Bioconjugate Chemistry

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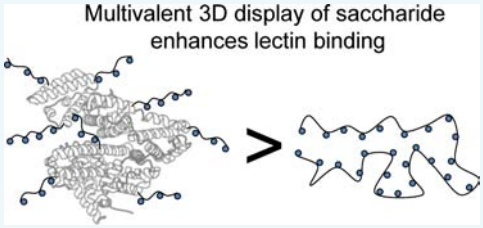
Multivalent 3D Display of Glycopolymer Chains for Enhanced Lectin Interaction

Kenneth Lin and Andrea M. Kasko*

Department of Bioengineering, University of California Los Angeles, California 90095, United States

Supporting Information

ABSTRACT: Synthetic glycoprotein conjugates were synthesized through the polymerization of glycomonomers (mannose and/or galactose acrylate) directly from a protein macroinitiator. This design combines the multivalency of polymer structures with 3D display of saccharides randomly arranged around a central protein structure. The conjugates were tested for their interaction with mannose binding lectin (MBL), a key protein of immune complement. Increasing mannose number (controlled through polymer chain length) and density (controlled through comonomer feed ratio of mannose versus galactose) result in greater interaction with MBL. Most significantly, mannose glycopolymers displayed in a multivalent and 3D configuration from the protein exhibit dramatically enhanced interaction with MBL compared to linear glycopolymer chains with similar total valency but lacking 3D display. These findings demonstrate the importance of the 3D presentation of ligand structures for designing biomimetic materials.



Oligo- and polysaccharides are polymers with saccharide repeat units connected through glycosidic bonds. Complex oligo- and polysaccharides fulfill a myriad of important roles in human health. These saccharide chains, whether alone or as a component of glycoproteins, are displayed at cell surfaces, incorporated into the extracellular matrix, mediate cell−cell and cell−matrix adhesion, imbue structural integrity to tissue (e.g., hyaluronic acid in cartilage), modulate signaling events at the cell surface (e.g., heparin and basic fibroblast growth factor), influence the presentation and lifetime of growth factors to cells, influence wound healing (e.g., selectin interactions and white blood cell extravasation into damaged tissue), and play crucial roles in our immune system, including pathogen recognition and viral entry.

Because of the many reactive functional groups on each saccharide monomer, the synthesis of even small oligosaccharides with well-defined structures is very tedious, and very few techniques exist to produce well-defined oligo- and polysaccharides on a reasonable scale. The lack of tools for the rapid synthesis and characterization of well-defined polysaccharides has hampered the investigation of their complex roles in biology, and limited their exploitation for biomedical applications. As an alternative, glycomimetic polymers can be synthesized in larger quantities and higher molecular weights. However, glycomimetic polymers only partially recapitulate the connectivity and branching seen in natural oligo- and polysaccharides. Despite the challenge of producing well-defined glycomimetic polymers, many structure−property relationships have been established for the interaction between oligo- and polysaccharides and glycomimetics and lectins, including the importance of valency,[1] saccharide identity,[2] density,[3] and branching.[4] However, the role of 3D presentation of saccharide chains on their interaction with lectins is not well-understood.

The carbohydrate binding activity of most lectins is thought to be mediated through multiple carbohydrate recognition domains (CRDs) comprising a limited number of amino acids. Lectins from diverse sources lack primary sequence homology but share similarities in their tertiary structure. The similarities in tertiary structure imply that the 3D presentation of multiple CRDs is crucial for lectin−saccharide interactions, especially since the per-residue affinity for saccharide−lectin interactions is quite low. While many examples of glycomimetic−lectin interactions have been reported, most examples investigate the interaction of soluble or adsorbed glycopolymers with lectins in solution or on a surface. This approach may not recapitulate natural lectin−polysaccharide encounters.

Proteins inherently exhibit 3D structure, so protein-based glycomimetics offer a convenient route to explore the role of 3D presentation of saccharide chains on lectin binding. Synthetic glycoproteins,[1] glycopeptides,[2] and protein glycoconjugates[3] have been reported in the literature.

Kiessling's group reported individual monosaccharides randomly conjugated to surface lysines of a protein (no chain valency), and the resulting modified protein was tested for its ability to bind and cluster lectins.[1] The protein−saccharide conjugate had higher avidity for lectin than an equimolar amount of monosaccharide. However, the conjugate did not facilitate lectin clustering well and exhibited significantly less binding than a glycopolymer with a similar valency of mannose (but no protein core).

Increased lectin binding has been observed with nanoparticles that present monomeric saccharides on their surface

Received: March 17, 2015
Revised: June 2, 2015
Published: June 25, 2015

DOI: 10.1021/acs.bioconjchem.5b00140
Bioconjugate Chem. 2015, 26, 1504−1512

compared to monomeric saccharides in solution.[4] The Akashi group reported that saccharide surface density is directly proportional to the size of the nanoparticle when size is adjusted by increasing the length of the hydrophobic block.[5] In addition, there is an optimal surface density for lectin binding, above which lectin interaction decreases.[5,6]

Since both 3D presentation and valency appear to enhance lectin−carbohydrate interactions, we hypothesize that synthesizing glycomimetics with 3D display of saccharide residues around a central structure can lead to an increase in carbohydrate binding to the lectin. In this report, we combine the 3D display of saccharide residues randomly arranged on a protein, with multivalent presentation through the use of glycopolymer chains. This type of glycomimetic−protein conjugate may have many important therapeutic applications as mimics of natural glycoproteins,[3c] for example, in vaccines.[3d]

In Nature, the glycan component of glycoproteins is incorporated either through stepwise addition of sugars (divergent) or through conjugation of oligosaccharide chains to the protein (convergent).[3e,f] Likewise, synthetic routes for glycopolymer−protein mimetics also have similar divergent and convergent approaches. The glycopolymer component can be added through a grafting-from technique where monomers are polymerized directly from a protein macroinitiator (divergent), or grafting-to where a premade polymer is conjugated to the protein (convergent). Many examples of convergent synthesis of protein−glycopolymer conjugates have been reported in the past few years, typically by conjugating a preformed glycopolymer to a protein to produce a diblock copolymer. Maynard has previously reported the polymerization of a *N*-acetyl glucosamine derived monomer from a peptide initiator[3b] (grafting from approach), but no examples of glycopolymerizations directly from a protein macroinitiator exist in the literature to our knowledge.

