Bioconjugate Chemistry

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Oligosaccharides and Peptide Displayed on an Amphiphilic Polymer Enable Solid Phase Assay of Hapten Specific Antibodies

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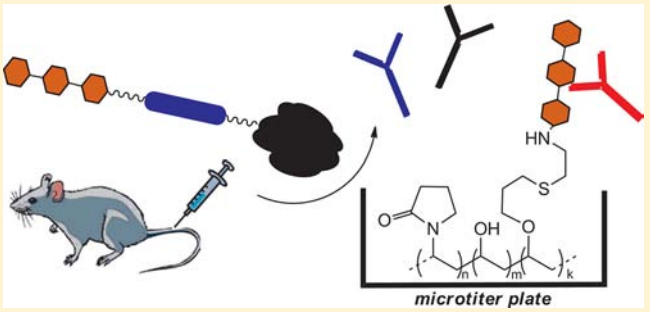
Supporting Information

ABSTRACT: Copovidone, a copolymer of vinyl acetate and *N*-vinyl-2-pyrrolidone, was synthesized via reversible addition−fragmentation chain transfer (RAFT) polymerization, and after deacetylation the polymer was functionalized by introduction of amino, azide, and alkyne pendant groups to allow attachment of glycans and peptide. *Candida albicans* β-mannan trisaccharides **1** and **2** and *M. tuberculosis* arabinan hexasaccharide **3** with appropriate tethers were conjugated to the polymers by squarate or click chemistry. *C. albicans* T-cell peptide **4** bearing a C-terminal ε-azidolysine was also conjugated to copovidone by click chemistry. The resulting conjugates provide convenient non-protein-based antigens that are readily adsorbed on ELISA plates, and display excellent characteristics for assay of antibody binding to the haptenic group of interest. Copovidone and BSA glycoconjugates exhibited similar adsorption characteristics when used to coat ELISA plates, and both conjugates were optimal when used as coating solutions at low nanogram/mL concentrations. Provided that the copovidone conjugated glycan is stable to acid, assay plates can be easily processed for reuse at least three times without detectable variation or degradation in ELISA readout.



INTRODUCTION

Conjugate vaccines constructed from the capsular polysaccharides of *Neisseria meningitidis* and *Streptococcus pneumoniae* and covalently attached to an immunogenic protein such as tetanus toxoid or diphtheria toxoid now rank as blockbuster prophylactic vaccines with annual sales exceeding several billion dollars.[1] The success of these vaccines has stimulated a broad area of research into other microbial diseases that might benefit from vaccines composed of cell wall oligosaccharides or polysaccharides conjugated to immunogenic proteins.[2−6] Therapeutic cancer vaccines containing tumor specific oligosaccharides and glycopeptides conjugated to immunogenic proteins and peptides are also under investigation in many laboratories.[7,8] Here we describe convenient chemistry that allows the antibodies produced in response to each component of a conjugate vaccine to be dissected into antibodies against the carbohydrate, the linker fragment, and peptide/protein components.

The design of conjugate vaccines ideally requires either the isolation and characterization of the cell wall polysaccharide to be coupled to protein, or identification of the minimum-sized oligosaccharide fragment of polysaccharide (the protective epitope), which when conjugated to protein is able to raise antibodies that kill bacteria,[4−6,9] or in the case of cancers, a tumor specific immune response.[7,8] The ability to employ oligosaccharide epitopes is attractive because it permits the use of chemical synthesis of relatively small oligosaccharides that are functionalized for conjugation to protein by well-defined chemistry.[3−6,9,10] The choice of protein (carrier protein) to which oligosaccharide is covalently attached is crucial because carbohydrates are nonimmunogenic, and to provide efficacious vaccines, they must first be conjugated to strongly immunogenic proteins, such as bacterial toxoids (tetanus or diphtheria toxoids[11]) or other candidates such outer membrane proteins[11] or keyhole limpet hemocyain (KLH).[7]

Measurement of antibody response following immunization with conjugate vaccines typically involves conjugation of the carbohydrate antigen to a protein that is different from that of the conjugate vaccine. This second glycoconjugate is used to coat ELISA plates and detect antibodies.[9,10] In this way the antibody response to the glycan component of the vaccine can be determined without confounding issues of antibodies that are directed to the carrier protein.

Our work on a *Candida albicans* glycoconjugate vaccine typically employed tetanus toxoid as the immunogenic protein, to which we attached oligosaccharide epitopes.[6,9,10] Antibodies to the oligosaccharide haptenic group are monitored by conjugating the same hapten to a heterologous protein such as BSA, and this conjugate is used to coat ELISA plates. The range of choices for a second protein for synthesis of coating

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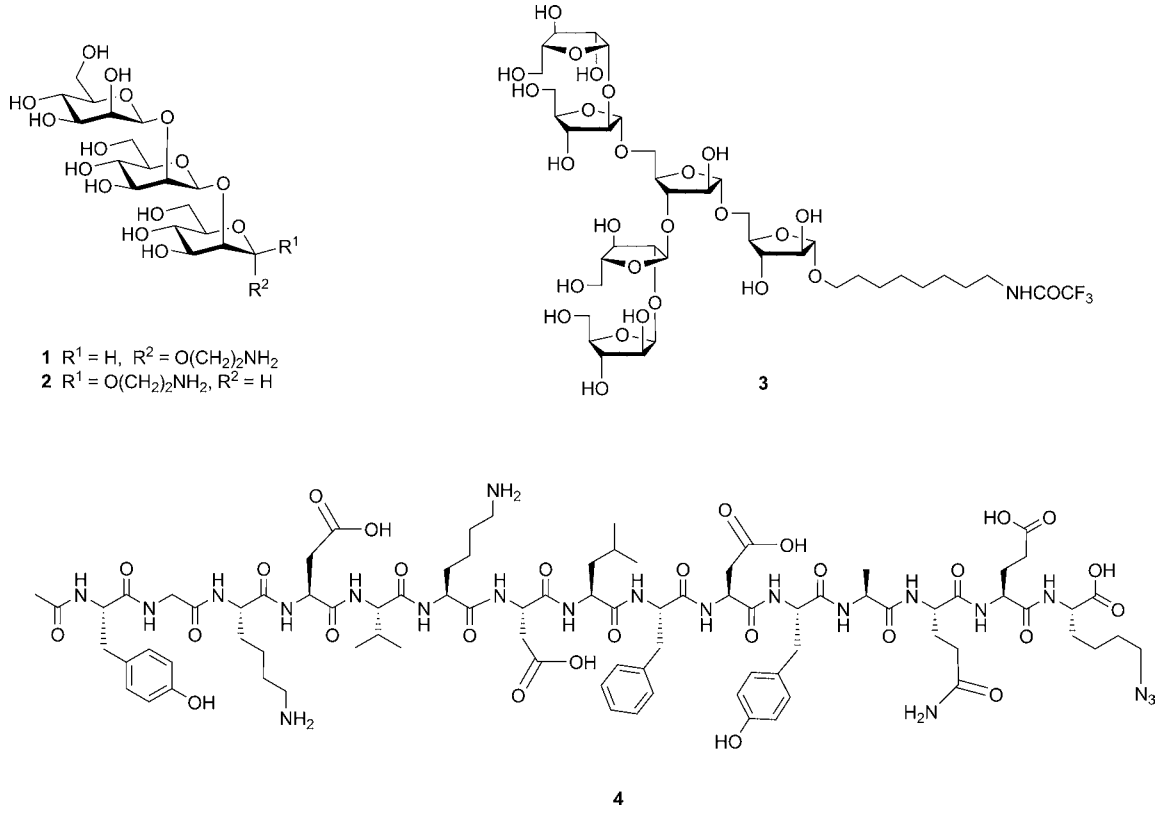

Figure 1. Oligosaccharides **1**−**3** and peptide **4** haptens for attachment to functionalized copovidone.

antigens is quite limited, because this protein should possess 10 or more sites, usually lysine residues, that permit facile conjugation of haptenic groups. The second protein should lack *N*- or *O*-glycosylation sites, exhibit good aqueous solubility before and after conjugation, and ideally the glycoconjugate should be soluble in water so that it may be freeze-dried to allow convenient preparation of coating antigens of known concentration. BSA fulfills these roles well and its conjugates readily dissolve in aqueous buffer when reconstituted after lyophilization. Use of BSA may be prohibited if antiserum contains BSA specific antibodies and especially if BSA or other serum albumin glycoconjugates are employed as carrier proteins in conjugate vaccines. These issues highlight the need for a suitable polymer that contains reactive groups for attachment of antigenic determinants while being sufficiently lipophilic to effectively coat plastic microtiter plates.

In search of a nonprotein, biocompatible polymer that would also be approved for use as a multivalent drug candidate, we identified copovidone, a widely used excipient in drug formulations.[13] It is a copolymer of vinyl acetate and *N*-vinyl-2-pyrrolidone with the attractive feature that it is compatible with a variety of chemical manipulations performed in both organic and aqueous media. These properties are retained after deacetylation and allow the introduction of side chain groups through which haptens may be readily attached.[14]

We report here the synthesis of copovidone with pendant groups terminated by amine, azide, and alkyne groups, and the conjugation of three oligosaccharides (**1**−**3**) and one peptide (**4**) to these polymer derivatives (Figure 1). Trisaccharides **1** and **2** are α and β 2-aminoethyl glycosides of a *Candida albicans* trisaccharide that has been shown to be a protective epitope present in the cell wall phosphomannan of this fungal pathogen.[15−18] The Fba peptide **4** is a T cell peptide derived from fructose-bisphosphate aldolase, a protein present in *C. albicans* during pathogenesis of human disseminated candidiasis.[19] A conjugate composed of Fba and a variant of trisaccharide **2** was shown to be an effective glycopeptide conjugate vaccine[20] and its conjugation to copovidone allows for convenient detection of peptide specific antibodies. The third oligosaccharide is a branched hexasaccharide from the cell wall of *Mycobacterium tuberculosis*, the crystal structure of which has been solved as a complex with the monoclonal antibody (mAb) CS35.[21,22]

We demonstrate here that copovidone conjugates are antigens that show excellent coating of microtiter plates at low nanogram/mL concentrations and that the ELISA data obtained with them exhibits low nonspecific binding of antibody to the copovidone coated plates. Moreover, when the oligosaccharide hapten is stable to acid solution used to stop color development, ELISA plates can be reused.

RESULTS

Reversible addition−fragmentation chain transfer polymerization (RAFT) of vinyl acetate and *N*-vinyl-2-pyrrolidone gave polymers of ∼40 kDa molecular weight. Saponification and subsequent derivatization at hydroxyl groups was used to create azido **P1** and amino **P2** substituted polymers as previously described[14] and an alkyne derivative **P3** (Figure 2).

Trisaccharide **1** was prepared by using glycosyl donor **5** according to chemistry developed by Crich.[23,24] 2-Azidoethyl α-D-mannopyranoside[25] **6** was glycosylated by **5**[23] to give disaccharide **7**, which was selectively deprotected to give acceptor **8** for a second glycosylation to afford trisaccharide **9**. Removal of the *p*-methoxybenzyl group gave **10** and a two-stage hydrogenation[26] gave the acetate salt of **1**. Reaction of **1** with dibutyl squarate gave the squarate half ester **11**, which may

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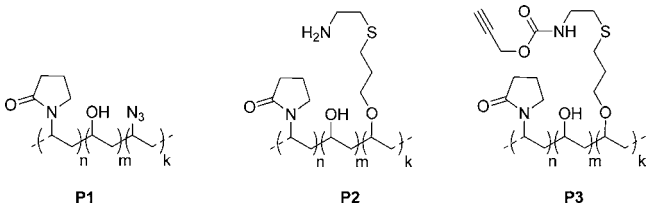


Figure 2. Functionalized copovidone with azide **P1**, amino **P2**, and alkyne **P3** pendant groups.

be used directly but here was isolated and characterized. Reaction of **11** with the aminated copovidone **P2** provided the conjugate **12** (Scheme 1).

