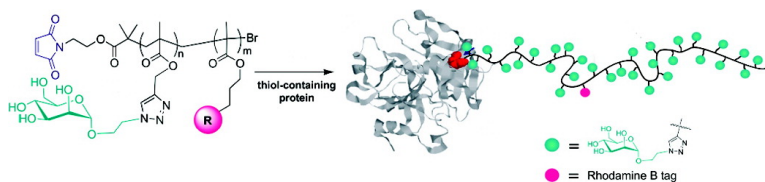Article

Site-Directed Conjugation of "Clicked" Glycopolymers To Form Glycoprotein Mimics: Binding to Mammalian Lectin and Induction of Immunological Function

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Site-Directed Conjugation of "Clicked" Glycopolymers To Form Glycoprotein Mimics: Binding to Mammalian Lectin and Induction of Immunological Function

Jin Geng,[†] Giuseppe Mantovani,*,[†] Lei Tao,[†] Julien Nicolas,[†,⊥] Gaojian Chen,[†]
Russell Wallis,[§] Daniel A. Mitchell,[‡] Benjamin R. G. Johnson,[‖]
Stephen D. Evans,[‖] and David M. Haddleton*,[†]

*Contribution from the Department of Chemistry, University of Warwick, CV4 7AL, Coventry,
United Kingdom; Clinical Sciences Research Institute, Warwick Medical School, University of
Warwick, CV2 2DX, Coventry, United Kingdom; Department of Infection, Immunity and
Inflammation, University of Leicester, Leicester, United Kingdom; and School of Physics and
Astronomy, University of Leeds, Leeds, United Kingdom*

Received April 29, 2007; E-mail: D.M.Haddleton@warwick.ac.uk

Abstract: Synthesis of well-defined neoglycopolymer−protein biohybrid materials and a preliminary study focused on their ability of binding mammalian lectins and inducing immunological function is reported. Crucial intermediates for their preparation are well-defined maleimide-terminated neoglycopolymers ($M_n = 8-30$ kDa; $M_w/M_n = 1.20-1.28$) presenting multiple copies of mannose epitope units, obtained by combination of transition-metal-mediated living radical polymerization (TMM LRP) and Huisgen [2+3] cycloaddition. Bovine serum albumin (BSA) was employed as single thiol-containing model protein, and the resulting bioconjugates were purified following two independent protocols and characterized by circular dichroism (CD) spectroscopy, SDS PAGE, and SEC HPLC. The versatility of the synthetic strategy presented in this work was demonstrated by preparing a small library of conjugating glycopolymers that only differ from each other for their relative epitope density were prepared by coclicking of appropriate mixtures of mannopyranoside and galactopyranoside azides to the same polyalkyne scaffold intermediate. Surface plasmon resonance binding studies carried out using recombinant rat mannose-binding lectin (MBL) showed clear and dose-dependent MBL binding to glycopolymer-conjugated BSA. In addition, enzyme-linked immunosorbent assay (ELISA) revealed that the neoglycopolymer−protein materials described in this work possess significantly enhanced capacity to activate complement via the lectin pathway when compared with native unmodified BSA.

Introduction

The post-genome era is one of the most challenging and exciting periods in the history of chemical and biological research. The study of oligosaccharides and lectins in the context of whole organisms—functional glycomics—is one of the most difficult yet crucial fields of post-genome analysis, with impact upon many biochemical and physiological processes such as pathogen−host interactions, cell adhesion, tissue integrity and homeostasis, and cell signaling.[1−7] For example, recognition of microbial mannose-rich glycans by lectins of the immune system mediates important host defense mechanisms such as pathogen neutralization and phagocytosis.[8] A major obstacle to rapid progress in the field of glycobiology and functional glycomics is the availability of purified oligosaccharides in sufficient quantities for inclusion in biological and biophysical analysis strategies.

Synthetic neoglycopolymers having multiple copies of monodentate sugar moieties are a class of macromolecular displays that have shown very promising results when employed as natural oligosaccharide mimics.[9−13] The ability of some of these materials to specifically recognize lectins and cell surfaces has

[†] Department of Chemistry, University of Warwick.
[‡] Warwick Medical School, University of Warwick.
[§] University of Leicester.
[‖] University of Leeds.
[⊥] Present address: Laboratoire de Physico-Chimie, Pharmacotechnie et Biopharmacie, UMR CNRS 8612, Univ Paris-Sud, 92296 Chatenay-Malabry Cedex, France.

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been the subject of extensive research, and now that the development of novel therapeutic strategies can be based on multivalent interactions a better understanding of these factors is required.[13−16]

A number of different strategies have been employed for the synthesis of the required multivalent carbohydrate ligands. Kiessling and co-workers have shown that polymers obtained by ring-opening metathesis polymerization (ROMP) can efficiently interact with both lectins[17−28] and cells.[29−34] Controlled radical polymerization techniques, in particular transition-metal-mediated living radical polymerization (TMM LRP, often called ATRP)[35−38] and radical addition−fragmentation chain-transfer (RAFT) polymerization,[39−42] have emerged as leading strategies to tailor-made neoglycopolymers with great control over a number of macromolecular features that include polymer architecture, chain length, and molecular weight distributions. One of the main advantages in using these techniques includes the high functional-group tolerance, which allows unprotected sugar monomers and polar protic and nonprotic solvents such as water and DMSO.[12] Cyanoxyl-mediated radical polymerization has also been successfully employed by Chaikof and co-

workers[43−50] in a range of applications that include the preparation of glycocalyx-mimetic surfaces[50] to the synthesis of lactose-sulfate-based synthetic heparin analogues potentially employable in areas related to therapeutic angiogenesis.[48] The synthesis of well-defined synthetic glycopolymers by TMM LRP[51−55] and RAFT polymerization[56−60] and their use in a number of specific applications have been described, and the number of reports on this field is now rapidly growing.

