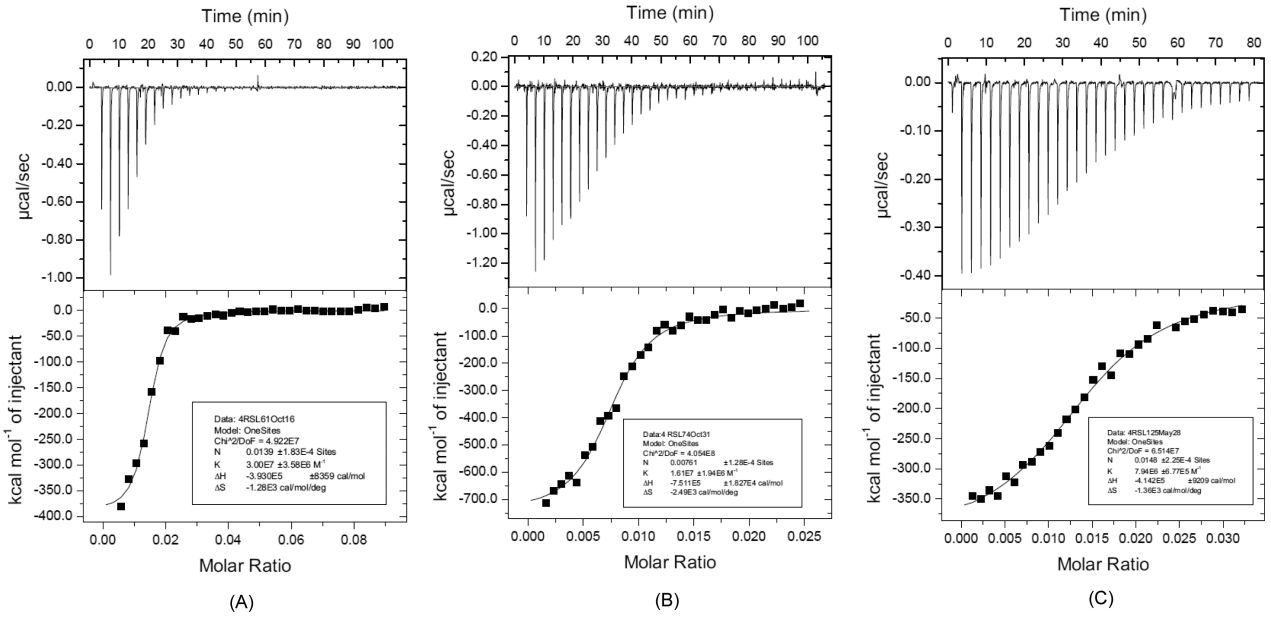


Figure 2. Calorimetric data for (A) homofunctional diantennary neoglycopolymer, **poly(Man-Man)**, (B) heterofunctional diantennary neoglycopolymer, **poly(Man-Glc)**, and (C) monoantennary glycopolymer, **poly(Man-OMe)**, binding of Con A protein. Top: raw data. Bottom: integrated data points with a best fit curve for one binding site.

allows for the rapid construction of novel and architecturally unique heterofunctional diantenary polymers.

C. Isothermal Titration Calorimetry Studies of Glycopolymers. With both the monoantennary and diantennary neoglycopolymers in hand, we next investigated their interactions with Con A using isothermal titration calorimetry (ITC).[48−50] Previous studies have showed that a lectin with a higher number of binding sites increases the possibility of precipitation.[33,35] To avoid this problem, we chose to examine the binding affinity of Con A to our neoglycopolymers at acidic pH, wherein Con A is in its dimeric form rather than tetrameric at higher pH. Even with use of dimeric Con A, there is evidence in Figure 2 that there is a small amount of noise in the baseline consistent with minimal aggregation. This is consistent with what has been observed by Cloninger and co-workers in the studies of interactions between carbohydrate-functionalized dendrimers and Con A.[33]