Trisaccharide **2** was prepared in analogous fashion with the exception that the donor employed was phenyl glycosyl sulfoxide **13** (Scheme 2).[23] It was used to glycosylate 2-azidoethanol to provide **14** and the starting acceptor **15** after removal of the *p*-methoxybenzyl group. Iterative cycles of glycosylation by **13** or **5**[23] gave first the disaccharide **16** and subsequently the disaccharide acceptor **17** and finally the deprotected trisaccharide **2** obtained from **18**. Acylation of **2** by the succinimide ester of glutaric acid propargylamide[27] gave **19**. This alkyne derivative was conjugated to azide bearing copovidone **P1** by a Huisgen 1,3-dipolar copper(I)-catalyzed cycloaddition reaction[28−31] to afford the conjugate **20**. Integration of ^{1}H NMR side chain resonances permits quantification of the degree of substitution. This procedure is described in detail in a prior publication.[14]

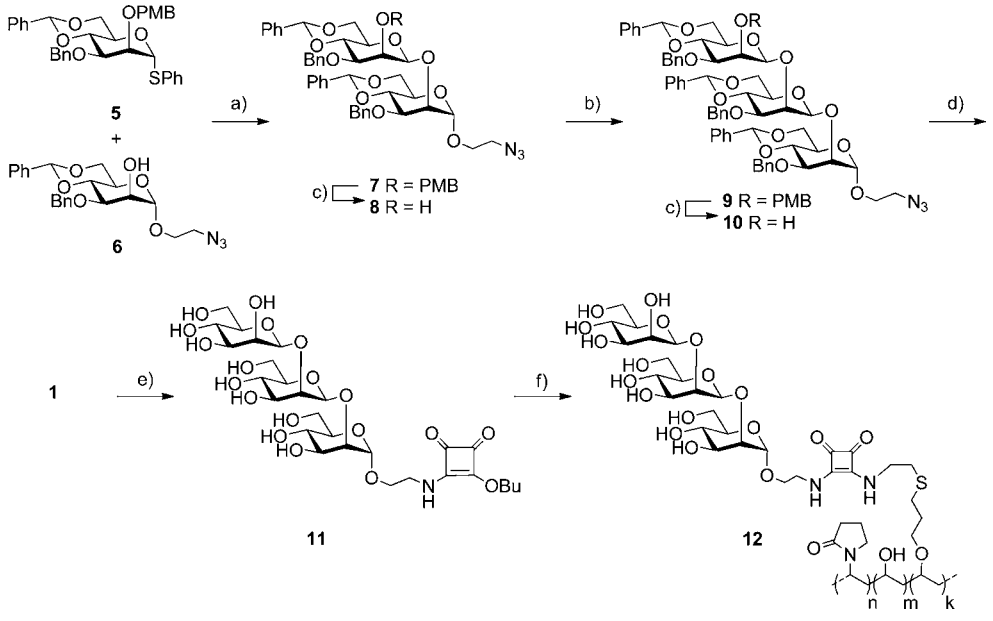
The branched Ara_6 hexasaccharide 8-trifluoroacetamido-octyl glycoside **3**[21] was treated with base to liberate the amine terminated tether, which was then acylated by the NHS ester of glutaric acid monopropargylamide[27] and the resulting compound **21** was conjugated to the azido substituted copovidone **P1** to give **22** (Scheme 3). Hexasaccharide **3** was also conjugated to BSA to give the glycoconjugate Ara_6-BSA (**23**).

Fba peptide **4** installed with an ε-azido-L-lysine residue at its C-terminus was conjugated to alkyne substituted copovidone polymer **P3** by a Huisgen 1,3-dipolar cycloaddition reaction to directly yield conjugate **24** (Scheme 4). The degree of peptide incorporation was estimated by the NMR method previously discussed[14] and also in this case by HPLC separation and integration of free and bound peptide (Supporting Information page 39).

In order to compare the properties of copovidone **22** and BSA **23** glycoconjugates, plates were coated with these Ara_6 antigens in the concentration range 10 μg/mL − 0.1 ηg/mL and titered against serial dilutions of CS35 antibody. The results are displayed as three-dimensional plots (Figure 3a and b). The data used to create the graphs are tabulated as Supporting Information Tables S1 and S2). The coating efficiency of the conjugate is most clearly shown by viewing two-dimensional slices showing absorbance versus antigen coating at constant antibody concentration (Figure 3c). Comparison of the coating efficiency for the two conjugates determined by ELISA response was similar across the concentration range and clearly demonstrates that each conjugate has an optimal coating concentration of approximately 100−10 ηg/mL (Figure 3c).

The general utility of povidone conjugates was established with specific mAbs and polyclonal sera as follows. Conjugates **12** and **20** were used to coat ELISA plates, and a titration assay was performed with serial dilutions of affinity purified *C. albicans* mAb C3.1.[16] C3.1 binds to both conjugates with similar affinity (Figure 4a). In other work,[18] we have shown that either α- or β-mannotriose represented by compound **1** or **2** are equally effective as inhibitors of this mAb, so the small difference observed in the two titration curves (Figure 4a) that could be interpreted as a preference for binding to the α-mannotriose conjugate **12** actually results from the higher epitope loading of this conjugate (payload 9.8% versus 6.8% for **20**). The titration curve obtained with CS35 mAb indicates that

Scheme 1[a]



[a]Reagents and conditions: (a) BSP, TTBP, Tf_2O, 4 Å MS, CH_2Cl_2; (b) Donor **5**, BSP, TTBP, Tf_2O, 4 Å MS, CH_2Cl_2; (c) DDQ, CH_2Cl_2; (d) (i) Pd/C, pyridine, H_2; (ii) $Pd/(OH)_2$, $AcOH/H_2O$, H_2; (e) dibutyl squarate, MeOH; (f) **P2**, Na_2CO_3 buffer pH 9.

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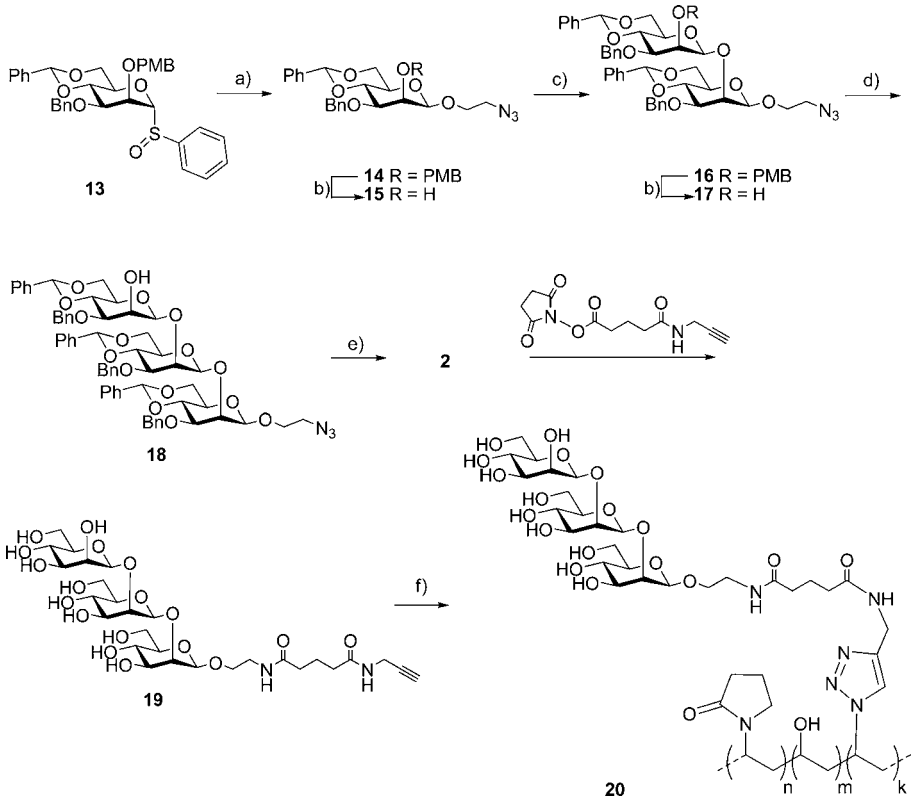
Scheme 2[a]



[a]Reagents and conditions: (a) 2-azidoethanol, TTBP, Tf_2O, 4 Å MS, CH_2Cl_2; (b) DDQ, CH_2Cl_2; (c) **13**, TTBP, Tf_2O, 4 Å MS, CH_2Cl_2; (d) (i) **5**, BSP, TTBP, Tf_2O, 4 Å MS, CH_2Cl_2; (ii) DDQ, CH_2Cl_2; (e) (i) Pd/C, pyridine, H_2; (ii) $Pd/(OH)_2$, $AcOH/H_2O$, H_2; (f) **P1**, $CuSO_4$, sodium ascorbate.

the copovidone conjugate **22** performs as well as the corresponding Ara_6-BSA glyconjugate **23** in ELISA (Figures 3c and 4b). When copovidone without pendant groups was used to coat plates, nonspecific absorption of mAb can be seen to be esentially zero across a wide range of antibody concentrations (Figure 4b).

Sera from a group of mice immunized with a glycopeptide $[Man_3\text{-}Fba]_{16}$-tetanus toxoid conjugate (unpublished results) were titered on a plate coated with the Fba-copovidone conjugate **24** (Figure 4c). On the same plate, polyclonal sera from mice immunized with alum were titered. Fba end point titers showed that the glycoconjugate raised peptide specific antibodies that titered to $\sim 10^5$. The control sera gave negligible background at dilution of 10^{-3} or higher.

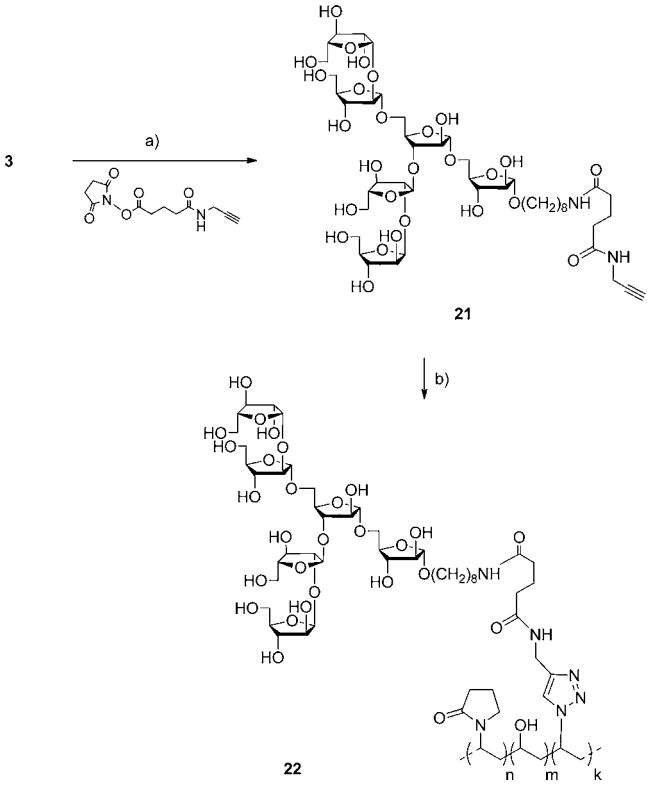
Under certain circumstances such as iterative on plate generation and screening protocols used to discover improved carbohydrate ligands for Shiga like toxins recently described by us,[32] it could be useful to repeat ELISA assays on the same plate. There have been several reports in the literature concerning reuse of ELISA plates.[33,34] In some of these methods extensive incubation with detergents and denaturing agents was necessary. We reasoned that arresting color development by addition of 1 M phosphoric acid would cause loss of bound antibody from antigen coated plates. This encouraged us to attempt reuse of plates because the developmental stage with horseradish peroxidase-conjugated second antibody employs 1 M phosphoric acid to stop color development. This is essentially an elution step for any bound protein. We observed that plates coated with copovidone conjugates **12** and **20** could be reused at least 3 times without any degradation in the reproducibility of end point titers or the magnitude of the signal at any serum or antibody dilutions (Figure 5 and Table S3 Supporting Information).

However, carbohydrate epitopes such as the arabinofuranose residues of conjugate **22** are known to be sensitive to hydrolysis dilute acid[35] and plates coated with antigen **22** show a decrease in binding capacity with repeated exposure to acid. Peptide copovidone conjugate **24** was not amenable to a recycling regime. Having established that plates coated with conjugates **12** and **20** could be recycled, we also observed that that ELISA plates coated with a mannotriose-BSA conjugate[10] prepared from **2** could also be recycled (Supporting Information, Table S4).

■ CONCLUSIONS

Originally, we selected copovidone as a biocompatible polymer to display ligands for potential application as oral therapeutics to absorb toxins such as the Shiga-like toxin of enterohemorrhagic *E. coli*.[14,32] We have shown that the polymer exhibits excellent characteristics for repeated cycles of on-plate chemistry and activity assays in the elaboration of focused libraries of toxin agonists.[32] Here we extend those observations and describe the application of copovidone as a convenient nonprotein polymer that is readily synthesized and subsequently modified with a variety of functional groups that allow for facile conjugation of saccharide and peptide haptens employing squarate and click chemistry, thereby permitting determination of epitope-specific antibody response to synthetic conjugate vaccines. The resulting conjugates display excellent characteristics in ELISA, and provided the hapten

Scheme 3[a]



[a]Reagents and conditions: (a) (i) MeOH, $NaOCH_3$; (ii) H^+ ion exchange resin; (b) **P1**, $CuSO_4$, sodium ascorbate.

groups are stable to strong acid at room temperature, the plates may be reused at least three times without degradation of response signals.