Here, we describe a grafting-from approach to polymerize glycomonomers (mannose and/or galactose residues) from multiple sites of a protein macroinitiator (derived from bovine serum albumin). This is the first report of glycopolymers grafted from a protein. The design of the polymer−protein conjugate results in the 3D presentation of saccharide ligands of varying valency to the multiple CRDs of lectins. This approach allows us to determine the effect of spatial orientation of multivalent glycomimetic chains on their interaction with lectins. Using this approach we can also compare the combined effects of valency and spatial orientation (by varying glyco chain length), and of carbohydrate density and spatial orientation (though copolymerization of nonbinding saccharide units).

RESULTS AND DISCUSSION

Synthesis of Mannose and Galactose Glycomonomers. Mannose binding lectin (MBL), a key protein of immune complement, binds to mannose and other sugars with equatorial 3- and 4-hydroxyls, but not to galactose, in which the 4-hydroxyl is in the axial position.[7] Our previous study demonstrated that glycopolymers of 6-*O*-acryloxy-β-mannopyranose are able to bind to MBL (depending on molecular weight and branching density), and incorporation of 6-*O*-acryloxy-galactopyranose into the polymer chain decreases the interaction with MBL.[8] Mannose and galactose were therefore chosen as the polymerizable saccharides for this study due to their well-characterized interaction (or lack thereof) with MBL. Glycopolymers with pendant mannose residues may also be of further interest as immune modulators due to their reactivity with other immune system proteins (macrophage mannose receptor,[9] dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin[10]). The mannose-based glycomonomer, 6-*O*-acryloxy-mannopyranose, was synthesized by deprotection of 6-*O*-acryloxy-1,2,3,4-tetraacetate β-mannopyranose[8] using sodium methoxide. Exposure of the sugar to the basic deprotection conditions was limited to 4 min to minimize cleavage of the acrylate group while promoting removal of the acetate protecting groups. The galactose based glycomonomer, 6-*O*-acryloxy-galactopyranose, was prepared from the galactose in three steps. The 1,2- and 3,4-hydroxyls were first protected with isopropylidene groups by stirring galactose in acetone with sulfuric acid, leaving the 6-position available for acrylation with acryloyl chloride. The isopropylidene protecting groups were then removed with acetic acid (Supporting Information (SI) Scheme S1).

Synthesis of Initiators. Bovine serum albumin (BSA) is an inexpensive model protein that has been used for previous polymer−protein conjugates. BSA exhibits a single accessible free thiol that is typically used as the polymer conjugation site using a grafting-to approach.[11] While this approach results in a single polymer chain at a known location, it also requires separation of unreacted protein as BSA typically exists as a mixture of species, only some of which contain a reactive free thiol.[12] Protein−polymer conjugates have also been formed using a grafting-from approach, for example, by modifying a single lysine residue to act as a polymerization initiating site.[13] In order to achieve 3D presentation of glycopolymers across the surface of the globular BSA, we transformed multiple lysine residues on BSA into initiating sites through reaction with 2-bromopropionic *N*-hydroxysuccinimidyl ester (Figure 1a).

While this approach sacrifices some uniformity of the resulting polymer−protein conjugates, it allows us to grow multiple polymer chains from one central protein structure and explore the effect of 3D presentation of glycopolymer chains on lectin interaction. It also more closely mimics the natural presentation of glycoresidues in nature, as many proteins are glycosylated in multiple locations. MALDI-TOF confirmed conjugation of the initiator group with disappearance of the original BSA peak at 66.4 kDa and appearance of a peak centered at 69.4 kDa, which corresponds to an average of 22 initiating sites per BSA.

Glycopolymerizations. Aqueous atom transfer radical polymerization (ATRP) of glycomonomers from the protein macroinitiator alone was unsuccessful. Sacrificial initiators, which have been used for surface polymerizations[14] as well as protein polymerizations,[11b] are necessary to generate sufficient concentration of deactivator (Cu(II)) and maintain control over the polymerization. A resin bound sacrificial initiator, synthesized by reaction of 2-bromopropionyl bromide with Wang resin (Figure 1b), was used to allow facile separation after polymerization. Mannose and galactose glycomonomers were successfully homopolymerized separately or copolymerized together from the BSA macroinitiator in the presence of this resin-bound sacrificial initiator via aqueous ATRP.

Polymerization was initiated by transferring a degassed solution of CuBr/PMDETA and monomer via cannula into a degassed flask containing resin bound sacrificial initiator (Figure 2a). After stirring the solution for 10 min (to generate sufficient Cu(II)), a degassed solution of protein macroinitiator and monomer was transferred via cannula. The solution was stirred for 4 h before quenching the polymerization. Complete conversion occurred, as no monomer was detected at the end