Recently,[28] we reported the synthesis of mannose- and/or galactose-containing neoglycopolymers by combination of TMM LRP and Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition,[61,62] a "click chemistry"[63] process. Our approach involved the grafting of sugar azides onto a polyalkyne "clickable" scaffold prepared by TMM LRP. Several azide-containing molecules could be simultaneously grafted in different proportions onto the same clickable scaffold, yielding libraries of different copolymers in a process that we named a "coclicking" approach.

The ability of neoglycopolymers to interact with lectins and cells is strongly dependent on their size, shape, structure, and binding epitope density (defined as "the mole fraction of specific binding epitopes incorporated into a multivalent backbone"[26]).[3,13−15] For ligand comparative studies it is therefore of importance to have a set of synthetic tools that allows for the synthesis of libraries of polymers having identical macromolecular features, with the relative density of epitope binding units being the only variable. Postpolymerization processes appear to be the only way for achieving this goal as long as extremely efficient grafting processes are available. In this respect, use of "clickable" polymers is very promising,[64] especially since a recent report by Munro and co-workers showed that some *N*-hydroxysuccinimide ester polymers, polymeric scaffolds often em-

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Scheme 1. Synthesis of the Glycoprotein Mimic Employed in This Study

Scheme 2 [a]

Path A

Path B
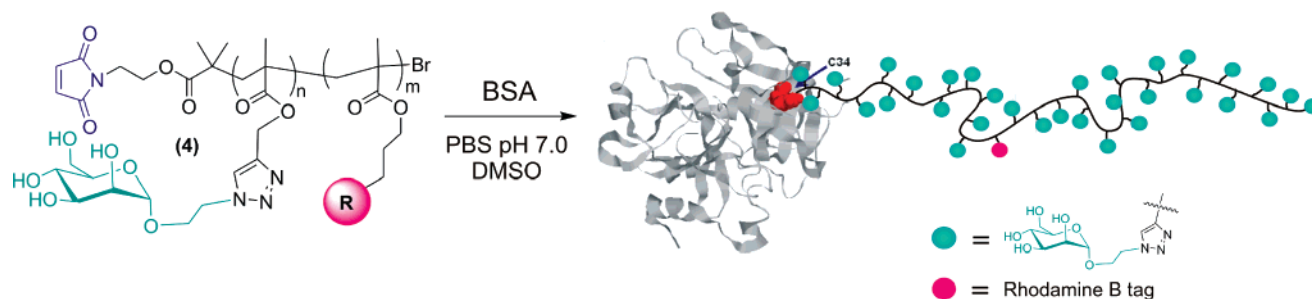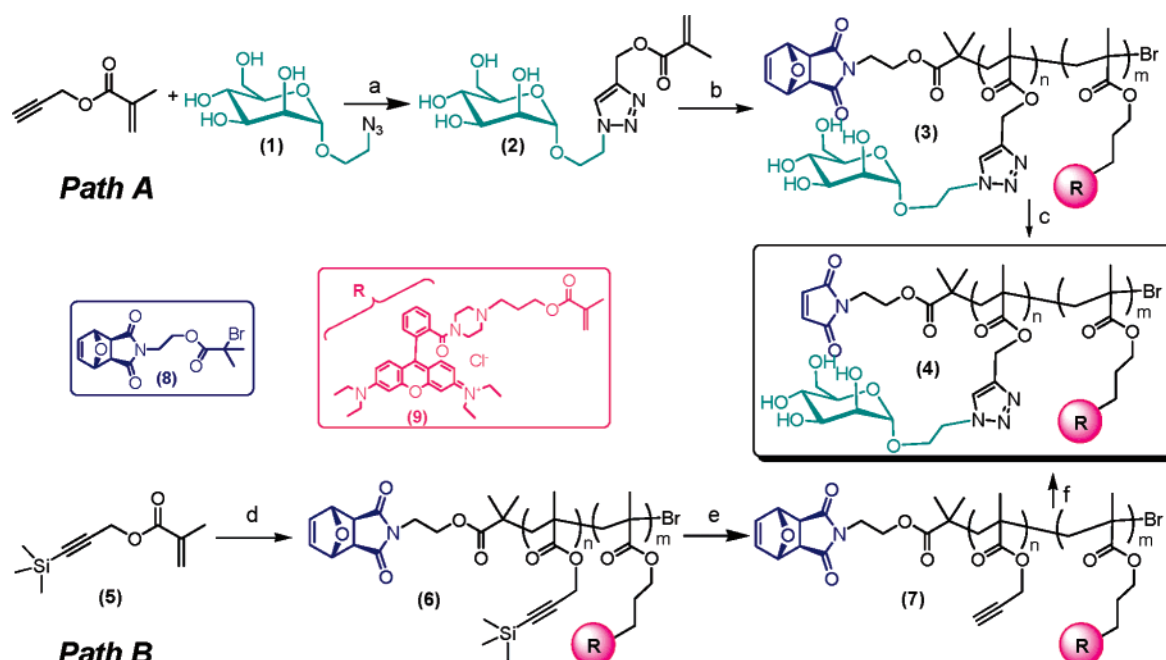
[a] Reagents and conditions: (a) CuSO$_4$, sodium ascorbate, methanol/H$_2$O (1:1 v/v), ambient temperature; (b) N-(ethyl)-2-pyridylmethanimide, Cu(I) Br, rhodamine B methacrylate **9**, initiator **8**, CH$_3$OH/H$_2$O 5:2 (v/v), ambient temperature; (c) toluene reflux, 18 h; (d) N-(ethyl)-2-pyridylmethanimide, Cu(I) Br, initiator **8**, rhodamine B methacrylate **9**, toluene, 30 °C; (e) TBAF·3H$_2$O, THF, 0 °C to ambient temperature, 12 h; (f) (i) **1**, (PPh$_3$)$_3$CuBr, Et$_3$N, DMSO, ambient temperature, 2 days; (ii) toluene reflux, 18 h.