EXPERIMENTAL PROCEDURES

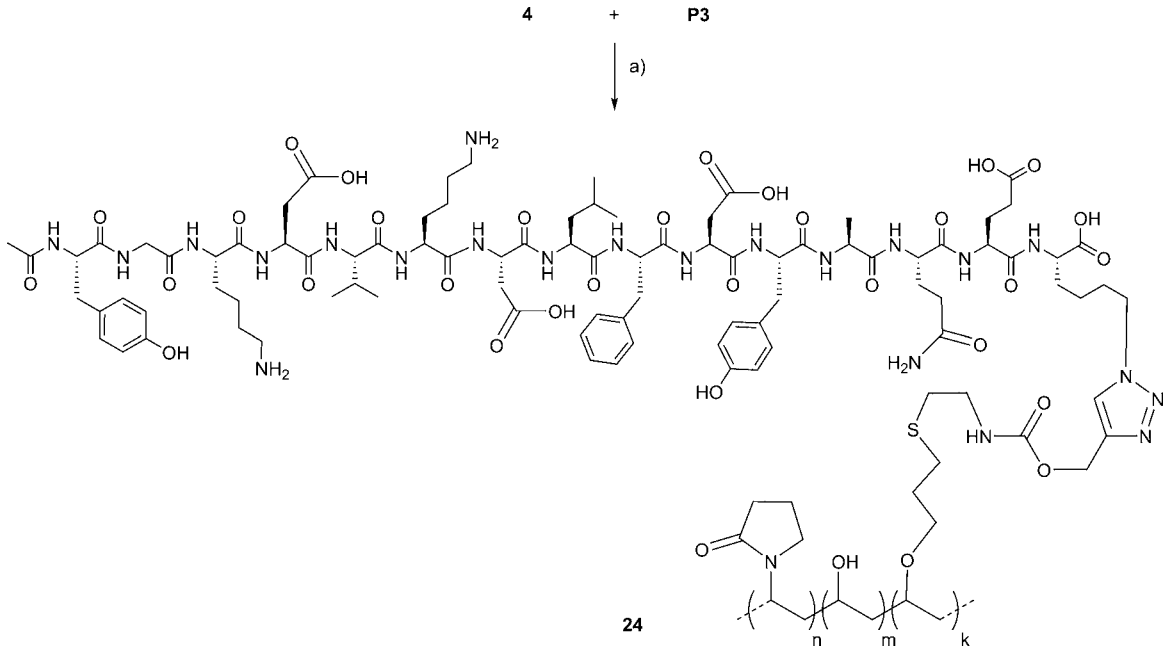
General Methods. ^1H NMR spectra were recorded at either 400, 500, or 600 MHz, and are referenced to the residual protonated solvent resonance: δ_H 7.24 ppm for solutions in $CDCl_3$, and 0.1% external acetone (δ_H 2.225) for solutions in D_2O. Mass analysis was performed by positive-mode electrospray ionization on a hybrid sector-TOF mass spectrometer and for protein glycoconjugates by MALDI mass analysis, employing 2,5-dihydroxybenzoic acid (DHB) as matrix. Analytical thin-layer chromatography (TLC) was performed on silica gel 60-F254 (Merck). TLC detection was achieved by charring with 5% sulfuric acid in ethanol. All commercial reagents were used as supplied. Column chromatography used silica gel (SiliCycle, Quebec City, Quebec, 230−400 mesh, 60 Å) and redistilled solvents. HPLC separations were performed on a Beckmann C18 semipreparative reversed-phase column with a combination of acetonitrile and water containing 0.1% acetic acid as eluent. Photoadditions were carried out using a Spectroline model ENF-260C UV lamp and cylindrical quartz vessels.

The ligand payload on copovidone polymers, compounds **12**, **20**, **22**, and **24**, were estimated by integration of ligand and polymer resonances and the degree of incorporation was calculated as reported,[13] where payload was calculated as the percentage of monomers (vinyl pyrrolidone and vinyl alcohol combined) substituted with the ligand. The ligand payload for **23** was also determined by HPLC of polymer bound versus free peptide.

Polymers. Copovidone derivatives **P1** and **P2** were synthesized as previously described.[13] The alkyne substituted polymer **P3** was obtained as follows.

Poly(prop-2-ynyl 2-(3-(vinyloxy)propylthio)-ethylcarbamate-co-N-vinyl-2-pyrrolidone) ***P3.*** To a solution of poly(2-(3-(vinyloxy)propylthio)ethanamine-co-*N*-vinyl-2-pyrrolidone)[13] (100 mg) in 1 M $NaHCO_3$ (3 mL), a solution of propargyl chloroformate (100 μL, 1 mmol) in methanol (1

Scheme 4[a]



[a]Reagents and conditions: (a) $CuSO_4$, sodium ascorbate, $(Et_3NH)HCO_3$ buffer.

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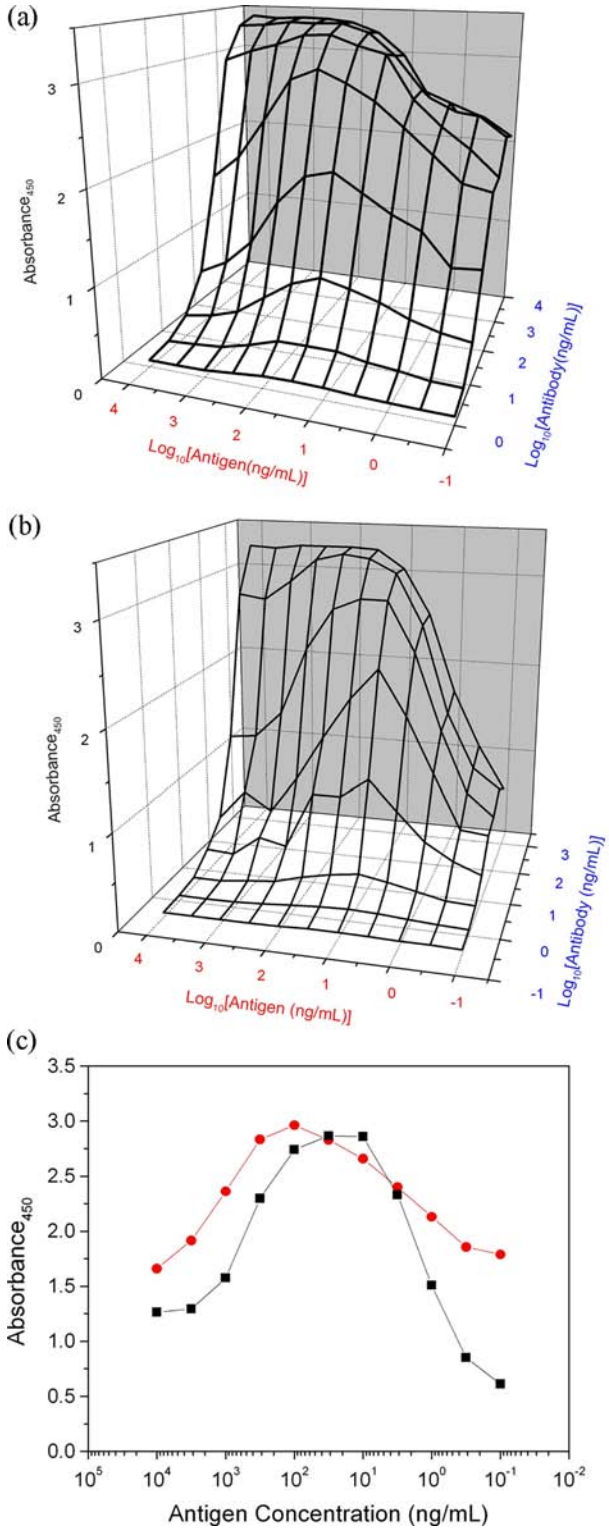

Figure 3. Comparison of ELISA response at different antigen coating and antibody concentrations. Two antigens were examined, copovidone conjugate **22** and its BSA counterpart **23**. (a) Three dimensional plot of the variation of ELISA absorbance plotted against serial dilutions of antigen **22** and antibody CS35. (b) Three-dimensional plot of the variation of ELISA absorbance plotted against serial dilutions of antigen **23** and antibody CS35. (c) Comparison of ELISA response at constant antibody concentration and variable antigen coating demonstrating the similar antigen coating effciency for maximum response.

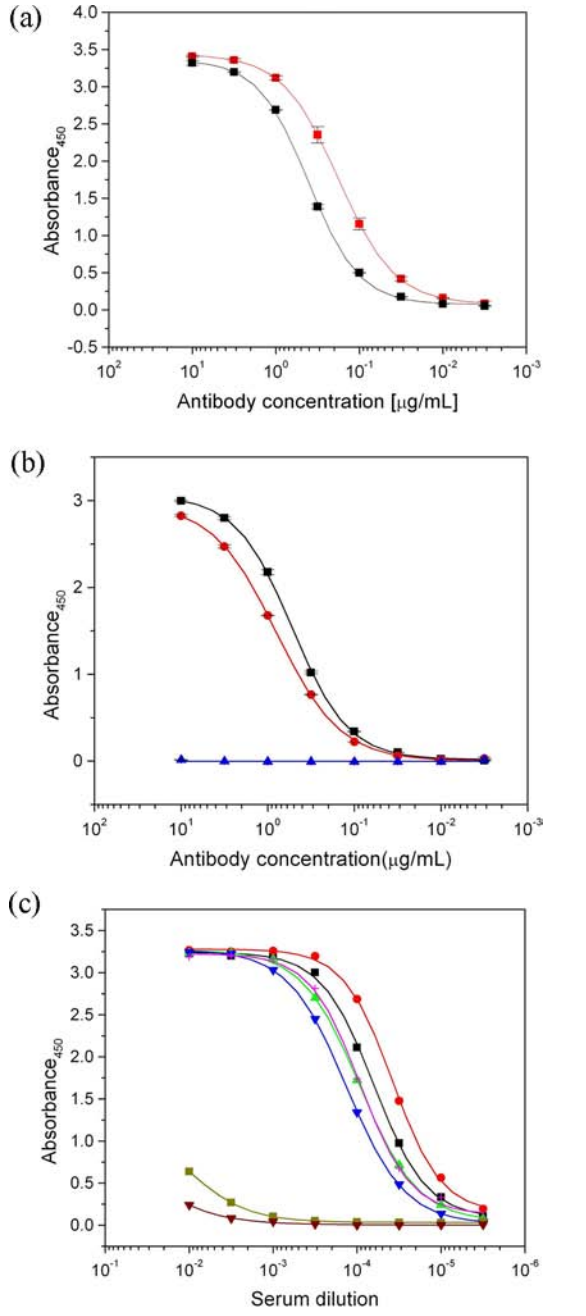

Figure 4. (a) Titration of *C. albicans* mAb C3.1[16] on plates coated with conjugates **12** (red ■) and **20** (black ■); (b) titration of the mAb CS35[22] on plates coated with conjugate **22** (red ●), Ara_6-BSA **23** (black ■), and unconjugated copovidone (blue ▲); (c) titration of polyclonal sera on plates coated with Fba-copovidone conjugate **24** (red ●, black ■, green ▲, red +, and blue ▼). Five mice were immunized with Man_3-Fba-tetanus toxoid glycoconjugate absorbed on alum and two control mice immunized with alum (gray ■, purple ▼). Two mice had superimposable titration curves (green ▲, red +).

mL) was slowly added and the solution was stirred for 1 h at room temperature. The product was dialyzed extensively against deionized water and freeze-dried to afford the title compound **P3** as a white foam (95 mg); ^{1}H NMR (600 MHz, D_2O) δ_H 4.8−4.6 (br m under water, CH_2 propargyl), 4.3−2.9 (br m, polymer backbone), 4.30−2.90 (br m, polymer backbone), 2.80−2.55 (br m, CH propargyl), 4.3−2.9 (br m, polymer backbone), 2.55−1.30 (br m, polymer backbone); IR

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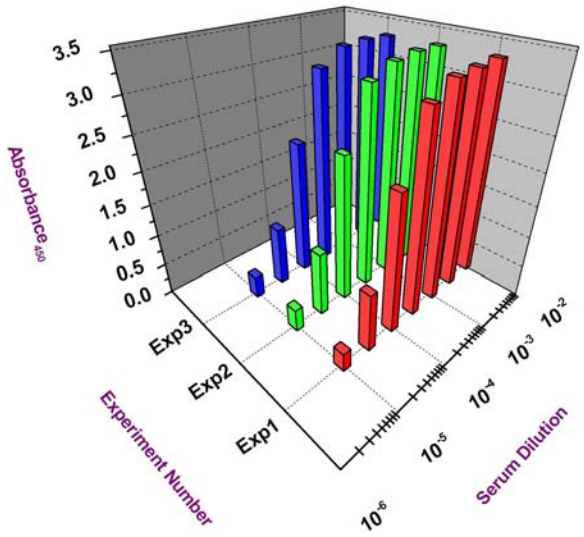

Figure 5. Reuse of ELISA plates coated with povidone conjugate conjugate **20**. Experiment 1 is the initial use of the ELISA plate followed by incubation with 1 M phosphoric acid, washing, and repetition of the assay, experiment 2. After recording absorbance incubation, washing and reuse gave data for experiment 3. Data are plotted as a bar graph for plates coated with conjugate **20**. The data recorded in triplicate and used to plot the graph can be found in Table S3 and for a BSA-Man_3 conjugate[10] Table S4. Data obtained with conjugates **22** and **24** (not reported) show poor consistency on reuse.