The results obtained from ITC for both glycomonomers and polymers are shown in Table 2. The binding affinity (K_a) of methyl mannose is comparable with previously reported values (K_a = 7.6 × 10^3 M^{-1}) (Table 2, entry 1).[33] Compared to α-methyl mannose, the K_a of deprotected glycomonomer, (Man-Man) **22b**, was increased about 2-fold (K_a = 17.3 × 10^3 M^{-1}) (entry 2). Given that two mannose units are present in **22b**, this result was expected. A small increase in binding affinity (K_a = 8.6 × 10^3 M^{-1}) was observed for deprotected glycomonomer, (Man-Glc) **25b** (entry 3). Upon examining the homofunctional neoglycopolymers **11**−**14** (Table 2, entry 4−7), which correspond to schematic monoantennary **1** shown in Figure 1, we found that increasing the valency resulted in higher binding affinity. For example, monoantennary neoglycopolymer **11** (entry 4) with K_a = 537 × 10^3 M^{-1} showed a 70 fold increase in binding affinity over methyl α-mannoside (entry 1). The increasing trend continues with polymers **12** (K_a = 3200 × 10^3 M^{-1}) and **13** (K_a = 3880 × 10^3 M^{-1}). However, the binding begins to level off with neoglycopolymers **14** (K_a = 2860 × 10^3 M^{-1}). We hypothesize that steric congestion due to the folding of larger neoglycopolymers **14** decreases the availability of α-mannose residue for binding to Con A. Upon comparison with excellent seminal work reported by the Cloninger group,[33] our synthetic neoglycopolymers of similar valency (DP) to the highly branched glycodendrimers have higher binding affinities.[33] For example, the third generation glycodendrimer which displayed 29 mannose units had less binding affinity (K_a = 300 × 10^3 M^{-1}) than that of monoantennary neoglycopolymer **11** (K_a = 537 × 10^3 M^{-1}). This may be due to higher conformational flexibility of linear polymer chains than branched dendrimers, and the higher flexibility imparts greater availability of α-mannose units.

Next, we compare the binding affinity between homofunctional neoglycopolymer **13** with diantennary homofunctional **24** and heterofunctional **26** neoglycopolymers in Table 2. For instance, diantennary poly(Man-Man) **24** (entry 8) (K_a = 30000 × 10^3 M^{-1}) demonstrated a nearly 10-fold increase in binding affinity versus monoantennary neoglycopolymer **13** (entry 6) (K_a = 3880 × 10^3 M^{-1}). The increase in binding affinity is attributed to the interactions of both mannose units with Con A binding sites. Importantly, when one of the strong binding mannose residues is substituted with a nonbinding β-glucose unit, generating heterodiantennary poly(Man-Glu) **26** (entry 9), we see a 5-fold increase in binding affinity (K_a = 16100 × 10^3 M^{-1}) from **13**. Although an enhancement in binding affinity is observed as going from **13** to **26**, the mechanism for this increase is unknown at this stage. We hypothesize that this may be due to an additional interaction of the β-glucose unit with the extended binding site of Con A. Furthermore, ITC studies of homofunctional diantennary poly(Glc-Glc) **28** (entry 10) determined that polymeric β-glucose does not, by itself, bind to Con A. This result also demonstrates the polymeric scaffold does not play a critical role in Con A binding. On the other hand, the change in linker from the monofunctional scaffold to the bifunctional counterpart did show 2-fold increase in binding when comparing monofunctional neoglycopolymer **13** (entry 6) with bifunctional

DOI: 10.1021/acs.biomac.5b01380
Biomacromolecules XXXX, XXX, XXX−XXX

poly(Man-OMe) **31** (entry 11, $K_a = 7940 \times 10^3\ M^{-1}$). This can possibly be attributed to the increase in linker length, which allows for increased flexibility and availability of pendant mannose moieties. It has also been speculated that the triazoles participate in binding to Con A protein.[16] In comparison, poly(Man-OMe) **31** did not bind as strongly as heterofunctional diantennary poly(Man-Glc) **26** (bearing a nonbinding β-glucose unit). In order to examine whether the close proximity of the mannose and glucose units in a highly ordered poly(Man-Glc) **26** result in higher avidity, ITC measurements were also taken with the copolymer poly(Man-OMe)-(Glc-OMe) (entry 12) and its binding affinity ($K_a = 2340 \times 10^3\ M^{-1}$) was much lower than that of poly(Man-Glc) **26** (entry 9). This result clearly illustrates the importance of the highly ordered diantennary architecture.