DOI: 10.1021/acs.bioconjchem.5b00140
Bioconjugate Chem. 2015, 26, 1504−1512

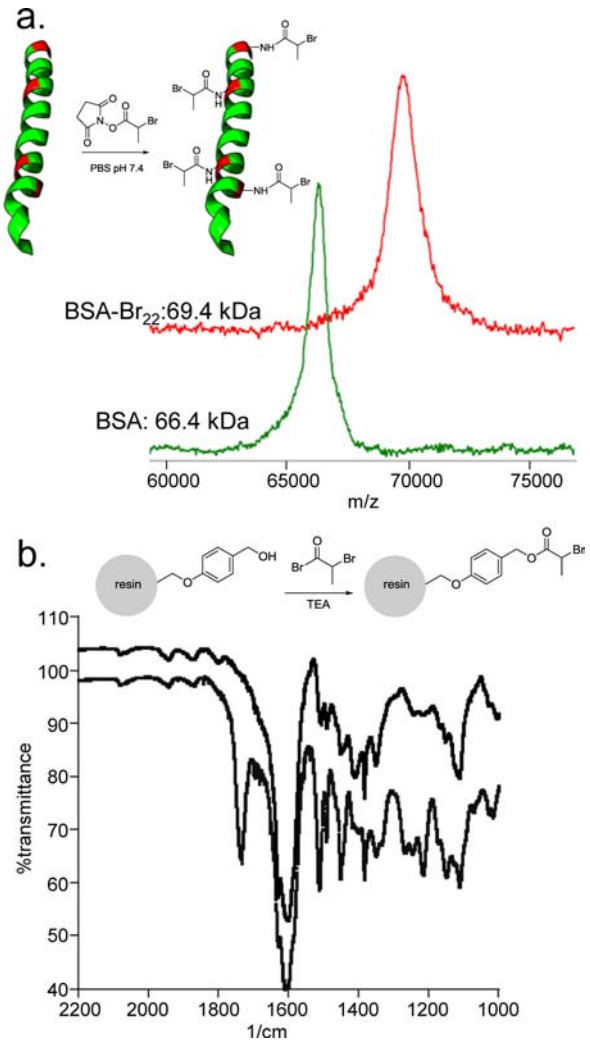


Figure 1. Synthesis and characterization of initiators necessary for glycopolymerizations from a protein macroinitiator. (a) Scheme (protein fragment shown here) and MALDI-TOF MS of BSA-macroinitiator (with peak at 69.4 kDa) versus BSA (with peak at 66.4 kDa). (b) Scheme and IR of resin sacrificial initiator (with ester peak at 1730 cm^{-1}, lower) versus Wang resin (upper).

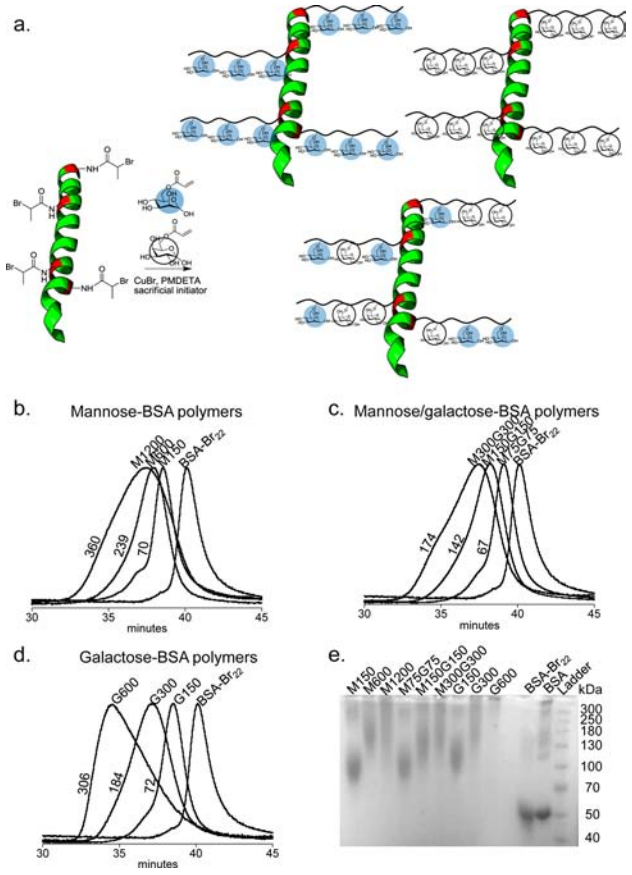


Figure 2. Synthesis and characterization of glycopolymer–protein from bovine serum albumin macroinitiator. (a) Glycopolymerizations of mannose and/or galactose acrylate from BSA macroinitiator in the presence of sacrificial initiator to form homopolymers or copolymers. (b) GPC traces of mannose-BSA polymers, (c) mannose/galactose-BSA polymers, (d) galactose-BSA polymers, and (e) SDS-PAGE of all glycopolymers and starting material proteins. The number of saccharide units per protein is displayed along the side of each corresponding GPC curve.

Table 1. Properties of BSA-Glycopolymers

entry	[mannose]:[galactose]:[Resin-Br]:[BSA-Br_{22}]:[CuBr]:[PMDETA]	peak elution time (min)	# saccharides/protein
M1200	1200:0:3:1:30:30	37.4	360 ± 35
M600	600:0:3:1:30:30	38.0	239 ± 21
M150	150:0:3:1:30:30	38.6	70 ± 19
MG300300	300:300:3:1:30:30	37.5	174 ± 17
MG150150	150:150:3:1:30:30	38.2	142 ± 13
MG7575	75:75:3:1:30:30	39.1	67 ± 3
G600	0:600:3:1:30:30	34.6	306 ± 76
G300	0:300:3:1:30:30	37.1	184 ± 43
G150	0:150:3:1:30:30	38.5	72 ± 4

of the polymerization. The glycomonomer to initiator feed ratio was systematically varied (M1200, M600, M150, G600, G300, G150) to generate samples to allow us to elucidate the effect of glycopolymer length on MBL binding. Mannose and galactose were also copolymerized (MG300300, MG150150, MG7575) to generate samples to allow us to elucidate the effect of mannose density on MBL binding (Table 1).

Protein–Glycopolymer Purification. All previously described glycopolymer–protein conjugates have been synthesized through the grafting-to route, where the glycopolymer component is conjugated to the protein post-polymerization. In these cases, post-conjugation purification is required to remove unreacted polymer or protein. Purification can be a relatively simple process if differences in affinity, molecular weight,[15] or solubility[11b] can be exploited to isolate the polymer–protein conjugate. In other cases, unreacted polymer and protein must be removed through affinity, ion-exchange, or size exclusion chromatography.[3d,16] In contrast to the costly chromatography techniques required in these previous examples, the grafting-from approach that we used allows for facile purification through dialysis or ultrafiltration to remove low-molecular-weight monomer and polymerization byproducts. Purification of our protein–glycopolymer conjugates is quite simple. After quenching, the resin bound polymer is removed through centrifugation. The supernatant of the initial centrifugation is then placed in an Amicon ultrafiltration device and centrifuged again to concentrate the glycopolymer–protein and remove any compounds with molecular weight below 3000 Da

DOI: 10.1021/acs.bioconjchem.5b00140
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(unreacted monomer, copper). No further purification techniques such as chromatography are necessary.