(cm^{-1}): 3226.79, 3049.4, 2951.04, 2921.97, 2879.45, 2120.27 (alkyne), 1724.52, 1678, 1533.63, 1493.36, 1460. 29, 1423.79, 1371.76, 1316.47, 1287.02, 1231.18, 1168.01, 1130.61, 1094.72, 1036.21, 1000.77, 932.78, 895.84, 844.39, 731.53, 698.48.

*2-Aminoethyl β-D-mannopyranosyl-(1→2)-β-D-mannopyranosyl-(1→2)-α-D-mannopyranoside (**1**).* Pd/C (10 wt %, 87 mg) was added to trisaccharide **10** (58 mg, 0.052 mmol) in pyridine (6 mL) and the reaction was stirred under H_2 atmosphere overnight.[26] The mixture was filtered through Celite and the solvent was coevaporated with toluene few times. The residue was further dried *in vacuo* for 30 min before being redissolved in 10:1 AcOH/H_2O (6 mL). $Pd(OH)_2$/C (10 wt %, 110 mg) was added and the reaction was stirred under H_2 atmosphere overnight. The mixture was filtered through Celite and the solvents were coevaporated with toluene several times. The residue was further dried *in vacuo* overnight to give the crude product as white solid. The crude was further purified by HPLC using TSK-GEL Amide-80 column to give the pure product **1** (22 mg, 69%) as white fluffy solid after lyophilization: R_f 0.24 (9:1:0.1 methanol−water−acetic acid); $[\alpha]_D$ = −21.1 (*c* 0.2, H_2O); ^1H NMR (600 MHz, D_2O) δ_H 4.96 (d, 1H, *J* = 1.4 Hz, H-1), 4.81 (s, 2H, H-1′, H-1″), 4.24 (d, 1H, *J* = 3.2 Hz, H-2′), 4.15 (dd, 1H, *J* = 3.1, 1.9 Hz, H-2), 4.11 (d, 1H, *J* = 3.1 Hz, H-2″), 3.95 (m, 1H, OCH_2), 3.82−3.89 (m, 4H, H-3, H-6a, H-6a′, H-6a″), 3.66−3.74 (m, 4H, H-6b, H-6b′, H-6b″, OCH_2), 3.50−3.63 (m, 6H, H-4, H-5, H-3′, H-4′, H-3″, H-4″), 3.34 (ddd, 1H, *J* = 9.2, 6.5, 2.5 Hz, H-5′/H-5″), 3.31 (ddd, 1H, *J* = 9.3, 6.9, 2.2 Hz, H-5′/H-5″), 3.18−3.26 (m, 2H, CH_2NH_2), 1.87 (s, 3H, CH_3); ^{13}C NMR (125 MHz, D_2O) δ_C 100.9 (C-1′/C-1″), 99.0 (C-1′/C-1″), 97.9 (C-1), 78.4 (C-2′), 77.6 (C-2), 76.4 (2C, C-5′, C-5″), 73.0, 72.9, 72.1 (C-5, C-3′, C-3″), 70.4 (C-2″), 69.4 (C-3), 67.1, 66.9, 66.8 (C-4, C-4′, C-4″), 63.5 (OCH_2), 61.1, 60.7, 60.4 (C-6, C-6′, C-6″), 39.1 (CH_2NH_3), 23.2 (CH_3). HRMS (ESI) calcd. for (M + H) $C_{20}H_{38}NO_{16}$: 548.2185. Found: 548.2181. FTIR: 3255.6 cm^{-1}.

*2-Aminoethyl β-D-mannopyranosyl-(1→2)-β-D-mannopyranosyl-(1→2)-β-D-mannopyranoside (**2**).* Pd/C (10 wt %, 100 mg) was added to trisaccharide **18** (87 mg, 0.078 mmol) in pyridine (8 mL) and the reaction was stirred under H_2 atmosphere overnight.[26] The mixture was filtered through Celite and the solvent was coevaporated with toluene a few times. The residue was further dried *in vacuo* for 30 min before being redissolved in 10:1 AcOH/H_2O (10 mL). $Pd(OH)_2$/C (10 wt %, 140 mg) was added and the reaction was stirred under H_2 atmosphere overnight. The mixture was filtered through Celite and the solvents were coevaporated several times with toluene. The residue was further dried *in vacuo* overnight to give the crude product as white solid. The crude was further purified by HPLC using TSK-GEL Amide-80 column to give the pure product **2** (30 mg, 64%) as fluffy white solid: $[\alpha]_D$ = −85.1 (*c* 0.4, H_2O); ^1H NMR (500 MHz, D_2O) δ_H 4.93 (s, 1H, H-1′), 4.91 (s, 1H, H-1″), 4.76 (s, 1H, H-1), 4.33 (d, 1H, *J* = 3.2 Hz, H-2′), 4.27 (d, 1H, *J* = 3.0 Hz, H-2), 4.12 (d, 1H, *J* = 3.0 Hz, H-2″), 4.08 (ddd, 1H, *J* = 10.0, 4.9, 4.9 Hz, OCH_2), 3.88−3.96 (m, 4H, H-6a, H-6a′, H-6a″, OCH_2), 3.46−3.79 (m, 9H, H-3, H-4, H-6b, H-3′, H-4′, H-6b′, H-3″, H-4″, H-6b″), 3.31−3.43 (m, 3H, H-5, H-5′, H-5″), 3.24 (t, 1H, *J* = 4.9 Hz, CH_2NH_2), 1.92 (s, 3H, CH_3); ^{13}C NMR (125 MHz, D_2O) δ_C 182.4 (C═O), 101.5(8), 101.5(5) (C-1′, C-1″), 101.1 (C-1), 79.1 (C-2), 78.9 (C-2′), 77.4, 77.2(8), 77.2(7) (C-5, C-5′, C-5″), 74.0, 73.2, 72.8 (3C, C-3/C-3′/C-3″/C-4/C-4′/C-4″), 71.4 (C-2″), 68.3, 67.9, 67.8 (3C, C-3/C-3′/C-3″/C-4/C-4′/C-4″), 67.3 (OCH_2), 62.1, 61.7, 61.6 (3C, C-6, C-6′, C-6″), 40.2 (CH_2NH_3), 24.3 (CH_3). HRMS (ESI) calcd. for (M + H) $C_{20}H_{38}NO_{16}$: 548.2185. Found: 548.2175. FTIR: 3313.0 cm^{-1}.

*Ac-Tyr-Gly-Lys-Asp-Val-Lys-Asp-Leu-Phe-Asp-Tyr-Ala-Gln-Glu-(ε-N_3-Nleu)-OH (**4**).* 2-Chlorotrityl chloride polystyrene resin (Nova Biochem) was derivatized with Fmoc-ε-N_3-Nleu-OH and immediately used for peptide synthesis according to standard Fmoc peptide synthesis procedures in an ABI 433A peptide synthesizer at 0.10 mmol scale. The protocol employed was the UV FastMoc protocol with single couplings and no postcoupling capping. Reagents used were *N,N,N′,N′*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate and *N*-hydroxybenzotriazole with *N,N*-diisopropylethylamine for coupling and 20% piperidine in *N*-methylpyrrolidin-1-one (NMP) for removal of N^{α}-Fmoc groups from the growing peptides' *N*-termini. After removal of the final N^{α}-Fmoc group from the fully elongated peptide, the terminal amino group was capped by treatment with 1 mmol acetic anhydride and 2 mmol pyridine in 3 mL NMP. The protected peptidyl resin was washed with 3 × 5 mL NMP, then 3 × 5 mL CH_2Cl_2. The peptide was cleaved from the resin and deprotected by gently shaking the peptidyl resin in a cocktail of H_2O (250 μL), triisopropyl silane (250 μL), and trifluoroacetic acid (TFA, 9.5 mL) for two hours. The resin was filtered from the peptide solution and washed with two 10 mL portions of TFA. The filtrates were combined in a round-bottomed flask and concentrated by rotary evaporation to a volume of approximately 1 mL. Toluene (5 mL) was added and the mixture concentrated again to give a film of crude peptide. Dry diethyl ether (15 mL) was added and the peptide film was scraped from the flask walls and crushed to give a suspension of fine white powdered peptide in the ether. The peptide precipitate was allowed to settle for five minutes and the supernatant ether

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was removed by decanting. The peptide was washed twice again with ether and the solid residue was left for two hours to dry in a fume hood. The dry peptide was dissolved in 3:7 acetonitrile:water (2.5 mL) and purified by reverse phase HPLC on a 250 mm × 21.2 mm Phenomenex Luna C18(2) preparative column, eluting with a linear gradient from 7:3 to 67.5:33.5 water:acetonitrile over 25 min, at a flow rate of 10 mL min^{-1}. The fractions collected from 20 to 23.5 min were collected, pooled, and lyophilized to give 13.1 mg of the title compound as a fluffy white solid. Analytical HPLC: t_R = 23.177 min, A_{280} = 98.30% (250 mm × 4.6 mm Phenomenex Luna C18(2), 1 mL min^{-1} H_2O:CH_3CN + 0.1% TFA 75:60 → 50:50, 60 min, A_{280}),. MALDI-TOF-MS (sinapinic acid matrix) *m*/*z* calcd. for (M + H) $C_{85}H_{125}N_{22}O_{27}$: 1886.91; Found: 1887.53. ^{1}H NMR (500 MHz, 5:2 D_2O:CD_3CN) δ 7.43−7.61 (m, 5 H, Phe), 7.38 (dd, *J* = 7.89, 1.85 Hz, 4 H, H_δ-Tyr), 7.06 (dd, *J* = 7.98, 4.68 Hz, 4 H, H_ε-Tyr), 4.84 (t, *J* = 6.5 Hz, 1 H), 4.43−4.57 (m, 5 H), 4.33−4.43 (m, 2 H), 4.27 (m, 1 H), 4.17 (d, *J* = 16.87 Hz, 1 H), 4.06 (d, *J* = 16.87 Hz, 1 H), 3.91 (s, 2 H), 3.53 (t, *J* = 6.88 Hz, 2 H), 3.25−3.40 (m, 3 H), 3.06−3.24 (m, 7 H), 2.65−3.01 (m, 6 H), 2.60 (t, *J* = 7.5 Hz, 2 H), 2.41−2.50 (m, 2 H), 2.31−2.40 (m, 2 H), 2.19−2.26 (m, 1 H), 2.17 (s, 3 H, H_{Ac}), 1.52−2.17 (m, 27 H), 1.17 (d, *J* = 4.65 Hz, 3 H, H_χ-Val), 1.19 (d, *J*=4.65 Hz, 3 H, H_χ-Val), 1.10 (d, *J* = 6.05 Hz, 3 H, H_δ-Leu), 1.03 (d, *J* = 6.05 Hz, 3 H, H_δ-Leu).

*2-Azidoethyl 3-O-benzyl-4,6-O-benzylidene-2-O-p-methoxybenzyl-β-D-mannopyranosyl-(1→2)-3-O-benzyl-4,6-O-benzylidene-α-D-mannopyranoside (**7**).* Mannose donor **5**[23] (670 mg, 1.2 mmol) and 4 Å molecular sieve (700 mg) in CH_2Cl_2 (15 mL) were stirred at room temperature under argon for 30 min. BSP[19] (304 mg, 1.4 mmol) TTBP (562 mg, 2.2 mmol) were added at −60 °C followed by the addition of Tf_2O (250 μL, 1.5 mmol) and the mixture was stirred at the same temperature for 30 min. Mannoside acceptor **6**[20] (370 mg, 0.87 mmol) in CH_2Cl_2 (3 mL) was added at −78 °C and the reaction was further stirred for 4 h. The reaction was then quenched with Et_3N and filtered through Celite. The filtrate was washed with sat. $NaHCO_3$, water and brine, and dried over anhydrous Na_2SO_4. The crude was concentrated and purified by chromatography (2:1 hexane−EtOAc) to give **7** (603 mg, 78%) as colorless oil: R_f 0.34 (2:1 hexane−EtOAc); $[\alpha]_D$ = −72.1 (*c* 2.5, CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 7.26−7.55 (m, 22H, ArH), 6.85−6.88 (m, 2H, ArH), 5.63 (s, 1H, PhCH), 5.55 (s, 1H, PhCH), 5.01 (d, 1H, *J* = 11.8 Hz, $PhCH_2$), 4.94 (d, 1H, *J* = 11.8 Hz, $PhCH_2$), 4.89 (d, 1H, *J* = 1.5 Hz, H-1), 4.81 (d, 1H, *J* = 12.2 Hz, $PhCH_2$), 4.76 (d, 1H, *J* = 12.2 Hz, $PhCH_2$), 4.74 (d, 1H, *J* = 12.6 Hz, $PhCH_2$), 4.66 (s, 1H, H-1′), 4.64 (d, 1H, *J* = 12.6 Hz, $PhCH_2$), 4.26−4.32 (m, 4H, H-2, H-6a, H-4′, H-6a′), 4.16 (dd, 1H, *J* = 9.8, 9.8 Hz, H-4), 4.02 (dd, 1H, *J* = 9.8, 3.3 Hz, H-3), 4.01 (d, 1H, *J* = 3.2 Hz, H-2′), 3.79−3.93 (m, 4H, H-5, H-6b, H-6b′, OCH_2), 3.79 (s, 3H, OCH_3), 3.60−3.65 (m, 2H, H-3′, OCH_2), 3.47 (ddd, 1H, *J* = 13.3, 7.4, 3.3 Hz, CH_2N_3), 3.33−3.41 (m, 2H, H-5′, CH_2N_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ_C 159.2 (Ar), 138.8 (Ar), 138.5 (Ar), 137.6 (Ar), 137.5 (Ar), 130.6 (Ar), 130.3 (2C, Ar), 128.9 (Ar), 128.8(7) (Ar), 128.3 (2C, Ar), 128.2(1) (2C, Ar), 128.2(0) (2C, Ar), 128.1 (2C, Ar), 127.5(8) (2C, Ar),, 127.5(5) (2C, Ar), 127.5(3) (Ar), 127.3(Ar), 126.1 (2C, Ar), 126.0(7) (2C, Ar), 113.6 (2C, Ar), 101.7 (PhCH), 101.4 (PhCH), 101.0 (C-1′, J_{C1-H1} = 155.9 Hz), 98.8 (C-1, J_{C1-H1} = 171.1 Hz), 78.5(4) (C-4/C-4′), 78.5(2) (C-4/C-4′), 77.7 (C-3′), 76.0 (C-3), 75.4 (C-2′), 75.0 (C-2), 74.1(4) ($PhCH_2$), 74.1(0) (C-3), 72.3 ($PhCH_2$), 71.6 ($PhCH_2$), 68.9 (C-6), 68.6 (C-6′), 67.8 (C-5′), 66.7 (OCH_2), 64.6 (C-5), 55.3 (OCH_3), 50.5 (CH_2N_3). HRMS (ESI) calcd. for (M + Na) $C_{50}H_{53}N_3O_{12}$: 910.3521. Found: 910.3505. FTIR: 2102.8 cm^{-1}.