Our data in Table 2 illustrated that the enthalpy (ΔH) increases almost linearly with valency, meaning there is very little variation in ΔH per mannose unit. However, $T\Delta S$increases unfavorably with additional valency, but is ultimately overcome by a strongly exothermic ΔH value, leading to a negative ΔG in all cases. Similar results have also been obtained for different systems by Dam and Wang.[34,35] The stoichiometry (n) defined as the amount of ligands per binding site is generally expressed as functional valency (N), which describes the binding sites or Con A proteins per polymer chain (Table 2). In all cases, the functional valency N is less than valency (DP) of each glycopolymer (entries 4−12), suggesting that not all of α-mannose units are participating in the binding. For the monofunctional neoglycopolymers **11**−**14** (Table 2), the functional valency (N) value, however, increases as valency (DP) increases up to $N = 54$ for **14** with a valency of 222 (Table 2, entry 7). In the case of diantennary neoglycopolymers, the increased ligand density and valency (DP = 2 × 195 = 380 mannose units per polymer chain) of poly(Man-Man) **24** (entry 8) potentially allowed for the binding of more Con A protein units per glycopolymer $N = 72$. Interestingly, poly(Man-Glc) **26** (entry 9) also showed the same functional valency $N = 72$, despite having half as many mannose residues per polymer chain. Overall, these results may suggest that only α-mannose moieties in both diantennary neoglycopolymers **24** and **26** are involved in binding. Although this is further evident by the similar functional valency ($N = 67$) for the poly(Man-OMe) **31** (entry 11), K_a values for **24**, **26**, and **31** are very different. As we mentioned earlier, the Brewal group has demonstrated that Con A shows greater affinity to the branched α-mannosyl trisaccharide than the α-mannopyranoside monomer due to the extended binding site that Con A has.[35] Similar functional valencies ($N \sim 70$, Table 2) for bifunctional glycopolymers poly(Man-Man) **24** and poly(Man-Glu) **26** and monofunctional glycoppolymer poly(Man-OMe) **31** imply that enhanced binding affinity for bifunctional glycopolymers **24** and **26** could be due to the interaction of α-mannose and/or β-glucose with the extended binding site of Con A. Even though β-glucose itself does not bind to Con A, we hypothesize that the α-mannose bound to Con A might facilitate interaction of β-glucose with the extended binding site of Con A due to the close proximity (13 Å) of β-glucose to α-mannose residues in the designed polymerizable scaffold.[46]

CONCLUSIONS

In summary, we have synthesized highly ordered homofunctional and heterofunctional diantennary neoglycopolymers consisting of strong binding α-mannose and nonbinding β-glucose units using ROMP. Interactions of these bifunctional neoglycopolymers with glycan binding protein, Con A, were investigated using ITC analysis. Binding affinity experiments were strategically carried at acidic pH to avoid aggregation because previous studies have showed that a lectin (e.g., Con A) with a higher number of binding sites increases the possibility of precipitation by ITC measurements.[33,35] We discovered that the heterobifunctional diantennary neoglycopolymers, bearing both strong binding α-mannose and nonbinding β-glucose residues, binds to Con A protein ($K_a = 16.1 \times 10^6\ M^{-1}$) comparably to homobifunctional diantennary neoglycopolymer ($K_a = 30 \times 10^6\ M^{-1}$) bearing only α-mannose residue. Although the exact mechanism for the high binding affinity of poly(Man,Glc) to Con A is unclear, we hypothesize that the α-mannose bound to Con A might facilitate interaction of β-glucose with the extended binding site of Con A due to the close proximity (13 Å) of β-glucose to α-mannose residues (measured by using solid state 3D-modeling software)[46] in the designed polymerizable scaffold.

The heterofunctional diantennary neoglycopolymer, poly(α-Man-β-Glc), can be utilized as an alternative to the synthetically challenging neoglycopolymers such as poly(α-Man-α-Glc) and poly(α-Glc) whose α-glucose residues are known to bind strongly to Con A.[19,20] In general, it is much easier to get access to a nonbinding β-glucoside than a strong binding α-glucoside. The synthesis of β-glucosides can be achieved by employing glucosyl electrophiles with a C(2)-participatory protecting group.[51,52] Despite the variety of methods available, the synthesis of α-glucosides remains challenging because it requires glucosyl electrophiles with a C(2)-nonparticipatory protecting group.[51,52] Finally, the ITC results of neoglycopolymer, poly(α-Man-β-Glc), clarify the importance of the highly ordered heterofunctional bivalent architecture in the overall binding affinity to Con A. These results provide a foundation for further molecular mechanistic studies of the heterodiantennary neoglycopolymer interactions with Con A protein and exploration with other types of heterobifunctional diantennary polymers by combining a strong binding α-mannose residue with other nonbinding monosaccharide units, a peptide, or a lipid. These studies will be reported in due course.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biomac.5b01380.

Experimental procedures and characterization data for all new compounds (PDF)

AUTHOR INFORMATION

Corresponding Author

*Tel.: 319-384-1887. E-mail: hien-nguyen@uiowa.edu.