ployed for polymer multiple functionalization, can undergo important side reactions during the final conjugation step that could potentially limit their applicability for the synthesis of multivalent therapeutics.[65]

Alternatively, controlled radical polymerization techniques allow for simple synthesis of well-defined glycopolymers by simply polymerization of appropriate sugar monomers. The main advantage of this approach is its simplicity, and the two synthetic strategies, postfunctionalization and direct polymerization of carbohydrate monomers, can now be regarded as complementary tools for the preparation of neoglycopolymers.

Another key advantage in using TMM LRP is that α-functional polymers can be easily prepared by simply choosing appropriate polymerization initiators; this has been exploited in the past for the synthesis of biohybrid materials by (poly)-peptide−polymer conjugation.[46,66−70]

In the present work we aimed to modify our "click" approach to glycopolymers for the synthesis of α-mannopyranoside-containing glycoprotein mimics suitable for probing interactions with mannose-binding lectin (MBL), a key mammalian lectin of the immune system. Mannoside structures are prevalent on the cell surfaces of many bacterial and fungal pathogens, their arrangements distinct from human mannoside glycans, thus being distinguishable from the human host. MBL is able to recognize these non-self-arrangements of mannoside residues and through binding in this way to pathogen surfaces is able to trigger the complement cascade, a powerful arm of the circulating innate immune system that subsequently opsonises the pathogens for phagocytosis as well as driving cellular pore formation that leads to pathogen cell destruction.

Essential polymeric starting materials are novel maleimide-terminated glycopolymers able to selectively react with the free-cysteine (Cys34) residue of bovine serum albumin (BSA)—an abundant model protein molecule.

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Free cysteine residues are rare in native polypeptides, and when present, they constitute an excellent attachment site for protein modification as they are present in very discrete number (typically one) and their location is often identifiable. When necessary, the protein of interest can be genetically modified in order to obtain free cysteine residues in specific positions of the protein surface. Development of protein—glycopolymer conjugates carries potential for the generation of therapeutic agents and biological probes. Through site-directed conjugate formation, defined biological molecules with novel dual properties can be formed very easily. Protein components can potentially confer functions such as highly defined ligand binding, enzyme activity, and sources of specific amino acid sequences in tertiary structural motifs or in the case of immunological antigen presentation as digested peptides loaded onto major histocompatibility complex molecules. Glycopolymers can confer selective lectin binding and thus engagement with the counter-glycome in plasma, cells, and tissues. Mannose polymers are of particular interest due to the fact that a number of mammalian lectins associated with immune function have been shown to bind mannose-containing structures expressed on the surface of pathogens such as Gram-positive and Gram-negative bacteria, fungal pathogens such as *Candida albicans*, and lethal viruses such as HIV, Ebola, and Hepatitis C.[71–74] In this study we focused upon modifying BSA, a protein that is inert with regard to the innate immune system, via conjugation of mannose glycopolymer in order to investigate binding to MBL, an acute phase C-type lectin of the immune system that triggers activation of the complement cascade—the fundamental and archetypal fluid-phase innate immune effector mechanism present in plasma, cerebrospinal fluid, and tissue mucosa.[75,76]

Results and Discussion

Synthesis of the α-Maleimide Neoglycopolymers. The required maleimide-terminated neoglycopolymers were prepared following two independent synthetic pathways (Scheme 2). Visibly fluorescent tag based on rhodamine B dye was introduced onto the polymers backbone in order to facilitate characterization of the relative protein conjugates and improve their traceability during the in vitro tests carried out in the present study.

In the first protocol (Path A) the mannose-containing monomer **2** was readily prepared by Huisgen 1,3-dipolar cycloaddition of the mannose azide **1** and propargyl methacrylate using $(PPh_3)_3CuBr$ as the catalyst. Polymerization of **2** in the presence of the maleimide-protected initiator **8** and fluorescent rhodamine B comonomer **9**,[77] using iminopyridine/Cu(I) Br as the catalytic system, afforded the macromolecular intermediate **3**. This was then suspended in refluxing toluene for 18 h in order to remove the furan protecting group by retro-Diels—Alder reaction, leading to the maleimide-terminated neoglycopolymer **4**. Alternatively, we found that the latter reaction occurred even

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Table 1. Polymers Prepared in This Study

polymer	DP (NMR)	M_n (NMR) (kDa)	M_w/M_n (SEC)
3a	70	26.1	1.20[c]
3b	20	7.5	1.22[c]
4a[a]	74	27.6	1.20[d]
4b[a]	22	8.2	1.23[d]
6	41	8.0	1.22[c]
7	40	5.0	1.25[c]
4c[b]	44	16.4	1.28[d]

[a] Prepared via path A. [b] Prepared via path B. [c] Determined by SEC using $CHCl_3/Et_3N$ 95:5 as the mobile phase. [d] Determined by aqueous SEC.