Protein−Glycopolymer Characterization. It is more difficult to characterize the glycopolymer chains in protein−polymer conjugates prepared using the grafting-from approach, compared to a grafting-to approach, in which the polymer chain can be characterized independently of the protein. However, the facile synthesis and purification of the glycopolymer−protein conjugates via the direct polymerization/grafting-from approach outweighs this limitation. Structural information about the glycopolymer chains can be obtained from a combination of techniques to examine the intact conjugate, as well as its degradation products.

The glycopolymer−protein samples were characterized with aqueous GPC and SDS-PAGE (Figure 2b-d, Table 1). GPC curves show decreasing elution time and increasing curve width as the ratio of monomer to initiator group is increased. These results suggest that molecular weight and polydispersity of the glycopolymer−protein conjugtates increase with the ratio of monomer to initiator group, although no absolute molecular weight values or polydispersities can be assigned due to the lack of comparable calibration standards. SDS-PAGE shows similar results with a decrease in migration distance and larger bands with increasing monomer to initiator group ratio. The large bands and presumably high polydispersity can be due to the variable number and/or length of polymer chains growing from each protein macroinitiator, but have also been attributed to increased friction between the glycopolymer−protein conjugate and the acrylamide gel.[17] It is also important to note that SDS-PAGE shows full conversion of the BSA macroinitiator in all polymerizations so that no separation of unreacted protein is required.

The number of saccharides per protein was determined with bicinchoninic acid assay and phenol-sulfuric acid assay[18] (Table 1). The glycopolymers contain from 70 to 360 saccharides. This number is higher than expected based on monomer to initiator group ratios, indicating that the resin bound initiator had lower initiation efficiency than the protein macroinitiator or that the resin bound chains terminated prematurely. Saccharide content of the copolymers was assumed to be a random distribution of mannose and galactose residues due to the similar structure and presumably equal reactivity of the two monomers.

The characterization methods described thus far do not provide any information on the average length of a glycopolymer chain. If we assume that the glycopolymers grew equally from all 22 initiating sites of the protein macroinitiator, each polymer chain statistically would have degrees of polymerization from 3 to 16. To investigate this assumption, we characterized the glycopolymer conjugates with FT-IR (SI Figure S1), ^{1}H (SI Figure S2) and ^{13}C NMR, BSA esterase (SI Figure S3), and tryptic digest assays to attempt to identify any unreacted initiator groups. If a significant amount of unreacted initiator group was found, the polymer chains would be much longer than the average calculated from assuming equal reactivity of all initiating sites. Of these assays, only the tryptic digest experiments provided significant information on the reactivity of various initiating sites. The other assays are summarized in Supporting Information.

Tryptic digests have been used previously with polymer−protein conjugates to discover the site of initiator conjugation.[11b,13] Trypsin cleaves proteins into peptides following lysines or, to a lesser extent, arginines. Modification of the lysines or arginines hinders cleavage by trypsin and results in a different digestion pattern. Our BSA-macroinitiator contains, on average, 22 initiating sites, so identifying the specific modified amino acids would be extraordinarily complex. For the samples in this report, tryptic digest can provide some information indicating whether all initiating sites are active and whether the polymer chains can sterically hinder trypsin activity.

BSA, BSA-Br_{22}, G600, G300, and G150 were cleaved with trypsin, and the resulting peptides were characterized by MALDI-TOF MS (SI Figure S4). The predominant peptide fragment after digestion of BSA is the peak at $[M + H]^+ = 1569$ which corresponds to peptide sequence DAFLGSFLYEYSR. This peak was present in all glycopolymer−protein samples as well, and was therefore used to normalize the results. Digestion of BSA-Br_{22} and the galactose conjugates resulted in peptide fragments at 1732 and 1776 m/z that were present across all the modified samples. Conjugation of the initiator to an amine results in a mass increase of 135 Da. This increase matches exactly with the peptide sequence HPEYAVSVLLRLAK with a modified lysine ($M = 1596 + 135 = 1731$, $[M + H]^+ = 1732$). A disodium adduct results in the peak at 1776, $[M + 2Na - H]^+$. Identification of a peptide fragment that contains an uninitiated bromide shows that at least one initiating site was not active in the polymerization.

The tryptic digest results also provide more information on the steric nature of the glycopolymer chains. As the polymer length increases, the ratio of peak height of 1569 m/z and 1732 m/z decreases (SI Figure S4f). The polymer chains sterically hinder trypsin from cleaving the peptide fragment at 1732 m/z (as well as 1776 m/z). The HPEYAVSVLLRLAK sequence is flanked by lysines 12 and 15 residues away (Lys346 and Lys386). While there is no information on whether there are initiating groups or polymer chains at those amino acids, it is a reasonable assumption that one or both of those sites have polymers that are large enough to disrupt trypsin from interacting with the protein, resulting in the decrease in tryptic activity as polymer length increases.

The poly(acrylic acid) backbones of the glycopolymer were also characterized by hydrolyzing the glycopolymer−protein conjugates with 6 M hydrochloric acid to cleave all amide and ester bonds, resulting in the polymer−protein being degraded to its component amino acids, saccharides, and poly(acrylic acid) backbones. If polymerization was initiated from just a few sites, the polymer chain would be long. Conversely, if most of the initiating sites were active, each polymer chain would be short.