*2-Azidoethyl 3-O-benzyl-4,6-O-benzylidene-β-D-mannopyranosyl-(1→2)-3-O-benzyl-4,6-O-benzylidene-α-D-mannopyranoside (**8**).* Disaccharide **7** (603 mg, 0.68 mmol) was dissolved in 4:1 CH_2Cl_2/H_2O (40 mL) and DDQ (192 mg, 0.85 mmol) was added. The reaction was stirred at room temperature overnight. The mixture was washed with sat. $NaHCO_3$ (2x) and brine, and dried over anhydrous Na_2SO_4. The crude was concentrated and purified by chromatography (3:2 hexane−EtOAc) to give **8** (406 mg, 78%) as colorless oil: R_f 0.16 (3:2 hexane−EtOAc); $[\alpha]_D$ = −44.4 (*c* 2.6, CH_2Cl_2); ^{1}H NMR (600 MHz, $CDCl_3$) δ_H 7.49−7.52 (m, 4H, ArH), 7.27−7.43 (m, 16H, ArH), 5.58 (s, 1H, PhCH), 5.51 (s, 1H, PhCH), 4.90 (d, 1H, *J* = 1.5 Hz, H-1), 4.87 (d, 1H, *J* = 12.2 Hz, $PhCH_2$), 4.82 (d, 1H, *J* = 11.8 Hz, $PhCH_2$), 4.79 (d, 1H, *J* = 11.7 Hz, $PhCH_2$), 4.78 (d, 1H, *J* = 11.5 Hz, $PhCH_2$), 4.77 (d, 1H, *J* = 1.2 Hz, H-1′), 4.45 (dd, 1H, *J* = 3.3, 1.4 Hz, H-2), 4.24−4.32 (m, 3H, H-6a, H-4′, H-6a′), 4.19 (d, 1H, *J* = 3.8 Hz, H-2′), 4.16 (dd, 1H, *J* = 9.8, 9.8 Hz, H-4), 4.03 (dd, 1H, *J* = 10.0, 3.5 Hz, H-3), 3.90 (ddd, 1H, *J* = 10.7, 5.9, 3.4 Hz, OCH_2), 3.78−3.86 (m, 3H, H-5, H-6b, H-6b′), 3.70 (dd, 1H, *J* = 9.0, 3.8 Hz, H-3′), 3.63 (ddd, 1H, *J* = 10.6, 7.1, 3.4 Hz, OCH_2), 3.37−3.47 (m, 3H, H-5′, CH_2N_3), 3.20 (br s, 1H, OH); ^{13}C NMR (125 MHz, $CDCl_3$) δ_C 138.2 (Ar), 138.1(9) (Ar), 137.5 (Ar), 137.4(8) (Ar), 128.9(3) (Ar), 128.9(2) (Ar), 128.8(4) (2C, Ar), 128.3 (2C, Ar), 128.2(4) (2C, Ar), 128.2(2) (2C, Ar), 127.8(7) (2C, Ar), 127.8(1) (2C, Ar), 127.8 (Ar), 127.7 (Ar), 126.0(9) (2C, Ar), 126.0(6) (2C, Ar), 101.5 (PhCH), 101.4 (PhCH), 98.9 (C-1), 98.4 (C-1′), 78.6 (2C, C-4, C-4′), 76.4 (C-3′), 74.4 (C-3), 72.9 (C-2), 72.6 ($PhCH_2$), 72.5 ($PhCH_2$), 69.6 (C-2′), 68.6(9) (C-6/C-6′), 68.6(8) (C-6/C-6′), 67.0 (C-5′), 66.7 (OCH_2), 64.4 (C-5), 50.4 (CH_2N_3). HRMS (ESI) calcd. for (M + Na) $C_{42}H_{45}N_3O_{11}$: 790.2932. Found: 790.2946. FTIR: 3491.0, 2102.9 cm^{-1}.

*2-Azidoethyl 3-O-benzyl-4,6-O-benzylidene-2-O-p-methoxybenzyl-β-D-mannopyranosyl-(1→2)-3-O-benzyl-4,6-O-benzylidene-β-D-mannopyranosyl-(1→2)-3-O-benzyl-4,6-O-benzylidene-a-D-mannopyranoside (**9**).* Mannose donor **5**[23] (653 mg, 1.1 mmol) and 4 Å molecular sieve (600 mg) in CH_2Cl_2 (14 mL) were stirred at room temperature under argon for 30 min. BSP (283 mg, 1.4 mmol) TTBP (420 mg, 1.7 mmol) were added at −60 °C followed by the addition of Tf_2O (240 μL, 1.4 mmol) and the mixture was stirred at the same temperature for 30 min.[23] Disaccharide acceptor **8** (386 mg, 0.50 mmol) in CH_2Cl_2 (3 mL) was added at −78 °C and the reaction was further stirred for 4 h. The reaction was then quenched with Et_3N and filtered through Celite. The filtrate was washed with sat. $NaHCO_3$, water and brine, and dried over anhydrous Na_2SO_4. The crude was concentrated and purified by chromatography (3:2 hexane−EtOAc) to give **9** (296 mg, 48%) as colorless oil: R_f 0.32 (3:2 hexane−EtOAc); $[\alpha]_D$ = −93.9 (*c* 2.8, CH_2Cl_2); ^{1}H NMR (600 MHz, $CDCl_3$) δ_H 7.26−7.54 (m, 32H, ArH), 6.76−6.78 (m, 2H, ArH), 5.64 (s, 1H, PhCH), 5.55 (s, 1H, PhCH), 5.44 (s, 1H, PhCH), 5.14 (s, 1H, H-1″), 4.92 (d, 1H, *J* = 1.3 Hz, H-1), 4.91 (d, 1H, *J* = 12.0 Hz, $PhCH_2$), 4.88 (d, 1H, *J* = 12.5 Hz, $PhCH_2$), 4.82 (d, 1H, *J* = 12.5 Hz, $PhCH_2$), 4.71 (d, 1H, *J* = 12.3 Hz, $PhCH_2$), 4.70 (d, 1H, *J* = 12.0 Hz, $PhCH_2$), 4.70 (d, 1H, *J* = 2.1 Hz, H-1′), 4.66 (d, 1H, *J* = 12.3 Hz, $PhCH_2$), 4.59 (d, 1H, *J* = 11.7 Hz, $PhCH_2$), 4.46 (d, 1H, *J* = 3.3 Hz, H-2″), 4.42 (d, 1H, *J* = 11.7 Hz, $PhCH_2$), 4.35−4.41 (m, 4H, H-2, H-2′, H-6a′, H-6a″), 4.40

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(dd, 1H, J = 10.2, 4.7 Hz, H-6a), 4.25 (dd, 1H, J = 9.5, 9.5 Hz, H-4″), 4.12 (dd, 1H, J = 9.7, 9.7 Hz, H-4′), 3.98−4.04 (m, 2H, H-3, H-6b″), 3.95 (dd, 1H, J = 9.4, 9.4 Hz, H-4), 3.83−3.94 (m, 2H, H-5,), 3.72−3.82 (m, 2H, H-6b, H-6b′), 3.70 (dd, 1H, J = 9.9, 3.1 Hz, H-3′), 3.65 (s, 3H, OCH_3), 3.61−3.66 (m, 1H, OCH_2), 3.57 (dd, 1H, J = 10.0, 3.3 Hz, H-3″), 3.36−3.50 (m, 4H, H-5′, H-5″, CH_2N_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ_C 158.8 (Ar), 138.6 (Ar), 138.4 (Ar), 138.3(5) (Ar), 137.6 (Ar), 137.4 (Ar), 137.1 (Ar), 131.5 (Ar), 129.7 (2C, Ar), 129.0 (2C, Ar), 128.8 (Ar), 128.1 (2C, Ar), 128.0(6) (3C, Ar), 128.0(2) (3C, Ar), 127.7 (2C, Ar), 127.5(6) (2C, Ar), 127.5(3) (2C, Ar), 127.5 (Ar), 127.4(6) (2C, Ar), 127.3(6) (2C, Ar), 127.2 (2C, Ar), 127.1(6) (Ar), 126.1(4) (2C, Ar), 126.1(1) (2C, Ar), 126.0(6) (2C, Ar), 113.4 (2C, Ar), 103.2 (C-1″, J_{C1-H1} = 160.5 Hz), 102.0 (PhCH), 101.7 (PhCH), 101.3 (PhCH), 99.8 (C-1′, J_{C1-H1} = 171.0 Hz), 98.2 (C-1, J_{C1-H1} = 157.8 Hz), 79.2 (C-3″), 78.8 (C-4), 78.2(9) (C-4′/C-4″), 78.2(0) (C-4′/C-4″), 76.3 (C-2′), 75.9 (C-3′), 75.6 (C-2″), 74.5 ($PhCH_2$), 74.3 (C-3), 73.8 (C-2), 72.1 ($PhCH_2$), 71.8 ($PhCH_2$), 71.1 ($PhCH_2$), 68.9 (C-6/C-6′/C-6″), 68.8 (C-6/C-6′/C-6″), 64.3 (C-5), 55.1 (OCH_3), 50.4 (CH_2N_3). HRMS (ESI) calcd. for (M + Na) $C_{70}H_{73}N_3O_{17}$: 1250.4832. Found: 1250.4822. FTIR: 2102.2 cm^{-1}.