Table 2. Maleimide-Terminated Polymers Featuring Different Mannose Density Prepared in This Study

polymer	% mannose	% galactose	DP (NMR)	M_n (NMR) (kDa)	M_w/M_n(SEC)[a]
23	100	0	139	52.3	1.26
24	75	25	141	53.1	1.25
25	50	50	139	52.3	1.25
26	25	75	136	51.1	1.26
27	0	100	138	51.9	1.25

[a] Obtained by SEC analysis using DMF as the mobile phase and RI detection.
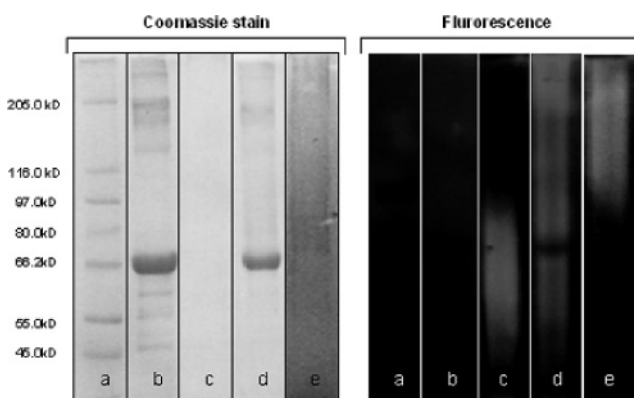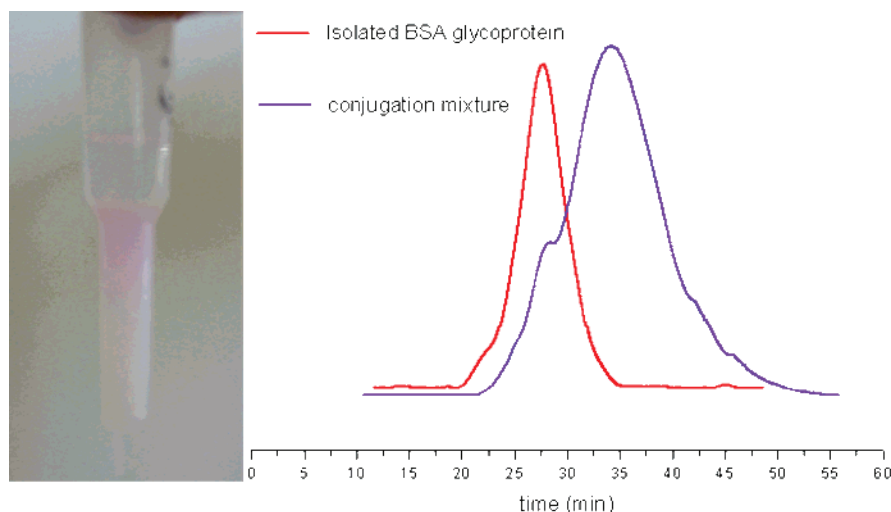

Figure 1. SDS-PAGE [Coomassie staining (left) and UV excitation at λ = 302 nm (right)] for the conjugation reaction of BSA with **4a**: (a) MW markers; (b) native BSA; (c) glycopolymer **4a**; (d) BSA with 10 equiv of **4a**: conjugation mixture; (e) **10** purified by SEC followed by affinity chromatography.

in the solid state by simply leaving a powder sample of **3** in a vacuum oven at 80 °C overnight, yielding directly pure **4** and avoiding use of organic solvents and the need for a further final precipitation step.

The second synthetic strategy relies on the ability of poly-(propargyl methacrylate) to act as a versatile "clickable" polymeric scaffold able to efficiently react with sugar azides (Path B). The first step involves polymerization of the trimethylsilyl-protected propargyl methacrylate **5** in toluene, using iminopyridine/Cu(I) Br as the catalyst, in the presence of the initiator **8**. Removal of the trimethylsilyl groups with TBAF in THF followed by clicking of the α-mannopyranoside azide **1** in DMSO and retro-Diels—Alder deprotection of the maleimide moiety afforded the expected maleimide-terminated mannose-containing polymer **4**.

In the polymerization steps the first-order kinetic plots showed some deviation from linearity, yet the linear increase of M_n with monomer conversion and the narrow molecular weight distributions indicate that polymerization occurred in a controlled fashion (see Supporting Information).

Both synthetic approaches developed led to the desired maleimide-terminated polymers (Tables 1 and 2). Particular

Figure 2. BSA + maleimide glycopolymer **4a** (10 equiv). (Left) Purification of conjugate **10** through an immobilized Concanavalin A column before eluting with 1.0 M mannose solution. (Right) SEC analysis of the conjugation reaction mixture (purple) and the final purified product **10**. UV detection (λ = 280 nm) was employed.

Scheme 3. Synthesis of the Glycoprotein Mimic **10** Obtained from Neoglycopolymer **4a**
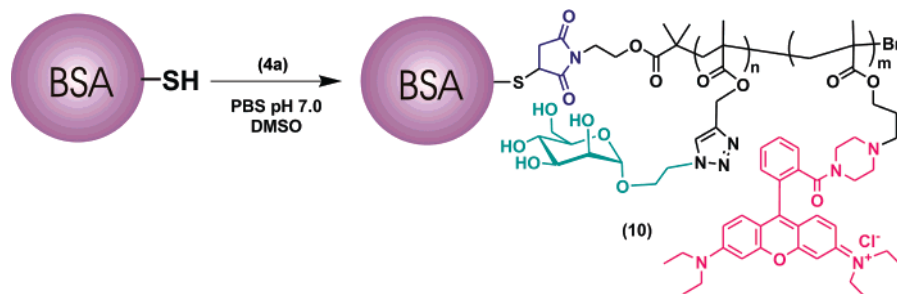
effort was spent in order to further simplify the synthetic protocol presented in this study. For example, mannose azide building block **1** was originally prepared following a well-established four-step procedure (see Supporting Information).[78] Subsequently, we employed an alternative two-step procedure starting from unprotected D-mannose by first reacting the sugar with 2-bromoethanol at 90 °C in the presence of Amberlite IR-120 acid catalyst and then converting the resulting α-2′-bromoethyl-D-mannopyranoside into the desired mannose azide **1** with sodium azide in refluxing water/acetone mixture.[79]