GPC of the hydrolyzed protein samples, shown in SI Figure S5, separates the poly(acrylic acid) backbone from the amino acids and saccharides. If the polymer only grows from a few initiator sites and therefore is high molecular weight, a polymer peak that elutes at a much earlier time would be expected. Instead, the GPC traces showed the elution of presumably poly(acrylic acid) chains followed very closely by the hydrolyzed amino acids and saccharides. This elution behavior is consistent with shorter polymer chains grown from most initiation sites.

When coupled with the tryptic digest experiments, these results indicate that while the protein macroinitiators are not initiated at every site, sufficient polymer chains are grown to result in relatively short chains. While quantifying the polymer length would be ideal, efforts to do so were unsuccessful. Poly(acrylic acid) standards were not available to measure the

DOI: 10.1021/acs.bioconjchem.5b00140
Bioconjugate Chem. 2015, 26, 1504−1512

molecular weight through GPC; additionally, GPC alone can not discern poly(acrylic acid) from peptide fragments, regardless of the calibration standard used. Efforts to quantify the molecular weight via MALDI were complicated by the high concentration of other compounds, combined with the expected small molecular weight of the polymers. Furthermore, because MALDI-TOF MS reports a mass to charge ratio, analysis of ionized polymers such as poly(acrylic acid) can be challenging. Nevertheless, the tryptic digest and acid hydrolysis results imply that most of the initiating sites were active. Although we were unable to absolutely determine the exact number of repeat units in each glycopolymer chain, based on the overall number of saccharide units per BSA initiator, the results of the tryptic digest, and the lack of any detectible high molecular weight material after acid hydrolysis, we estimate the chain lengths to be relatively short.

Interaction with Mannose Binding Lectin. Previously, we reported the effect of chain length, branching, and incorporation of a saccharide unit in the backbone on the interaction between mannose-based glycopolymers and mannose binding lectin (MBL), the protein that activates the MBL pathway of complement. Briefly, avidity of poly(6-*O*-acryloxy-mannopyranose) for MBL increases with increasing molecular weight but plateaus after about 40 repeat units (no significant difference was observed between DP = 42 and DP = 75). The avidity also increases with increasing branching density, and is higher for branched polymers that incorporate mannose in the branching repeat unit compared to those that incorporate either galactose, or no saccharide residue.[8] Here, we tested the glycopolymer–protein conjugates for their ability to interact with MBL via direct enzyme linked lectin assay (ELLA) and using an inhibitory ELLA as described previously[8] and compare their avidity to mannose-containing glycopolymers.

Direct ELLA. Direct ELLAs quantify the ability of an adsorbed polymer to bind lectin in solution. Our previous study demonstrated that a linear mannose-based glycopolymer binds significantly less MBL than mannan (direct ELLA normalized to repeat unit of mannose; $DP_{glycopolymer}$ = 42 binds less than 40% MBL compared to mannan, SI Figure S6). That is, glycopolymer alone does not fully recapture the lectin-binding properties of naturally occurring mannan.

Bovine serum albumin (BSA), the BSA macroinitiator, and a BSA-galactose polymer with 184 galactoses all had negligible binding to recombinant human MBL (rMBL) when compared to the polysaccharide mannan, as seen in Figure 3a. When mannose content was incorporated into the glycoprotein conjugate, we observed similar binding as seen with the positive control, mannan, with the greatest interaction seen with the largest polymers, M1200 and M600 with 360 and 239 mannoses, respectively (Figure 3b). Decreasing mannose content to 70 with M150 resulted in a statistically significant drop in interaction. MG300300, a mannose/galactose copolymer with 174 total saccharides (87 mannoses assuming even split of mannose and galactose), was not significantly different from M150. These results suggest that the total number of reactive saccharides is the controlling factor in rMBL binding rather than their distribution, as MG300300 has roughly the same number of reactive mannoses as M150, but they are distributed randomly in a statistical copolymer with galactose.

As shown in Figure 3b, all glycopolymer–protein conjugates exhibit activity at least 70% of that of mannan, much higher than the glycopolymer alone. Haddleton reported the single-site conjugation of a linear mannose-based glycopolymer with

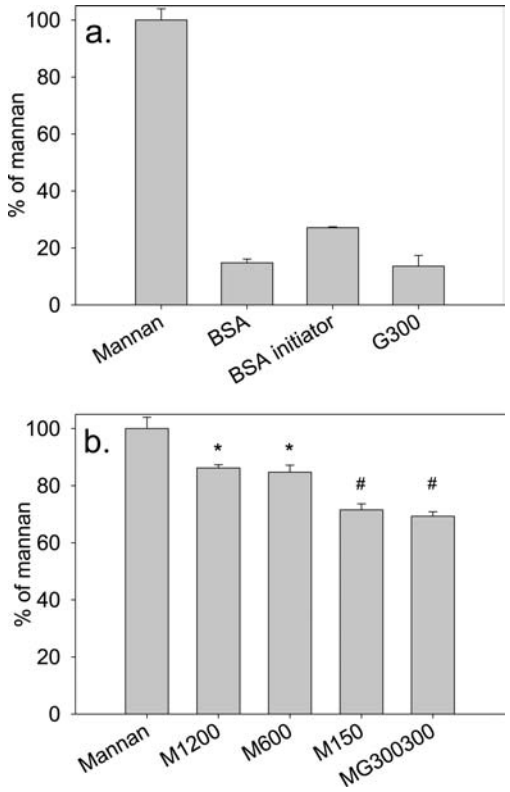

Figure 3. Direct enzyme linked lectin assay of protein glycopolymers show that (a) BSA, BSA initiator, and BSA-galactose have negligible binding with rMBL and (b) increasing mannose content increases binding. * Denotes statistically significant difference between groups with a * symbol and groups without a * symbol. # denotes statistically significant difference between groups with a # symbol and groups without a # symbol.

BSA, and observed complement activation activity that is about 50% of native mannan.[16a] Although this assay measures a different outcome (complement activation) than the direct ELLA (which measures MBL interaction), it indicates that a single linear chain of mannose-based glycopolymer is not sufficient to match the native activity of mannan, whereas the glycopolymer–protein conjugates reported here approach the activity of native mannan.