*2-Azidoethyl 3-O-benzyl-4,6-O-benzylidene-β-D-manno-pyranosyl-(1→2)-3-O-benzyl-4,6-O-benzylidene-(1→2)-3-O-benzyl-4,6-O-benzylidene-α-D-mannopyranoside (**10**).* Tri-saccharide **9** (240 mg, 0.20 mmol) was dissolved in 4:1 CH_2Cl_2/H_2O (25 mL) and DDQ (90 mg, 0.40 mmol) was added. The reaction was stirred at room temperature overnight. The mixture was washed with sat. $NaHCO_3$ (2x) and brine, and dried over anhydrous Na_2SO_4. The crude was concentrated and purified by chromatography (3:2 hexane−EtOAc) to give **10** (153 mg, 71%) as white solid: R_f 0.22 (3:2 hexane−EtOAc); $[\alpha]_D$ = −91.8 (c 0.3, CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 7.16−7.56 (m, 30H, ArH), 5.63 (s, 1H, PhCH), 5.61 (s, 1H, PhCH), 5.55 (s, 1H, PhCH), 5.13 (s, 1H, H-1″), 4.91 (s, 1H, H-1), 4.82 (s, 2H, $PhCH_2$), 4.75 (d, 1H, J = 11.9 Hz, $PhCH_2$), 4.70 (s, 1H, H-1′), 4.67 (d, 1H, J = 11.8 Hz, $PhCH_2$), 4.65 (d, 1H, J = 11.4 Hz, $PhCH_2$), 4.62 (d, 1H, J = 12.0 Hz, $PhCH_2$), 4.43 (d, 1H, J = 3.2 Hz, H-2′), 4.20−4.42 (m, 7H, H-2, H-6a, H-4′, H-6a′, H-2″, H-4″, H-6a″), 3.81−4.01 (m, 6H, H-3, H-4, H-5, H-6b′, H-6b″, OCH_2), 3.74 (dd, 1H, J = 10.2, 10.2 Hz, H-6b), 3.70 (dd, 1H, J = 9.9, 3.3 Hz, H-3′), 3.63 (ddd, 1H, J = 10.8, 7.6, 3.2 Hz, OCH_2), 3.57 (dd, 1H, J = 10.2, 3.2 Hz, H-3″), 3.35−3.51 (m, 4H, H-5′, H-5″, CH_2N_3), 2.92 (br s, 1H, OH); ^{13}C NMR (125 MHz, $CDCl_3$) δ_C 138.4 (Ar), 138.3 (Ar), 138.2 (Ar), 137.5 (Ar), 137.4 (Ar), 137.2 (Ar), 129.0 (Ar), 128.8(9) (Ar), 128.8(6) (Ar), 128.4 (2C, Ar), 128.3(3) (3C, Ar), 128.3(1) (3C, Ar), 128.2(5) (3C, Ar), 128.2 (2C, Ar), 128.1(6) (2C, Ar), 127.7 (2C, Ar), 127.6(6) (Ar), 127.6(0) (2C, Ar), 127.5 (1C, Ar), 126.1(9) (2C, Ar), 126.1(5) (2C, Ar), 126.1(2) (2C, Ar), 101.8 (PhCH), 101.5 (PhCH), 101.4(6) (PhCH), 100.8 (C-1″), 99.4 (C-1′), 98.1 (C-1), 78.5 (C-4/C-4″), 78.3 (C-4/C-4″), 78.1 (C-4′), 77.5 (C-3″), 76.3 (C-3′), 74.0 (C-2′), 73.8 (C-3), 73.7 (C-2), 72.2 ($PhCH_2$), 71.8 ($PhCH_2$), 71.6 ($PhCH_2$), 68.9 (C-2″), 68.8 (2C, C-6/C-6′/C-6″), 68.4 (C-6/C-6′/C-6″), 67.8 (C-5′), 67.3 (C-5″), 66.8 (OCH_2), 64.3 (C-5), 50.4 (CH_2N_3). HRMS (ESI) calcd. for (M + Na) $C_{62}H_{65}N_3O_{16}$: 1130.4257. Found: 1130.4255. FTIR: 3504.6, 2102.9 cm^{-1}.

*2-(2-Butoxycyclobutene-3,4-dione-1-ylamino)ethyl-β-D-mannopyranosyl-(1→2)-β-D-mannopyranosyl-(1→2)-α-D-mannopyranoside (**11**).* To the deprotected trimannoside **1** (7.4 mg, 12 μmol) in MeOH (1.0 mL), dibutyl squarate (7.5 μL, 24 μmol) was added and the reaction was stirred for 1 h. The pH of the reaction mixture was carefully adjusted with Et_3N to slightly above pH 7. The reaction mixture was then concentrated and purified on an Iatrobead-packed column. Excessive squarate reagent was washed out with DCM−MeOH (9:1) and the desired product was eluted with MeOH−H_2O (9:1) to yield the trimannoside squarate half-ester **11** (6.9 mg, 81%) as white fluffy solid after lyophilization: R_f 0.53 (9:1 MeOH−H_2O); ^{1}H NMR (500 MHz, D_2O) δ_H 4.94, 4.93 (s, 1H, H-1), 4.82 (s, 1H, H-1″), 4.79, 4.77 (s, 1H, H-1′), 4.72 (t, 1H, J = 6.3 Hz, $OCH_{2\text{-squarate}}$), 4.67 (t, 1H, J = 6.1 Hz, $OCH_{2\text{-squarate}}$), 4.23 (br s, 1H, H-2′), 4.13 (d, 1H, J = 2.9 Hz, H-2″), 4.04, 4.02 (br s, 1H, H-2), 3.46−3.92 (m, 17H), 3.28−3.38 (m, 2H), 1.70−1.82 (m, 2H, $CH_{2\text{-squarate}}$), 1.34−1.48 (m, 2H, $CH_{2\text{-squarate}}$), 0.87−0.95 (m, 3H, $CH_{3\text{-squarate}}$); ^{13}C NMR (125 MHz, D_2O) δ_C 101.0 (C-1″), 99.0 (C-1′), 97.6, 97.3 (C-1), 78.5 (C-2′), 77.8 (C-2), 76.4 (2C, C-5′, C-5″), 74.5 ($OCH_{2\text{-squarate}}$), 73.0 (C-3″), 72.9, 72.2 (C-3′), 70.4 (C-2″), 69.6 (C-3), 67.1, 66.9, 66.8, 66.6 (1C, OCH_2), 61.2, 60.6, 60.4 (C-6, C-6′, C-6″), 44.2, 43.8 (1C, CH_2NH), 31.4 (CH_2), 18.2 (CH_2), 12.9 (CH_3). HRMS (ESI) calcd. for (M + Na) $C_{28}H_{45}NO_{19}$: 722.2478. Found: 722.2468.

*β-D-Mannopyranosyl-(1→2)-β-D-mannopyranosyl(1→2)-α-D-mannopyranoside−copovidone Glycoconjugate (**12**).* Poly(prop-2-ynyl 2-(3-(vinyloxy)propylthio)ethylcarbamate-co-*N*-vinyl-2-pyrrolidone) (9.6 mg) **P2**[14] and 2-(2-butoxy-3,4-dioxocyclobut-1-enylamino)ethyl 2-*O*-[(2-*O*-β-D-mannopyranosyl)-β-D-mannopyranosyl)]-α-D-mannopyranoside (9.2 mg) **11** were dissolved in water (100 μL) and pH was adjusted to 9 with 1 M Na_2CO_3. The mixture was stirred overnight at room temperature, then dialized against deionized water and freeze-dried to give **12** as a white powder (14.1 mg); from ^{1}H NMR the estimated payload[8] is 9.8% corresponding to ∼66% m/m of the ligand/polymer conjugate composition. ^{1}H NMR (500 MHz, D_2O) δ_H 4.96 (br s, H-1), 4.82 (br s, H-1″), 4.78 (br s, H-1′), 4.23 (d, J = 2.5, H-2′), 4.12 (d, J = 2.6 Hz, H-2″), 4.03 (br s, 1H, H-2), 4.39−2.9 (br m, H-3−6, H-3′-6′, H-3″-6″, polymer backbone), 2.80−2.55 (br m, CH_2S linker), 2.55−1.30 (br m, polymer backbone).

*2-Azidoethyl 3-O-benzyl-4,6-O-benzylidene-2-O-p-methoxybenzyl-β-D-mannopyranoside (**14**).* A mixture of mannosyl sulfoxide **13**[23] (3.5 g, 5.9 mmol), TTBP (3.0 g, 12 mmol), and 4 Å molecular sieve (3.5 g) was dissolved in CH_2Cl_2 (180 mL) and stirred for 30 min at −78 °C. Tf_2O (1.1 mL, 6.6 mmol) was added to the mixture and stirred at the same temperature. After 10 min, 2-azidoethanol (1.0 g, 12 mmol) was added and the reaction was further stirred for 20 min. The reaction was slowly warmed to −30 °C, quenched with MeOH, and filtered through Celite. The filtrate was washed with sat. $NaHCO_3$ and brine, and dried over anhydrous Na_2SO_4. The crude was concentrated and purified by chromatography (2:1 hexane−EtOAc) to give **14** (2.2 g, 68%) as colorless syrup: R_f 0.43 (2:1 hexane−EtOAc); $[\alpha]_D$ = −89.37 (c 3.9, CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 7.50−7.52 (m, 2H, ArH), 7.27−7.41 (m, 10H, ArH), 6.85−6.87 (m, 2H, ArH), 5.63 (s, 1H, PhCH), 4.92 (d, 1H, J = 11.8 Hz, $PhCH_2$), 4.81 (d, 1H, J = 11.9 Hz, $PhCH_2$), 4.67 (d, 1H, J = 12.5 Hz, $PhCH_2$), 4.56 (d, 1H, J = 12.5 Hz, $PhCH_2$), 4.53 (d, 1H, J = 0.8 Hz, H-1), 4.30 (dd, 1H, J = 10.4, 4.9 Hz, H-6a), 4.21 (app t, 1H, J = 9.6 Hz, H-4), 4.09−4.13 (m, 1H, OCH_2), 3.99 (app d, 1H, J = 3.1 Hz, H-2), 3.94 (app t, 1H, J = 10.3 Hz, H-6b), 3.80 (s, 3H, OCH_3), 3.65 (ddd, 1H, J = 10.5, 8.7, 3.3 Hz, OCH_2), 3.59 (dd, 1H, J =

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9.9, 3.1 Hz, H-3), 3.53−3.59 (m, 1H, CH_2N_3), 3.32−3.37 (m, 2H, H-5, CH_2N_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ_C 159.3 (Ar), 138.3 (Ar), 137.6 (Ar), 130.5 (Ar), 130.3 (2C, Ar), 128.9 (Ar), 128.3 (2C, Ar), 128.2 (2C, Ar), 127.5(9), 127.5(6) (3C, Ar), 126.1 (2C, Ar), 113.6 (2C, Ar), 102.3 (C-1, J_{C1-H1} = 156.6 Hz), 101.5 (PhCH), 78.5 (C-4), 77.8 (C-3), 75.4 (C-2), 74.7 ($PhCH_2$), 72.3 ($PhCH_2$), 68.7 (OCH_2), 68.6 (C-6), 67.7 (C-5), 55.3 (OCH_3), 50.9 (CH_2N_3). HRMS (ESI) calcd. for (M + Na) $C_{30}H_{33}N_3O_7$: 570.2211. Found: 570.2197. FTIR: 2105.2 cm^{-1}.

*2-Azidoethyl 3-O-benzyl-4,6-O-benzylidene-β-D-mannopyranoside (**15**).* Monosaccharide **14** (2.2 g, 3.9 mmol) was dissolved in 17:1 CH_2Cl_2/H_2O (120 mL) and DDQ (2.0 g, 9.0 mmol) was added at 0 °C. The reaction was slowly warmed back to room temperature and stirred overnight. The reaction was then washed with sat. $NaHCO_3$ (2x) and brine, and dried over anhydrous Na_2SO_4. The crude was concentrated and purified by chromatography (3:2 hexane−EtOAc) to give **15** (1.3 g, 79%) as white solid: R_f 0.22 (3:2 hexane−EtOAc); $[\alpha]_D$ = −57.8 (*c* 0.9, CH_2Cl_2); ^1H NMR (500 MHz, $CDCl_3$) δ_H 7.49−7.51 (m, 2H, ArH), 7.27−7.41 (m, 8H, ArH), 5.61 (s, 1H, PhCH), 4.86 (d, 1H, *J* = 12.2 Hz, $PhCH_2$), 4.78 (d, 1H, *J* = 12.2 Hz, $PhCH_2$), 4.58 (s, 1H, H-1), 4.32 (dd, 1H, *J* = 10.4, 5.0 Hz, H-6a), 4.14−4.18 (m, 2H, H-2, H-4), 4.09 (ddd, 1H, *J* = 10.7, 4.9, 3.9 Hz, OCH_2), 3.89 (app t, 1H, *J* = 10.4 Hz, H-6b), 3.74 (ddd, 1H, *J* = 10.7, 8.2, 3.7 Hz, OCH_2), 3.66 (dd, 1H, *J* = 9.5, 3.3 Hz, H-3), 3.54 (ddd, 1H, *J* = 12.1, 8.2, 3.7 Hz, CH_2N_3), 3.34−3.40 (m, 2H, H-5, CH_2N_3), 2.53 (d, 1H, *J* = 1.5 Hz, OH); ^{13}C NMR (125 MHz, $CDCl_3$) δ_C 137.8 (Ar), 137.4 (Ar), 129.0 (Ar), 128.5 (2C, Ar), 128.2 (2C, Ar), 127.8(9), 127.8(7) (3C, Ar), 126.0 (2C, Ar), 101.6 (PhCH), 100.6 (C-1), 78.3 (C-4), 76.6 (C-3), 72.6 ($PhCH_2$), 69.8 (C-2), 68.6 (OCH_2), 68.5 (C-6), 67.0 (C-5), 50.6 (CH_2N_3). HRMS (ESI) calcd. for (M + Na) $C_{22}H_{25}N_3O_6$: 450.1636. Found: 450.1629. FTIR: 3500.8, 2104.1 cm^{-1}.