Synthesis of BSA Glycoprotein Mimics. Bovine serum albumin (BSA) is a commercially available 66 kDa protein that is often chosen as a model single-thiol-containing substrate. Its free Cys34 residue is generally regarded as the attachment site of choice for BSA site-specific conjugation. In the past both we[68] and Maynard[67] have shown that BSA can be conjugated with functional polymers obtained by controlled radical polymerization bearing chain ends able to react selectively with free thiol-containing (poly)peptides. In addition, BSA had been employed as a protein carrier for the chemoenzymatic synthesis of *N*-glycan-containing neoglycoproteins,[80] and it appeared, therefore, to be a suitable substrate to be employed in our study.

(78) Kleinert, M.; Roeckendorf, N.; Lindhorst, T. K. *Eur. J. Org. Chem.* **2004**, *18*, 3931−3940.

(79) To the best of our knowledge, two examples of direct conversion of D-mannose into its corresponding 2′-bromoethyl-glycoside have been previously reported: Smith, E. A.; Thomas, W. D.; Kiessling, L. L.; Corn, R. M. *J. Am. Chem. Soc.* **2003**, *125* (20), 6140−6148 (acid catalyst, camphorsulfonic acid). Mukhopadhyay, B.; Maurer, S. V.; Rudolph, N.; Van Well, R. M.; Russell, D. A.; Field, R. A. *J. Org. Chem.* **2005**, *70* (22), 9059−9062 (catalyst, HClO₄ immobilized on silica)

In the present work, glycoconjugate **10** was obtained by reaction of maleimide-terminated display **4a** with BSA in 1:1 DMSO:PBS (50 mM, pH 7.0) (Scheme 3).

Several reaction conditions and purification protocols were explored for the conjugation reaction. In particular, an excess of both glycopolymer **4a** and BSA protein, 10:1 and 1:10 mol/mol, respectively, was successfully employed.

In the former case, the excess of unreacted maleimide polymer **4a** was removed by repeated SEC purifications, while unreacted BSA and BSA dimer were separated by affinity chromatography using an agarose-immobilized Concanavalin A (Con A) column. Con A is a 26 kDa lectin (normally forming dimers or tetramers depending on the pH) extracted from Jack Beans that specifically recognize α-mannopyranoside sugar moieties. When the mixture obtained from SEC purification was passed through the affinity chromatography column, only the bioconjugate **10** was able to selectively interact with the Con A while the unreacted BSA and BSA dimer were rapidly eluted (Figure 1). **10** was then released from the column by simply washing the latter with a 1.0 M solution of D-mannose, a monovalent competitive ligand for Con A.

This protocol allowed for isolation of the pure **10** biohybrid material as confirmed by SDS-PAGE and SEC analysis (Figure 1). It was evident from SDS-PAGE analysis of the crude product that some unreacted BSA was still present at the end of the conjugation reaction. This result agreed well with previous

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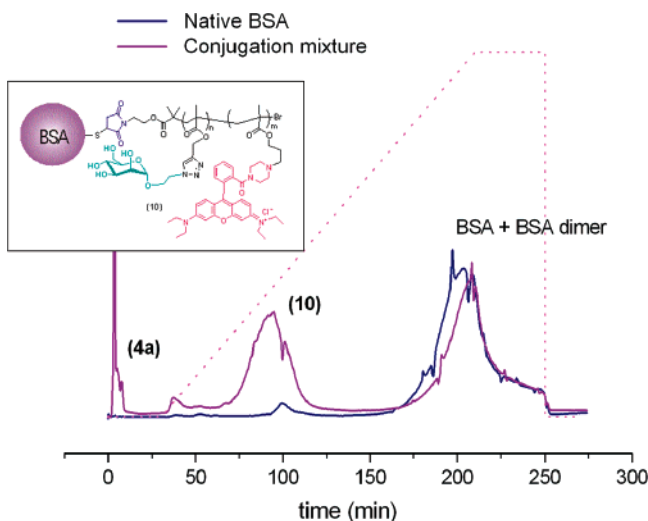
Figure 3. BSA (10 equiv) + maleimide glycopolymer **4a**. FPLC anion-exchange chromatography purification of the conjugate **10** using Source Q column in 20 mM TRIS buffer at pH 9.0 as the mobile phase. A gradient of NaCl (pink dotted line) was used in order to elute the protein-based products.

reports that indicated that only 36−69% of the BSA molecules present in commercially available samples have the free cysteine thiol required for protein conjugation.[68,81−83] SDS-PAGE analysis also showed the absence of both BSA and BSA dimer in the final purified sample. SEC chromatography analysis of the purified conjugate **10** confirmed the presence of a fluorescent product with a molecular mass higher than BSA, in full agreement with the SDS-PAGE results (Figure 2).

The ability of **10** to interact strongly with the Con A lectin during affinity chromatography was in itself a preliminary direct evidence of the ability of this biohybrid material to act as a multivalent ligand for lectin recognition.