Inhibitory ELLA. To further investigate this interaction of the glycopolymer–protein conjugates with MBL, we performed inhibitory ELLA, in which the glycopolymer–proteins competitively inhibit rMBL from binding with mannan. Higher inhibition of rMBL binding from mannan can be interpreted as increased interaction of the glycopolymer–protein with rMBL. The inhibition results were normalized to the concentration of saccharide. Data sets were considered statistically significant if their plotted 95% confidence bands did not overlap[19] (SI Figure S7).

As expected, unmodified BSA and BSA-macroinitiator exhibit no inhibitory effect on rMBL binding with mannan and BSA-galactose glycopolymer exhibits only minimal inhibition with an inhibition plateau at 10% (SI Figure S8). These results demonstrate that the protein structure alone has no effect on rMBL binding and the assay is specific for mannose containing structures.

While a BSA-mannose glycopolymer that bound MBL and activated complement has been previously synthesized through a grafting-to approach of a *single* polymer chain to the free thiol

DOI: 10.1021/acs.bioconjchem.5b00140
Bioconjugate Chem. 2015, 26, 1504–1512

of BSA,[16a] no other structures were reported, so structure–property relationships have not been established. Systematic variation of the monomer to initiator group ratio and of the mannose:galactose feed ratio allows us to produce samples to investigate the effects of glycochain length and binding residue density (glycocomposition) in conjunction with 3D presentation on the interaction between glycopolymers and lectins.

Effect of Chain Length. Glycopolymer–BSA conjugates with higher mannose content exhibit better inhibition of binding of MBL to mannan than glycopolymer–BSA conjugates with lower mannose content. For example, M600 (239 mannoses per protein) has a relative potency six times higher than M150 (70 mannoses per protein) when comparing IC_{50} values (Figure 4a, Table 2). The increase in potency from M150 to M600 is consistent with the cluster glycoside effect, where multivalent structures of saccharides show enhancement of activity compared to a corresponding amount of monosaccharide.[20] However, when mannose content is increased with M1200 (360 mannoses per protein), there was no statistically significant difference when comparing the IC_{50} of M1200 with M150 or M600. This shows that at sufficiently high number of mannose residues, the cluster glycoside effect plateaus or even decreases possibly due to steric hindrance as mannose content increases. We have previously observed a similar plateau using mannose glycopolymers without protein content.[8] The inhibition behavior of M150 may also be near the lower boundary of the cluster glycoside effect for this glycopolymer–protein system, as M150s individual polymer chains are, on average, quite short (three repeat units per chain if polymer grows from all initiating sites).

Effect of Binding Residue Density. We decreased the density of pendant mannoses by copolymerizing nonreactive galactose derived monomers. After normalizing for the amount of binding residues (mannoses), M150 (70 mannoses per protein) exhibits a relative potency seven times higher than MG300300 (174 saccharides per protein, 87 mannoses per protein) despite having roughly the same number of mannose saccharides (Figure 4a, Table 2). This decrease in inhibition can be attributed to the decreased density of binding mannose units. These results are similar to those seen by Kiessling and co-workers when they copolymerized mannose and galactose in varying ratios and tested binding versus Concanavalin A (Con A), a mannose specific lectin.[1] Polymers with higher binding epitope (mannose) density also had higher affinity for Con A. It is important to note that this relationship between binding epitope density and activity is specific for each epitope/lectin pair, and not a universal structure/property relationship applicable to the interaction of any glycopolymer with any lectin. In another study by Kiick and co-workers, cholera toxin was inhibited most effectively by polymers with the lowest galactose density.[21] The authors hypothesized that for polymers with low galactose density, the distance between galactose epitopes increases and could approximately reflect the spacing between binding sites on the toxin.

Effect of 3D Presentation. We examined the effect of multivalent 3D presentation of the glycopolymer chains from the protein compared to glycopolymers with no protein attachment. Inhibition of rMBL binding by a mannose glycopolymer with 75 pendant mannose residues was 260 times weaker than inhibition by M150 with 70 mannoses per protein (Figure 4b, Table 2). These results are even more striking when considering that the average degree of polymerization for M150 is 3 repeat units (if all 22 initiation sites are reactive). In comparison, inhibition curves by a linear glycopolymer with 14 repeat units were no different than monosaccharide mannose. These multivalent 3D presentation results add a new dimension to the traditional view of the cluster glycoside effect, as the presence of multiple short mannose chains attached to one protein clearly results in increased interaction between the glycopolymer and MBL as compared to individual longer polymer chain. That is, a linear glycopolymer with 75 repeat units exhibits an IC_{50} value three orders of magnitude higher than a BSA-glycopolymer conjugate with a similar number of mannose residues (70) distributed as