*2-Azidoethyl 3-O-benzyl-4,6-O-benzylidene-2-O-p-methoxybenzyl-β-D-mannopyranosyl-(1→2)-3-O-benzyl-4,6-O-benzylidene-β-D-mannopyranoside (**16**).* A mixture of mannosyl sulfoxide **13**[23] (280 mg, 0.48 mmol), TTBP (240 mg, 0.97 mmol), and 4 Å molecular sieve (300 mg) were dissolved in CH_2Cl_2 (18 mL) and stirred for 30 min at −78 °C. Tf_2O (88 μL, 0.52 mmol) was added to the mixture and stirred at the same temperature. After 10 min, glycosyl acceptor **15** (97 mg, 0.23 mmol) was added and the reaction was further stirred for 4 h. The reaction was then quenched with MeOH and filtered through Celite. The filtrate was washed with sat. $NaHCO_3$ and brine, and dried over anhydrous Na_2SO_4. The crude was concentrated and purified by chromatography (3:2 hexane−EtOAc) to give **16** (138 mg, 69%) as a colorless syrup: R_f 0.42 (3:2 hexane−EtOAc); $[\alpha]_D$ = −105.7 (*c* 1.5, CH_2Cl_2); ^1H NMR (500 MHz, $CDCl_3$) δ_H 7.22−7.52 (m, 22H, ArH), 6.80−6.84 (m, 2H, ArH), 5.59 (s, 1H, PhCH), 5.44 (s, 1H, PhCH), 5.00 (d, 1H, *J* = 11.9 Hz, $PhCH_2$), 4.92 (d, 1H, *J* = 11.9 Hz, $PhCH_2$), 4.79 (d, 1H, *J* = 12.6 Hz, $PhCH_2$), 4.78 (s, 1H, H-1′), 4.75 (d, 1H, *J* = 12.5 Hz, $PhCH_2$), 4.63 (d, 1H, *J* = 12.6 Hz, $PhCH_2$), 4.54 (d, 1H, *J* = 12.5 Hz, $PhCH_2$), 4.52 (s, 1H, H-1), 4.31 (dd, 1H, *J* = 10.3, 4.8 Hz, H-6a′), 4.25−4.30 (m, 2H, H-2, H-6a), 4.20 (app t, 1H, *J* = 9.6 Hz, H-4), 4.12 (d, 1H, *J* = 3.4 Hz, H-2′), 4.04−4.10 (m, 2H, H-4, OCH_2), 3.89 (app t, 1H, *J* = 10.3 Hz, H-6b′), 3.76 (app t, 1H, *J* = 10.4 Hz, H-6b), 3.74 (s, 3H, OCH_3), 3.61−3.69 (m, 2H, H-3, OCH_2), 3.56 (dd, 1H, *J* = 10.0, 3.1 Hz, H-3′), 3.26−3.37 (m, 3H, H-5, H-5′, CH_2N_3), 3.20 (ddd, 1H, *J* = 13.6, 5.0, 3.1 Hz, CH_2N_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ_C 159.0 (Ar), 138.6(2) (Ar), 138.6(0) (Ar), 137.7 (Ar), 137.4 (Ar), 131.1 (Ar), 130.2 (2C, Ar), 128.1(9), 128.1(4), 128.1(3) (5C, Ar), 127.5(9), 127.5(6), 127.5(3), 127.5(1), 127.4(5), 127.3(8), 127.3(6) (7C, Ar), 126.0(4), 126.0(3), 125.9(8), 125.9(6) (8C, Ar), 113.4 (2C, Ar), 103.8 (C-1′, J_{C1-H1} = 160.4 Hz), 101.5(9) (PhCH), 101.5(6) (PhCH), 101.3 (C-1, J_{C1-H1} = 157.5 Hz), 78.4 (C-4′), 78.1 (C-4), 76.7 (2C, C-2, C-3′), 76.0 (C-3), 75.2 (C-2′), 74.1 ($PhCH_2$), 72.1 ($PhCH_2$), 71.1 ($PhCH_2$), 68.9, 68.6(8), 68.6(5) (3C, C-6, C-6′, OCH_2), 67.7, 67.5 (2C, C-5, C-5′), 55.2 (OCH_3), 50.9 (CH_2N_3). HRMS (ESI) calcd. for (M + Na) $C_{50}H_{53}N_3O_{12}$: 910.3521. Found: 910.3502. FTIR: 2105.0 cm^{-1}.

*2-Azidoethyl 3-O-benzyl-4,6-O-benzylidene-β-D-mannopyranosyl-(1→2)-3-O-benzyl-4,6-O-benzylidene-β-D-mannopyranoside (**17**).* Disaccharide **16** (140 mg, 0.16 mmol) was dissolved in 17:1 CH_2Cl_2/H_2O (9 mL) and DDQ (77 mg, 0.34 mmol) was added at 0 °C. The reaction was slowly warmed back to room temperature and stirred overnight. The reaction was then washed with sat. $NaHCO_3$ (2x) and brine, and dried over anhydrous Na_2SO_4. The crude was concentrated and purified by chromatography (3:2 hexane−EtOAc) to give **17** (82 mg, 68%) as colorless syrup: R_f 0.33 (1:1 hexane−EtOAc); $[\alpha]_D$ = −116.0 (*c* 0.2, CH_2Cl_2); ^1H NMR (500 MHz, $CDCl_3$) δ_H 7.49−7.52 (m, 4H, ArH), 7.26−7.42 (m, 16H, ArH), 5.59 (s, 1H, PhCH), 5.58 (s, 1H, PhCH), 4.94 (s, 1H, H-1′), 4.85 (d, 1H, *J* = 12.5 Hz, $PhCH_2$), 4.81 (d, 1H, *J* = 12.5 Hz, $PhCH_2$), 4.78 (d, 1H, *J* = 12.5 Hz, $PhCH_2$), 4.74 (d, 1H, *J* = 12.5 Hz, $PhCH_2$), 4.54 (s, 1H, H-1), 4.44 (d, 1H, *J* = 3.3 Hz, H-2), 4.27−4.34 (m, 3H, H-6a, H-2′, H-6a′), 4.25 (app t, 1H, *J* = 9.4 Hz, H-4′), 4.07−4.10 (m, 2H, H-4, OCH_2), 3.88 (app t, 1H, *J* = 10.4 Hz, H-6b′), 3.84 (app t, 1H, *J* = 10.2 Hz, H-6b), 3.64−3.70 (m, 3H, H-3, H-3′, OCH_2), 3.32−3.47 (m, 3H, H-5, H-5′, CH_2N_3), 3.26 (ddd, 1H, *J* = 13.5, 4.9, 3.0 Hz, CH_2N_3), 2.93 (s, 1H, OH); ^{13}C NMR (125 MHz, $CDCl_3$) δ_C 138.4 (Ar), 138.2 (Ar), 137.6 (Ar), 137.4 (Ar), 128.9 (Ar), 128.8(7) (Ar), 128.3 (3C, Ar), 128.2(5) (3C, Ar), 128.2 (2C, Ar), 127.9 (2C, Ar), 127.6 (Ar), 127.6(0) (Ar), 127.5 (2C, Ar), 126.1 (2C, Ar), 126.0(5) (2C, Ar), 101.5 (3C, C-1, PhCH), 100.6 (C-1′), 78.4 (C-4′), 77.9 (C-4), 76.6 (C-3′), 76.2 (C-3), 73.2 (C-2), 72.3 ($PhCH_2$), 71.6 ($PhCH_2$), 69.5 (C-2′), 69.1 (OCH_2), 68.7 (C-6), 68.4 (C-6′), 67.6 (C-5′), 67.0 (C-5), 50.8 (CH_2N_3). HRMS (ESI) calcd. for (M + Na) $C_{42}H_{45}N_3O_{11}$: 790.2946. Found: 790.2936. FTIR: 3496.2, 2105.2 cm^{-1}.

*2-Azidoethyl 3-O-benzyl-4,6-O-benzylidene-β-D-mannopyranosyl-(1→2)-3-O-benzyl-4,6-O-benzylidene-β-D-mannopyranosyl-(1→2)-3-O-benzyl-4,6-O-benzylidene-β-D-mannopyranoside (**18**).* Mannose donor **5**[23] (817 mg, 1.4 mmol) and 4 Å molecular sieves (1 g) in CH_2Cl_2 (14 mL) were stirred at room temperature under argon for 30 min. BSP (380 mg, 1.8 mmol) TTBP (570 mg, 2.3 mmol) were added at −60 °C followed by the addition of Tf_2O (314 μL, 1.9 mmol) and the mixture was stirred at the same temperature for 30 min.[23] Disaccharide **17** (710 mg, 0.92 mmol) in CH_2Cl_2 (3 mL) was added at −78 °C and the reaction was further stirred for 4 h. The reaction was then quenched with Et_3N and filtered through Celite. The filtrate was washed with sat. $NaHCO_3$ and brine, and dried over anhydrous Na_2SO_4. The crude was concentrated and partially purified by chromatography (3:2 hexane−EtOAc; R_f 0.32) to give trisaccharide as pale yellow syrup. The resulting intermediate was then dissolved in 4:1 CH_2Cl_2/H_2O (50 mL) and DDQ (160 mg, 0.71 mmol) was added. The reaction was stirred overnight before being washed with sat. $NaHCO_3$ (2x) and brine, and dried over anhydrous Na_2SO_4. The crude was

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concentrated and purified by chromatography (3:2 hexane−EtOAc) to give **18** (370 mg, 45% over two steps) as foamy white solid: R_f 0.26 (3:2 hexane−EtOAc); $[\alpha]_D$ = −134.5 (*c* 0.5, CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 7.48−7.52 (m, 4H, ArH), 7.26−7.44 (m, 23H, ArH), 7.18−7.21 (m, 2H, ArH), 7.11−7.12 (m, 1H, ArH), 5.60 (s, 2H, PhCH), 5.42 (s, 1H, PhCH), 5.11 (s, 1H, H-1″), 4.87 (s, 1H, H-1′), 4.69−4.78 (m, 6H, $PhCH_2$), 4.53 (d, 1H, *J* = 3.2 Hz, H-2′), 4.51 (s, 1H, H-1), 4.42 (d, 1H, *J* = 3.2 Hz, H-2″), 4.28−4.35 (m, 4H, H-2, H-4, H-6a, H-6a′), 4.25 (dd, 1H, *J* = 10.4, 4.8 Hz, H-6a″), 4.18 (app t, 1H, *J* = 9.6 Hz, H-4′), 4.07 (ddd, 1H, *J* = 10.7, 5.3, 3.0 Hz, OCH_2), 3.89−3.95 (m, 3H, H-6b, H-6b′, H-4″), 3.60−3.71 (m, 3H, H-3, H-3′, H-3″, H-6b″, OCH_2), 3.26−3.43 (m, 5H, H-5, H-5′, H-5″, CH_2N_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ_C 138.5 (Ar), 138.2 (2C, Ar), 137.5(5) (Ar), 137.5(0) (Ar), 137.2 (Ar), 128.8(8) (Ar), 128.8(7) (Ar), 128.8(4) (Ar), 128.2(9), 128.2(5), 128.2(1), 128.1(8), 128.1(6) (13C, Ar), 127.7 (2C, Ar), 127.6(7) (2C, Ar), 127.5(8) (2C, Ar), 127.5(4) (Ar), 127.5(0) (Ar), 126.0(7) (2C, Ar), 126.0(4) (2C, Ar), 126.0(0) (2C, Ar), 102.4 (C-1′, J_{C1-H1} = 162.3 Hz), 101.7 (C-1, J_{C1-H1} = 157.6 Hz), 101.5 (PhCH), 101.4(6) (PhCH), 101.3(8) (PhCH), 101.2 (C-1″, J_{C1-H1} = 155.1 Hz), 78.5 (C-4), 78.2 (C-4″), 78.0 (C-4′), 77.2 (C-3″), 76.4 (C-3′), 75.8 (C-3), 75.7 (C-2), 74.1 (C-2′), 72.2 ($PhCH_2$), 71.6 ($PhCH_2$), 71.5 ($PhCH_2$), 69.2 (OCH_2), 69.0 (C-2″), 68.8, 68.5(1), 68.4(9) (3C, C-6, C-6′, C-6″), 67.7, 67.5, 67.2 (3C, C-5, C-5′, C-5″), 50.9 (CH_2N_3). HRMS (ESI) calcd. for (M + Na) $C_{62}H_{65}N_3O_{16}$: 1130.4257. Found: 1130.4250. FTIR: 3512.8, 2105.3 cm^{-1}.