Ion-exchange chromatography was used for the case in which a 1:10 glycopolymer **4a**:BSA molar ratio was employed in an attempt to simplify the purification process of the glycoprotein mimic **10**. Optimum results were obtained by anion-exchange chromatography using a Source Q column in 20 mM TRIS buffer at pH 9.0. Under these experimental conditions the unreacted maleimide-terminated polymer **4a** was not retained, while **10** showed a retention time between that of free polymer and BSA and BSA dimer (Figure 3). SEC analysis of the conjugate **10** isolated using this method confirmed that the two purification protocols developed were equivalent in terms of purity of the desired glycoprotein mimic **10** (see Supporting Information), although use of ion-exchange chromatography appeared to be more suitable for large-scale purification processes. The two purification processes are suitable for purification of crude mixtures obtained from both conjugation protocols with the SEC + affinity chromatography protocol that appeared to be less indicated for the case in which an excess of conjugating glycopolymer was employed due to the rather tedious SEC purification of the excess of unreacted glycopolymer from the polymer−protein conjugate **10**.

Glycoprotein Mimics with Different Mannose Binding Unit Density. Recognition of lectin receptors is dependent on

(81) Ellman, G. L. *Arch. Biochem. Biophys.* **1959**, *82* (1), 70−77.
(82) Grassetti, D. R.; Murray, J. F., Jr. *Arch. Biochem. Biophys.* **1967**, *119*, 41−49.
(83) Riener, C. K.; Kada, G.; Gruber, H. J. *Anal. Bioanal. Chem.* **2002**, *373* (4−5), 266−276.
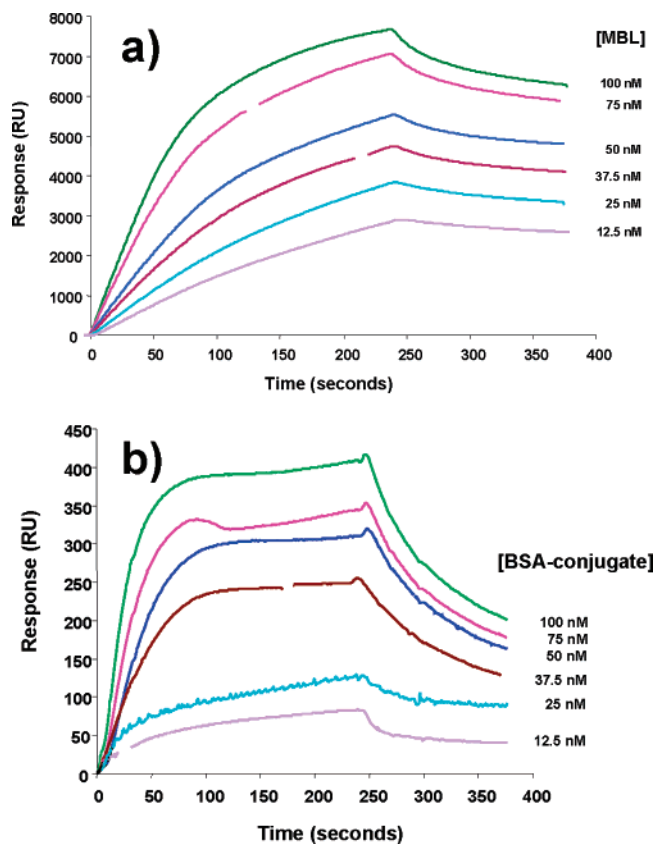
Figure 4. Surface plasmon resonance analysis of immobilized BSA−glycopolymer conjugate **10** with fluid phase mannose binding lectin (MBL) (a), and immobilized MBL with fluid phase BSA−glycopolymer conjugate (b). Due to the naturally heterogeneous nature of MBL oligomers, concentrations of fluid-phase MBL on immobilized BSA−conjugate are indicated assuming a molecular weight of 75 kD. (b) An average molecular weight of 100 kDa is assumed for indications of fluid-phase BSA−conjugate.

the length of the polydentate ligand counterpart[21] as well as the density of the binding epitopes present in the neoglyco-polymer.[26,28] This makes the development of efficient and reliable strategies for the synthesis of polyvalent ligands bearing multiple binding epitopes of particular importance.

Once the necessary synthetic pathways and purification processes for the glycoconjugates were developed, a library of conjugating neoglycopolymers differing from each other for the relative density of mannose epitopes was prepared following a coclicking approach[28] (Scheme 4) in order to further demonstrate the efficiency of the strategy presented in this work. As reported previously, libraries of glycopolymers identical in terms of macromolecular features (M_n, PDi, macromolecular architecture) can be obtain by simply introducing appropriate mixtures of different sugar azides in the reaction feed. BSA glycoconjugates **23**−**27** were then prepared using a 10:1 BSA to glycopolymer molar ratio and purified by anion-exchange chromatography.

Circular dichroism (CD) analysis of the conjugates **23**−**27** was performed in order to evaluate the influence of the conjugated glycopolymer onto the secondary structure of BSA. α-Helix and β-sheet contents were found to be 51−65% and 0−2%, respectively, values that do not significantly differ from 54% α-helix and 1% β-sheet that was found for native BSA under identical conditions.