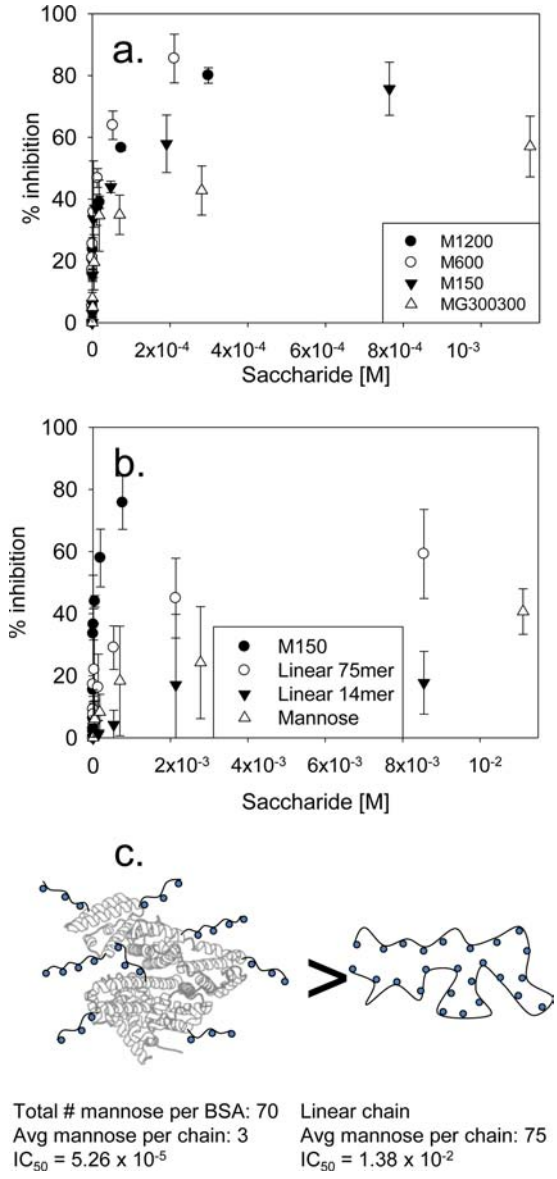

Figure 4. Binding assays show rMBL inhibition increases with mannose content and 3D presentation. (a) Inhibition of rMBL increases with increasing mannose polymer chain length (M150 vs M600) and decreases as galactose content is added (MG300300 vs M150). (b) M150, a BSA-mannose glycopolymer with 70 mannoses arranged around the protein with 3D presentation, inhibits rMBL better than a linear glycopolymer with 75 repeat units. A linear glycopolymer with 14 repeat units has similar inhibition as monomeric mannose (IC_{50} reported as M saccharide). (c) Idealized structures of the polymer–protein conjugate compared to the linear polymer.

DOI: 10.1021/acs.bioconjchem.5b00140
Bioconjugate Chem. 2015, 26, 1504–1512

Table 2. Summary of MBL Inhibition Data

entry	# mannose/ protein	# mannose/ chain	IC_{50}[c] (M saccharide)
M1200	360	16	2.35×10^{-05}[a,b]
M600	239	11	8.43×10^{-06}[a]
M150	70	3	5.26×10^{-05}[b]
MG300300	87	4	3.48×10^{-04}
Linear 75mer	N/A	75	1.38×10^{-02}
Linear 14mer	N/A	14	N/A
Mannose	N/A	1	N/A

[a]Difference between entries M1200 versus M600 not statistically significant. [b]Difference between entries M1200 versus M150 not statistically significant. [c]All other comparisons were statistically significant.

short chains over the surface (higher IC_{50} indicates weaker interaction).

Kiessling also synthesized a protein with 3D presentation of individual saccharides through modification of up to ten amino acids, each with a single mannose.[1] The saccharide modified protein inhibited Con A binding 38 times better than αMeMan. In comparison, a 12-mer linear mannose polymer synthesized through ring-opening metathesis polymerization had greater inhibition with 1000 times more binding than αMeMan. Compared to Kiessling's results, we found the magnitude of enhancement of interaction due to 3D presentation to be much larger.

Our system specifically combines multiple glycoside residues with 3D presentation, while Kiessling utilized a large aggregate decorated with a limited number of residues (Figure 5). Another group linked a streptavidin−oligosaccharide conjugate to biotinylated BSA resulting in 11 streptavidins and 140 oligosaccharides bound to BSA on average.[22] This conjugate, while synthesized through a different method, also attaches multiple saccharide chains to a central protein structure (Figure 5).

In binding tests with Chinese hamster ovary cells, the BSA-streptavidin-oligosaccharide conjugate bound 42 times better than individual streptavidin−oligosaccharide structures, and 3500 times better than free oligosaccharides. While these results are not directly comparable to our results due to differences between the assays, they do reinforce the importance of both multivalent structures and 3D presentation of polymer chains to promote lectin binding.

Finn's group grafted glucose-containing glycopolymer chains ($M_n \sim 13\,000$) to the surface of cowpea mosaic virus,[23] and observed strong interaction of these particles with Con A, which is qualitatively consistent with our results. Compared to Finn's results, we found that short chains were sufficient to dramatically increase the interaction with the binding lectin, compared to the much longer chains used in their study.

We hypothesize that the basis behind the binding enhancement seen with 3D structures is due to the close proximity of multiple polymer chains from the same central protein component. This allows rMBL to more easily span and bind multiple polymer chains with a resulting increase in avidity. The 3D presentation of polymer chains also mirrors the multivalent display of multiple carbohydrate recognition domains on lectins. Further work is still required to elucidate the mechanistic basis behind these enhancements.

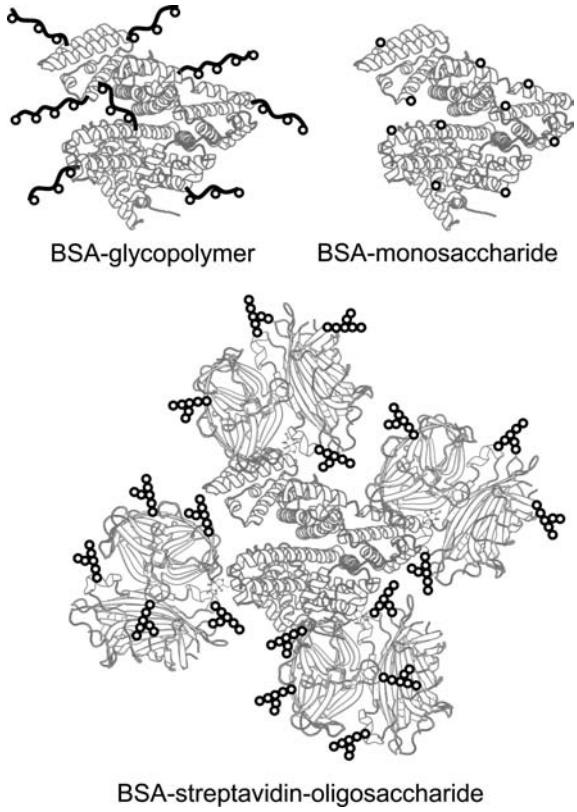

Figure 5. Our BSA−glycopolymer conjugate displays multivalent saccharides in 3D while the BSA−monosaccharide conjugate[5] displays single saccharides in 3D. The BSA-streptavidin-oligosaccharide conjugate[22] displays oligosaccharides from streptavidin.