Author Contributions

†These authors contribute equally.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We are grateful for the financial support from National Institutes of Health (R01 GM098285). We thank Professor Jon Houtman for assistance with ITC analysis and Professor Ned Bowden for assistance with GPC analysis.

DOI: 10.1021/acs.biomac.5b01380
Biomacromolecules XXXX, XXX, XXX−XXX

REFERENCES

(1) Etzler, M. E. *Annu. Rev. Plant Physiol.* **1985**, *36*, 209−234.
(2) Van Breedam, W.; Pöhlmann, S.; Favoreel, H. W.; de Groot, R. J.; Nauwynck, H. J. *FEMS Microbiol. Rev.* **2014**, *38*, 598−632.
(3) Klemm, P.; Schembri, M. A. *Int. J. Med. Microbiol.* **2000**, *290*, 27−35.
(4) Esko, J. D.; Selleck, S. B. *Annu. Rev. Biochem.* **2002**, *71*, 435−471.
(5) Lundquist, J. J.; Toone, E. J. *Chem. Rev.* **2002**, *102*, 555−578.
(6) Sacchettini, J. C.; Baum, L. G.; Brewer, C. F. *Biochemistry* **2001**, *40*, 3009−3015.
(7) Mann, D. A.; Kanai, M.; Maly, D. J.; Kiessling, L. L. *J. Am. Chem. Soc.* **1998**, *120*, 10575−10582.
(8) Dam, T. K.; Roy, R.; Pagé, D.; Brewer, C. F. *Biochemistry* **2002**, *41*, 1359−1363.
(9) van Kooyk, Y.; Rabinovich, G. A. *Nat. Immunol.* **2008**, *9*, 593−601.
(10) Kwon, D. S.; Gregorio, G.; Bitton, N.; Hendrickson, W. A.; Littman, D. R. *Immunity* **2002**, *16*, 135−144.
(11) De Silva, R. A.; Appulage, D. K.; Pietraszkiewicz, H.; Bobbitt, K. R.; Media, J.; Shaw, J.; Valeriote, F. A.; Andreana, P. R. *Cancer Immunol. Immunother.* **2012**, *61*, 581−585.
(12) Capila, I.; Linhardt, R. J. *Angew. Chem., Int. Ed.* **2002**, *41*, 390−412.
(13) Rao, J.; Lahiri, J.; Isaacs, L.; Weis, R. M.; Whitesides, G. M. *Science* **1998**, *280*, 708−711.
(14) Kiessling, L. L.; Pohl, N. L. *Chem. Biol.* **1996**, *3*, 71−77.
(15) Becer, C. R. *Macromol. Rapid Commun.* **2012**, *33*, 742−752.
(16) Ting, S. R. S.; Chen, G.; Stenzel, M. H. *Polym. Chem.* **2010**, *1*, 1392−1412.
(17) Miura, Y. *J. Polym. Sci., Part A: Polym. Chem.* **2007**, *45*, 5031−5036.
(18) Cloninger, M. J. *Curr. Opin. Chem. Biol.* **2002**, *6*, 742−748.
(19) Hild, W. A.; Breunig, M.; Göpferich, A. *Eur. J. Pharm. Biopharm.* **2008**, *68*, 153−168.
(20) Gao, X.; Wang, T.; Wu, B.; Chen, J.; Chen; Yue, Y.; Dai, N.; Chen, H.; Jiang, X. *Biochem. Biophys. Res. Commun.* **2008**, *377*, 35−40.
(21) Kikkeri, R.; Lepenies, B.; Adibekian, A.; Laurino, P.; Seeberger, P. H. *J. Am. Chem. Soc.* **2009**, *131*, 2110−2112.
(22) Bonduelle, C.; Lecommandoux, S. *Biomacromolecules* **2013**, *14*, 2973−2983.
(23) Pati, D.; Shaikh, A. Y.; Das, S.; Nareddy, P. K.; Swamy, M. J.; Hotha, S.; Gupta, S. S. *Biomacromolecules* **2012**, *13*, 1287−1295.
(24) Chien, Y.-Y.; Jan, M.-D.; Adak, A. K.; Tzeng, H.-C.; Lin, Y.-P.; Chen, Y.-J.; Wang, K.-T.; Chen, C.-T.; Chen, C.-C.; Lin, C.-C. *ChemBioChem* **2008**, *9*, 1100−1109.