*2-(2-(5-Oxo-5-(prop-2-ynylamino)pentanamido)ethyl) 2-(N-propargyl glutariamidyl)ethyl β-D-mannopyranosyl-(1→2)-β-D-mannopyranosyl-(1→2)-β-D-mannopyranoside (**19**).* To the deprotected trimannoside **2** (14 mg, 23 μmol) in 0.1 M sodium bicarbonate, pH 8.3 (1.0 mL), succinimidyl *N*-propargyl glutariamidate[26] (25 mg, 92 μmol) was added and the reaction was stirred for 2 h. The desired product was purified by HPLC (Luna C18 from Phenomenex) to give the propargylated trimannoside **19** (8.7 mg, 54%) as fluffy white solid after lyophilization: $[\alpha]_D$ = −53.2 (*c* 0.7, H_2O); ^{1}H NMR (500 MHz, D_2O) δ_H 4.91 (s, 1H, H-1), 4.85 (s, 1H, H-1′), 4.75 (s, 1H, H-1″), 4.30 (d, 1H, *J* = 3.0 Hz, H-2′), 4.19 (d, 1H, *J* = 3.1 Hz, H-2″), 4.13 (d, 1H, *J* = 2.8 Hz, H-2), 3.87−3.98 (m, 6H, H-6a, H-6a′, H-6a″, $CH_{2\text{-alkyne}}$, OCH_2), 3.52−3.77 (m, 9H, H-3, H-4, H-6b, H-3′, H-4′, H-6b′, H-3″, H-6b″, OCH_2), 3.30−3.50 (m, 6H, H-5, H-5′, H-4″, H-5″, CH_2NH_2), 2.60 (t, 1H, *J* = 2.5 Hz, alkyne), 2.29 (t, 4H, *J* = 7.6 Hz, CH_2), 1.88 (quin, 2H, *J* = 7.8 Hz, CH_2); ^{13}C NMR (125 MHz, D_2O) δ_C 176.8 (C═O), 176.5 (C═O), 101.8 (2C, C-1, C-1′), 101.2 (C-1″), 79.4 (C-2′), 79.2 (C-2″), 77.3 (2C), 77.2 (C-5, C-5′, C-5″), 73.9 (C-3), 73.2 (C-3′), 72.9 (C-3″), 71.4 ($CH_{2\text{-alkyne}}$), 69.2 (C-2), 68.3 (OCH_2), 68.0 (C-4″), 67.7 (2C, C-4′, C-4), 62.1, 61.7 (2C) (C-6, C-6′, C-6″), 40.2 (CH_2NH), 35.9 (CH_2CO), 35.6 (CH_2CO), 29.7 ($C_{-alkyne}$), 22.7 ($C_{-alkyne}$). HRMS (ESI) calcd. for (M + Na) $C_{28}H_{46}N_2O_{18}$: 721.2638. Found: 721.2636. FTIR: 3290.4 cm^{-1}.

*β-D-Mannopyranosyl-(1→2)-β-D-mannopyranosyl(1→2)-β-D-mannopyranoside−copovidone Glycoconjugate (**20**).* Propargylated trimannoside (8.7 mg, 12 μmol) **19** and polymer **P1**[14] (18 mg, 9.1 μmol) were dissolved in degassed water (1 mL). The mixture was adjusted to ∼ pH 8 with $NaHCO_3$ followed by the addition of 0.05 M $CuSO_4$ (50 μL) and 1.0 M sodium ascorbate (25 μL, freshly made).[30,31] The reaction was left at room temperature for two days and quenched with 0.5 M EDTA (30 μL). The reaction mixture was diluted 2-fold with water and dialyzed against 1 mM EDTA in water (2x) and deionized water (2x). The final product **20** was lyophilized as pale yellow solid (24 mg); from ^{1}H NMR the estimated payload is 6.8% corresponding to ∼49% m/m of the ligand/polymer conjugate composition.[14] ^{1}H NMR (500 MHz, D_2O) δ_H 4.90 (s, 1H, H-1), 4.84 (s, 1H, H-1′), 4.70 (s, 1H, H-1″), 4.42 (br s, 1H), 4.29 (d, 1H, *J* = 2.9 Hz, H-2′), 4.18 (d, 1H, *J* = 2.8 Hz, H-2″), 4.11 (d, 1H, *J* = 2.7 Hz, H-2), 2.80−3.97 (m, 49H), 1.45−2.50 (m, 136H).

*Ara_6-Copovidone Conjugate (**22**).* Hexasaccharide **3** (13 mg) was dissolved in MeOH (2.25 mL) and 1 M $NaOCH_3$ (0.25 mL) was added. The reaction was stirred at room temperature until TLC indicated completion (checked via TLC using ninhydrin reagent). The solution was then neutralized with Amberlite 120 (H^+), filtered, and concentrated. Without further purification, the crude product was redissolved in of $NaHCO_3$ buffer (1 mL 0.1 M, pH 8.3), and succinimidyl *N*-propargyl glutariamidate[25] (13.5 mg) was added and the reaction stirred overnight. The mixture was then purified by HPLC using a TSK gel Amide-80 column (21.5 mm ID × 30 cm, 10 μm; from TOSOH BioScience LLC, Japan) with a nonlinear gradient (Waters gradient 8) acetonitrile:water gradient from 80:20 to 65:35 over 45 min at a flow rate of 10 mL/min. Fractions collected were lyophilized and analyzed by high resolution MS (ESI). The final product, propargylated Ara_6 **21**, (7.0 mg, 54% theoretical yield) was identified at an *m*/*z* of 1111.5 for M+Na^+ [calculated mass for M = $C_{46}H_{76}N_2O_{27}$: 1088.46]. Alkyne **21** (0.97 mg, 0.89 μmol) was used without further characterization by dissolution in water with 2-poly(azidoethene-*co*-*N*-vinyl-2-pyrrolidone (2.97 mg) and the mixture was lyophilized. The resulting solid was dissolved in 0.2 M NH_4HCO_3 buffer (pH: 8; 0.4 mL) and 1 M sodium L-ascorbate (10 μL) and 0.05 M copper sulfate (20 μL) were added.[25,27] The reaction mixture was left on a tumbler. After 3 days the solution was diluted with water (2 mL) and concentrated by centrifugation (5000 rpm) using a Millipore membrane (10,000 MWCO). The concentrate was diluted with water (2 mL) and concentrated again. Then the concentrate was diluted with water and filtered through a Millipore syringe filter (0.02 μm) and lyophilized to provide the conjugate **22** as white foam (3.08 mg). From ^{1}H NMR the estimated payload[8] is 2.65% corresponding to ∼28% m/m of the ligand/polymer conjugate composition. ^{1}H NMR (600 MHz, D_2O): δ 8.00−7.60 (m, 1 H, CH); 5.23, 5.16, 5. 13, 5.12, 5.09, and 4.99 (6 br s, H-1_a, H-1_b, H-1_c, H-1_d, H-1_e, H-1_f), 4.44 (br. s.), 4.32−4.25 (m), 4.22−4.16 (m), 4.16−2.9 (br m, remaining carbohydrate signals, polymer backbone, linker), 2.55−1.25 (br m, polymer backbone, linker).

*Ara_6-BSA Conjugate (**23**).* To a solution of Ara_6-amine[21] related to trifluoracetamide **3** (5.1 mg) and 3,4-diethoxy-3-cyclobutene-1,2-dione (2 μL) in ethanol−water (1:1, 1.5 mL) was added sat aq sodium bicarbonate solution (30 μL). The solution was then stirred at room temperature for 1 h, when TLC (CH_3OH:CH_2Cl_2, 1:2) showed that almost all of the Ara_6-amine was consumed. The mixture was directly concentrated on a rotary evaporator and the residue was purified by column chromatography (CH_3OH:CH_2Cl_2, 1:3) to give the squaric acid monoester (5.3 mg, 92%). To this solution was added BSA (16.6 mg) followed by borate buffer (pH 9.0, 120 μL) and the reaction was left for 36 h at room temperature. The reaction solution was then diluted with deionized water (2−3 mL) and transferred to a membrane bag for dialysis

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against changes of deionized water (3 times over 36 h). The solution from the membrane bag was then transferred to a glass vial, and lyophilized to afford the Ara_6-BSA conjugate **23** as a white fluffy solid (20 mg). Analysis by MALDI mass spectrometry indicated a loading of 14.7 mol of Ara_6 per mol of BSA.

*Ac-Fba-Copovidone Conjugate (**24**).* To propargyl copovidone polymer **P3** (10.0 mg) dissolved in water (250 μL) and methanol (250 μL) in a 1.5 mL microcentrifuge tube was added 1 M sodium ascorbate (25 μmol, 25 μL) 0.05 M $CuSO_4$ (2.5 μmol, 50 μL), peptide **4** (3.0 μmol, 5.6 mg), and 1 M triethylammonium bicarbonate (100 μmol, 100 μL pH 8.5). The mixture was vortexed for 21 h on a benchtop shaker. The reaction mixture was neutralized by addition of trifluoroacetic acid (104 μmol, 8 μL). The solution was filtered through a 13 mm PVDF filter with 0.45 μm pore size and divided into three portions for separation. The polymer and peptide were efficiently separated from each other and other reaction mixture components by gel filtration on a Superdex Peptide 300/10 GL column eluted with 70:30 H_2O:CH_3CN containing 0.1% TFA at a flow rate of 0.8 mL min^{-1}. Peptide-containing eluent was selectively detected by absorbance at 280 nm and consisted of two fractions; the first, from 6.4 to 9.6 mL, contained polymer-bound peptide as determined by ^{1}H NMR. The second, from 11.0 to 13.0 mL, contained free peptide as determined by MALDI-MS. The gel chromatography peak areas were further compared with the peak area obtained upon gel filtration of a known amount (0.46 mg) free peptide, and corresponded to 3.6 mg and 0.34 mg polymer-linked peptide and free peptide, or 91% conversion, and a combined recovery of 70%. The fractions from 6.4 to 9.6 mL were combined and lyophilized, rendering 10.5 mg of peptide-bearing copovidone polymer, or 70% of the quantity expected from a 91% conversion. The estimated loading is 34% m/m (0.18 mmol peptide per gram of polymer); by ^{1}H NMR the estimated payload[14] is 1.6% corresponding to ∼33% m/m of the peptide/polymer conjugate composition. ^{1}H NMR (500 MHz, D_2O) δ 7.96−7.90 (m, CH), 7.80 (s), 7.23−7.12 (m), 7.07−6.98 (m), 6.74−6.65 (m), 5.14−5.05 (m), 4.90−4.44 (m under water), 4.40−0.7 (br m, peptide signals, polymer backbone, linker).

Antigen Coating Efficiency. Optimal antigen coating for ELISA plates was established by applying increasing dilutions of antigens **22** or **24** across the rows of an ELISA plate. Antigen solution was prepared at 10 μg/mL and successive wells across the plate were incubated with $\sqrt{10}$ dilutions of the antigen. This resulted in 12 wells with antigen coat beginning at 10 μg/mL and ending at 0.1 ηg/mL. Coated plates were then incubated with $\sqrt{10}$ dilutions of antibody CS35 such that each row received the same dilution of antibody. Bound antibody was detected as described below and the resulting three-dimensional matrix is recorded in Tables S1 and S2 and plotted as a three-dimensional graph (Figure 3).

ELISA. Antisera and mouse monoclonal antibodies were as follows: rabbit polyclonal antisera were raised to a synthetic β-mannose trisaccharide tetanus toxoid conjugate;[10] mouse immune sera were raised to a mannose trisaccharide glycopeptide conjugated to tetanus toxoid (unpublished data); and two mAbs were used: C3.1 specific for the *Candida albicans* β-mannan[16] and mAb CS35 specific for the *M. tuberculosis* cell wall antigen.[21,22] Antigen stock solutions were prepared by dissolving copovidone or BSA glycoconjugates in PBS (1 mg/mL). Microtiter plates were coated with antigen solution (10 μg/mL, 100 μL/well) by incubation at 4 °C for 18 h, then washed 5 times with PBST (0.05% Tween-20 in phosphate buffer saline, PBS). Plates were blocked using 1% BSA in PBS buffer and incubated at RT for 45 min, then washed with PBST prior to use. Serial $\sqrt{10}$ dilutions of immune sera or monoclonal antibodies were made in PBST containing 0.1% BSA (rabbit and mouse sera were used at a starting dilution of 1:100 and mAb was used at a starting concentration of 10 μg/mL). The solutions were distributed in triplicate on coated microtiter plates and incubated at room temperature for 2 h. Plates were washed with PBST (5 times) and goat anti-mouse IgG or goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (Kirkegaard & Perry Laboratories; 1:5000 dilution in 0.1% BSA/PBST; 100 μL/well) was added. The mixture was then incubated for 30 min at room temperature, and then washed (5 times) with PBST. Peroxidase substrate, 3,3′,5,5′-tetramethylbenzidine (TMB) with H_2O_2 (1:1 mixture of 3,3′,5,5′-tetramethylbenzidine (0.4 g/L) and 0.02% H_2O_2 solution, Kirkegaard & Perry Laboratories) was added (100 μL/well). After 15 min, the reaction was quenched by addition of 1 M phosphoric acid (100 μL/well). Absorbance was read at 450 nm.

End point titers are recorded as the dilution giving an absorbance 0.2 above background.

Repeated Use of Coated ELISA Plates. Assays were performed in an identical manner to that described above. After plates were read at 450 nm, they were stored with quenching solution (i.e., TMB + acid) overnight at RT. The following day, plates were washed with PBST, blocked, and ELISA was performed as before. After the plates had been read, they were again stored overnight and reused in the same manner. In total, 3 sequential assays were performed on plates coated with each antigen.

■ ASSOCIATED CONTENT

Supporting Information

Detailed ELISA data, NMR spectra for all new compounds, and where appropriate mass spectra and HPLC profiles for peptide **4** and its conjugate **23**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

Ara_6, arabinose hexasaccharide; BSA, bovine serum albumin; BSP, 1-benzenesulfinyl piperidine; DDQ, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone; mAb, monoclonal antibody; Man_3, 1,2-β-mannotriose; MS, molecular sieve; PMB, *p*-methoxybenzyl; TTBP, 2,4,6-tri*tert*-butylpyrimidine; Tf_2O, trifluoromethanesulfonic anhydride

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