■ CONCLUSIONS

We report a bovine serum albumin−glycopolymer (mannose and/or galactose) conjugate synthesized through the grafting-from approach. The bovine serum albumin provided a convenient platform to generate 3D structures of saccharide chains. The conjugates were tested for their ability to interact with mannose binding lectin of immune complement. Increasing number and density of mannose were found to result in more interaction with MBL, and 3D presentation of multiple polymer chains had superior interaction with MBL than compared to linear glycopolymer chains with no protein content. These enhanced binding results using small quantities of saccharides arranged in a multivalent 3D presentation can have a profound impact in how glycopolymer conjugates are constructed, particularly when using compounds available in short supply. The combination of this saccharide presentation method with a protein component could potentially lead to biomimetic materials with therapeutic activity. Glycomimetic−protein conjugates such as the ones reported in this manuscript may have important therapeutic applications as mimics of natural glycoproteins, for example, in vaccines.

■ EXPERIMENTAL SECTION

Example Polymerization. Glycopolymer−protein conjugates were synthesized as described in the following example. Resin sacrificial initiator (3.5 mg, 3.5 μmol Br) was added to reaction flask 1. CuBr (5.1 mg, 35 μmol), PMDETA (7.3 μL, 35 μmol), 6-*O*-acryloxy-mannopyranose (21 mg, 89 μmol), 6-*O*-acryloxy-galactopyranose (21 mg, 89 μmol), and water (826

DOI: 10.1021/acs.bioconjchem.5b00140
Bioconjugate Chem. 2015, 26, 1504−1512

μL) were added to reaction flask 2. BSA macroinitiator solution (8.72 mL, 1.2 μmol), 6-*O*-acryloxy-mannopyranose (62 mg, 265 μmol), and 6-*O*-acryloxy-galactopyranose (62 mg, 265 μmol) were added to reaction flask 3. The three flasks were degassed via freeze−pump−thaw. The contents of flask 2 were added to flask 1 via cannula and the solution stirred. After 10 min, the contents of flask 3 were added to flask 1 via cannula and the solution stirred. After 4 h, the polymerization was stopped by opening the flask to atmosphere. The resin sacrificial initiator was removed through centrifugation. The supernatant of that centrifugation was then centrifuged through an Amicon ultrafiltration device with 3000 Da cutoff. Saturated ammonium chloride solution was added to help solubilize and remove copper. Two rounds of centrifugation with saturated ammonium chloride followed by two rounds of centrifugation with water were performed to collect a viscous liquid that was later characterized by bicinchoninic acid assay and phenol-sulfuric acid assay to determine the protein and saccharide concentration.

Direct Enzyme Linked Lectin Assay. 100 μL of 10 μg/mL polymer of interest (glycoprotein conjugate or mannan) in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$) was incubated at room temperature overnight in immunosorp wells. The solution was removed and replaced with blocking buffer (5 mg/mL BSA, 10 mM TrisCl, 145 mM NaCl) for 2 h at 37 °C. Recombinant human mannose binding lectin (rMBL) diluted 1:2000 (final concentration 5 μg/mL) was added to the wells and incubated overnight at 4 °C. The wells were then washed three times with washing buffer and then 100 μL of mouse anti-rMBL diluted 1:5000 in washing buffer was added to each well and incubated for 2 h at room temperature. Wells were washed again, then 100 μL of HRP goat anti-mouse diluted 1:1500 in washing buffer was added to each well and incubated for 2 h at room temperature. Following a final wash, 100 μL of TMB was added to each well and developed for 25 min before stopping with 2 M sulfuric acid and read at 450 nm on a plate reader.

Inhibitory Enzyme Linked Lectin Assay. 100 μL of 10 μg/mL mannan in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$) was incubated at room temperature overnight in immunosorp wells. The solution was removed and replaced with blocking buffer (5 mg/mL BSA, 10 mM TrisCl, 145 mM NaCl) for 2 h at 37 °C. While wells were blocking, the inhibitor (glycopolymer of interest) was dissolved in lectin binding buffer (20 mM TrisCl, 1 M NaCl, 0.05% Triton X-100, 10 mM $CaCl_2$, 1 mg/mL BSA) to 2 mg/mL, and then serially diluted. An equal volume of rMBL, also in lectin binding buffer, was added to result in overall lectin concentration of 5 μg/mL and concentrations of inhibitor from 1 mg/mL to 0.008 mg/mL. For the no-inhibitor solutions, a 10 μg/mL solution of rMBL was diluted with an equal volume of lectin binding buffer. Inhibitor and serum were preincubated for 30 min at room temperature. The wells were washed three times with washing buffer (TBS + 0.05% Tween 20 and 5 mM $CaCl_2$) and then 100 μL of preincubated inhibitor and rMBL were added and incubated overnight at 4 °C. Wells were washed with washing buffer and then 100 μL of mouse anti-rMBL diluted 1:5000 in washing buffer was added to each well and incubated for 2 h at room temperature. Wells were washed again and then 100 μL HRP goat anti-mouse diluted 1:1500 in washing buffer was added to each well and incubated for 2 h at room temperature. Following a final wash, 100 μL of TMB was added to each well and developed for 25 min before stopping development with 2 M sulfuric acid and reading the plate at 450 nm. % inhibition was calculated as $100 - 100\%Abs_{inhibitor}/Abs_{noinhibitor}$. Sample analysis accounted for the different saccharide concentrations of each compound.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplementary methods, figures, and additional characterization of glycopolymers. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00140.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: akasko@ucla.edu.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This material is based on work supported by the National Science Foundation under CHE-1112490. K.L. was supported by a fellowship from the NIH Biotechnology Training Program, T32GM067555. We gratefully acknowledge H.D. Maynard for informative discussions and consultations on this project.

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DOI: 10.1021/acs.bioconjchem.5b00140
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