(25) Song, E.-H.; Osanya, A. O.; Petersen, C. A.; Pohl, N. L. B. *J. Am. Chem. Soc.* **2010**, *132*, 11428−11430.
(26) Reynolds, M.; Marradi, M.; Imberty, A.; Penadés, S.; Pérez, S. *Chem. - Eur. J.* **2012**, *18*, 4264−4273.
(27) Kiessling, L. L.; Grim, J. C. *Chem. Soc. Rev.* **2013**, *42*, 4476−4491.
(28) Lin, K.; Kasko, A. M. *Biomacromolecules* **2013**, *14*, 350−357.
(29) Malins, E. L.; Amabilino, S.; Yilmaz, G.; Isikgor, F. H.; Gridley, B. M.; Becer, C. R. *Eur. Polym. J.* **2015**, *62*, 347−351.
(30) Gestwicki, J. E.; Cairo, C. W.; Strong, L. E.; Oetjen, K. A.; Kiessling, L. L. *J. Am. Chem. Soc.* **2002**, *124*, 14922−14933.
(31) Schwarz, F. P.; Puri, K. D.; Surolia, A. *J. Biol. Chem.* **1993**, *268*, 7668−7677.
(32) Mammen, M.; Choi, S.-K.; Whitesides, G. M. *Angew. Chem., Int. Ed.* **1998**, *37*, 2754−2794.
(33) Mangold, S. L.; Cloninger, M. J. *Org. Biomol. Chem.* **2006**, *4*, 2458−2465.
(34) Wang, X.; Matei, E.; Gronenborn, A. M.; Ramström, O.; Yan, M. *Anal. Chem.* **2012**, *84*, 4248−4252.
(35) Dam, T. K.; Roy, R.; Das, S. K.; Oscarson, S.; Brewer, C. F. *J. Biol. Chem.* **2000**, *275*, 14223−14230.
(36) Castro, S.; Duff, M.; Snyder, N. L.; Morton, M.; Kumar, C. V.; Peczuh, M. W. *Org. Biomol. Chem.* **2005**, *3*, 3869−3872.
(37) Kwart, H.; Burchuk, I. *J. Am. Chem. Soc.* **1952**, *74*, 3094−3097.
(38) Rule, J. D.; Moore, J. S. *Macromolecules* **2002**, *35*, 7878−7882.
(39) Lee, S.-G.; Brown, J. M.; Rogers, C. J.; Matson, J. B.; Krishnamurthy, C.; Rawat, M.; Hsieh-Wilson, L. C. *Chem. Sci.* **2010**, *1*, 322−325.
(40) Liang, Q.; De Brabander, J. K. *Tetrahedron* **2011**, *67*, 5046−5053.
(41) Binboga, N.; Kisakurek, D.; Baysal, B. M. *J. Polym. Sci., Polym. Phys. Ed.* **1985**, *23*, 925−931.
(42) Zimm, B. H. *J. Chem. Phys.* **1948**, *16*, 1099−1116.
(43) Johnson, J. A.; Lu, Y. Y.; Burts, A. O.; Xia, Y.; Durrell, A. C.; Tirrell, D. A.; Grubbs, R. H. *Macromolecules* **2010**, *43*, 10326−10335.
(44) Yang, J.; Cooper-Vanosdell, C.; Mensah, E. A.; Nguyen, H. M. *J. Org. Chem.* **2008**, *73*, 794−800.
(45) Mckay, M. J.; Naab, B. D.; Mercer, G. J.; Nguyen, H. M. *J. Org. Chem.* **2009**, *74*, 4705−4711.
(46) Sletten, E. T.; Svec, R. L.; Nguyen, H. M. *Tetrahedron Lett.* **2015**, *56*, 3473−3476.
(47) Mandal, D. K.; Kishore, N.; Brewer, C. F. *Biochemistry* **1994**, *33*, 1149−1156.
(48) Ladbury, J. E. *Biochem. Soc. Trans.* **2010**, *38*, 888−893.
(49) Velazquez Campoy, A.; Freire, E. *Biophys. Chem.* **2005**, *115*, 115−124.
(50) Wiseman, T.; Williston, S.; Brandts, J. F.; Lin, L. N. *Anal. Biochem.* **1989**, *179*, 131−137.
(51) Schmidt, R. R.; Kinzy, W. *Adv. Carbohydr. Chem. Biochem.* **1994**, *50*, 21−123.
(52) Zhu, X.; Schmidt, R. R. *Angew. Chem., Int. Ed.* **2009**, *48*, 1900−1934.

DOI: 10.1021/acs.biomac.5b01380
Biomacromolecules XXXX, XXX, XXX−XXX