Pseudo-esterase activity of the protein part of our conjugate was assessed using *p*-nitrophenol acetate as model ester substrate

Scheme 4 [a]
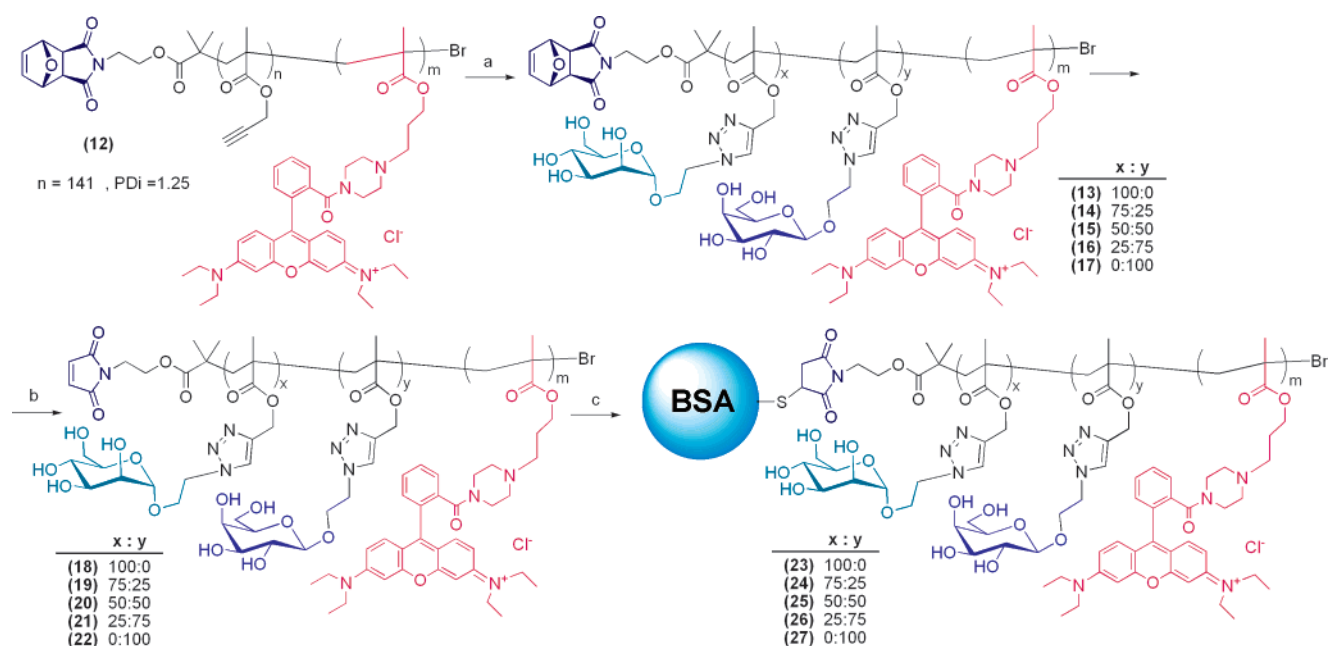
[a] Reagents and conditions: (a) α-2′-azidoethyl-D-mannopyranoside **1**/β-2′-azidoethyl-D-galactopyranoside **28**, (PPh₃)₃CuBr, Et₃N, DMSO, ambient temperature, 2 days; (b) reduced pressure, 80 °C, solid state, 12 h; (c) BSA (10 equiv), DMSO:PBS (50 mM, pH 7.0).

Scheme 5 [a]
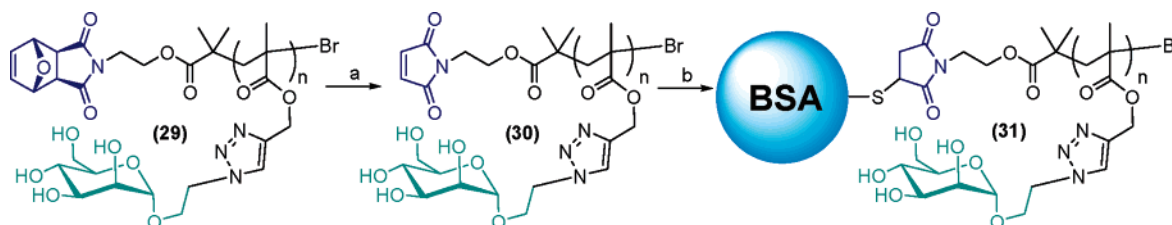
[a] Reagents and conditions: (a) toluene reflux, 18 h; (b) BSA (10 equiv) DMSO:PBS (50 mM, pH 7.0).

in 50 mM PBS, pH 8.5. Since this assay relies on the spectophotometric detection ($\lambda = 405$ nm) of the *p*-nitrophenolate ion produced by hydrolysis of the ester substrate, our rhodamine B-tagged conjugates appeared to be unsuitable for such a test due to their non-negligible absorbance at 405 nm. A non-rhodamine-tagged biohybrid material, **31**, was therefore prepared by direct polymerization of the mannose methacrylate monomer **2** in methanol/water 5:2 v/v at ambient temperature in the presence of the initiator **8** (Scheme 5). Retro-Diels–Alder removal of the furan protecting group followed by protein conjugation using an excess of BSA afforded the desired conjugate **31**.

Pseudo-esterase assay[84–86] revealed that **31** had an activity comparable with that of the BSA starting material (see Supporting Information), which further confirmed that the experimental conditions employed for both conjugation and purification of our biohybrid materials did not damage the protein part of the conjugates.

Binding studies using surface plasmon resonance (BIAcore) were carried out to examine glycopolymer–BSA conjugate interactions with recombinant rat mannose-binding lectin (MBL).

Conjugates and unmodified BSA were immobilized in equal quantities on parallel flow cells within a CM5 sensor chip and sensorgrams recorded with MBL in the fluid-phase at concentrations indicated in Figure 4. Correction for background binding was achieved through subtraction of parallel sensorgram readings from hemeoglobin-coated flow cells. Clear and dose-dependent MBL binding to glycopolymer-conjugated BSA was observed within the physiological range, which is typically 20 nM, in stark contrast to plain BSA where no signal was observed (data not shown). Although MBL exists in vitro and in vivo as a heterogeneous collection of oligomers, average kinetic values were calculated for MBL binding to immobilized polymer giving a k_{on} rate of $1.2 \pm 0.45 \times 10^5$ M^{-1} s^{-1} and a k_{off} rate of $7.3 \pm 2.3 \times 10^{-4}$ s^{-1} with a K_D of $7.9 \pm 4.5 \times 10^{-9}$. Binding of fluid-phase glycopolymer-conjugated BSA to immobilized MBL was positive but gave lower absolute signal and showed different curve characteristics with a substantially faster dissociation curve (Figure 4b). This indicates the likely effects of multiple protein–sugar contacts required to increase avidity, but we acknowledge that immobilization of MBL on the CM5 surface may substantially effect its binding properties. Binding was not observed in either case when D-mannose was introduced into the analyte at 10 mM (data not shown).

Functional analysis of the glycopolymer–BSA conjugates was performed using a protocol designed to measure comple-

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(85) Boyer, C.; Bulmus, V.; Liu, J.; Davis Thomas, P.; Stenzel Martina, H.; Barner-Kowollik, C. *J. Am. Chem. Soc.* **2007**, *129* (22), 7145–7154.
(86) Liu, J.; Bulmus, V.; Herlambang David, L.; Barner-Kowollik, C.; Stenzel Martina, H.; Davis Thomas, P. *Angew. Chem., Int. Ed.* **2007**, *46* (17), 3099–103.
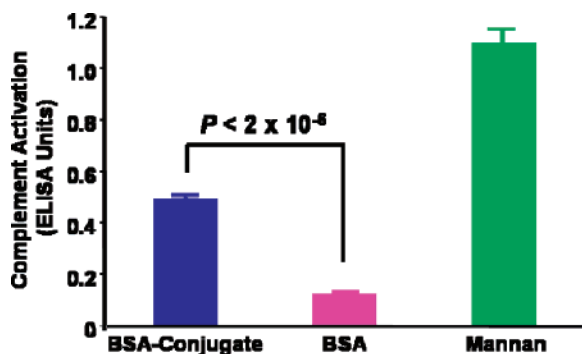
Figure 5. Detection of complement system activation. BSA−conjugate **10**, BSA, and yeast mannan were immobilized on microwell plates and exposed to diluted human serum. Values indicate the level of activated complement C9 deposition resulting from positive cascade triggering, as determined by a subsequent ELISA-style assay involving *p*-nitrophenol detection at $\lambda = 405$ nm. ELISA units obtained as direct absorbance at 405 nm values with reagent blank values deducted

ment system activation. Compared with BSA, immobilized glycopolymer−BSA conjugate showed significantly enhanced capacity to activate complement via the lectin pathway as assessed by quantitative measurement of pathway-dependent complement protein deposition by enzyme-linked immunosorbent assay (Student's *t* test, $P < 2 \times 10^{-6}$; Figure 5). Thus, incorporation of the glycopolymer-modified BSA imparts the inert protein with innate immune system interaction properties.

Conclusions

Glycoproteins are potent biological molecules active through the union of structural and chemical properties imparted by both specific protein and carbohydrate components. Neoglycopolymers represent very attractive molecules through which defined macrocarbohydrate properties can be generated rapidly and with high yield, providing a realistic alternative in many cases to scarce and prohibitively expensive natural oligosaccharides.

In this work we introduced a simple and general method for the synthesis of glycoprotein mimics. Well-defined maleimide-terminated neoglicopolymers ($M_n = 8-30$ kDa; $M_w/M_n = 1.20-1.28$) having multiple copies of D-mannose epitopes have been obtained following two independent synthetic pathways that involve a combination of copper-catalyzed living radical polymerization and alkyne−azide [2+3] cycloaddition ("click"). These coupled with model thiol-containing protein BSA and the resulting bioconjugates were first purified following two independent purification protocols and then characterized by SEC HPLC, SDS PAGE, and CD analysis. These biohybrid materials appear to be particularly intriguing as they could potentially combine features proper of their protein component, such as enzymatic activity, ligand affinity, or the ability of acting

as source of specific aminoacid sequences, with lectin binding properties, with consequent engagement with the counter-glycome in plasma, cells, and tissues, typical of its neoglyco-polymer component. The versatility of the approach was further demonstrated by preparing a library of BSA−neoglycopolymer hybrid materials featuring different relative amounts of D-mannose and D-galactose binding epitopes following a coclick-ing approach.

Surface plasmon resonance (BIAcore) tests carried out in the presence of the model mammalian lectin, recombinant rat mannose-binding lectin (MBL), revealed a significant and dose-dependent binding of the latter to the BSA−neoglycopolymer conjugates. ELISA tests also revealed that our biohybrid materials were also able to activate the complement system via the lectin pathway.

In summary, through a simple and efficient conjugation step, a non-glycosylated model protein, BSA, has been site-specifi-cally modified with a mannoside neoglycopolymer leading to a substantial addition of function with regard to engagement of a critical arm of mammalian innate immunity. This indicates the principle of how proteins can be effectively modified with neoglycopolymers in a highly defined fashion, providing a gateway for rapid, diverse, and cost-effective neoglycoprotein development−a phenomenon that could have profound value in the deconvolution and exploitation of the functional glycome in a broad range of biological species.

Note Added after ASAP Publication: There was a produc-tion error in the version published on the Internet on November 17, 2007. In the print version and in the version published on the Internet on November 19, 2007, the correct version of Scheme 4 is present.

Supporting Information Available: Experimental section, including characterization of maleimide polymers and all intermediates, bioconjugation experiments, and in vitro test procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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