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

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1.0 General

1.1 Reagents

1.2 Analysis

2.0 Synthesis of monomers, sugar azides and fluorescent dyes

1.0 General

1.1 Reagents

Copper(I) bromide (Aldrich, 98%) was purified according to the method of Keller and Wycoff[1]. *N*-(*n*-Propyl)-2-pyridylmethanimine[2], $[(PPh_3)_3CuBr]$[3], trimethylsilyl-protected propargyl methacrylate monomer **(5)**[4] and rhodamine B methacrylate **(9)**[5] were prepared as described earlier. Triethylamine (Fischer, 99%) was stored over sodium hydroxide pellets. Anhydrous THF (Romil "Hi-Dry", 99.99%) was stored over activated 4 Å molecular sieves under dry nitrogen. Tetra *n*-butylammonium fluoride (TBAF) in methanol (0.20 M) from Aldrich and D-(+)-Mannose from Lancaster were used as received. Bovine Serum Albumin (BSA) (Fluka, ≥95%) and was used as received and stored at 4ºC. All other reagents and solvents were obtained at the highest purity available from Aldrich Chemical Company and used without further purification unless stated.

CAUTION! *: Although we had never experienced any adverse event when working with these products, organic azides are potentially explosive compounds and they should be handled with utmost care!!*

1.2 Analysis

All reactions were carried out using standard Schlenk techniques under an inert atmosphere of oxygen-free nitrogen, unless otherwise stated. TLC performed using pre-coated silica gel 60 F254 and developed in the solvent system indicated. Compounds were visualized by use of UV light (254 nm) or a basic solution (10 % w/w K_2CO_3 in water) of $KMnO_4$. Merck 60 (230-400 mesh) silica gel was used for column chromatography. Molar mass distributions were measured using size exclusion chromatography (SEC), on a system equipped with two PL aquagel-OH 8 μm mixed columns (300 x 7.5 mm) with differential refractive index detection using water (containing $NaNO_3$ (53 g), NaH_2PO_4 (3 g) and NaN_3 (25 mg) per 2.5 L) at 1.0 mL min^{-1} as the eluent. Poly(ethyleneglycol) standards ($1.6 \cdot 10^5$-1. 10^3 g mol^{-1}) were used to calibrate the SEC. NMR spectra were obtained on a Bruker DPX300 and Bruker DPX400 spectrometer. All chemical shifts are reported in ppm (δ) relative to tetramethylsilane, referenced to the chemical shifts of residual solvent resonances (^1H and ^{13}C). The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, dd = doublet of doublets, t = triplet, td = triplet of doublets, m = multiplet. The molecular weight of the polymers M_n(NMR) were calculated by comparing the integrals of the chain-end signals and appropriate peaks related to the polymer backbone. Infrared absorption spectra were recorded on a Bruker VECTOR-22 FTIR spectrometer using a

Golden Gate diamond attenuated total reflection cell. Mass spectra were recorded using a Micromass Autospec apparatus. The yields are not optimized.

Two different SEC-HPLC systems were employed in this work. The first one was fitted with two BioSep-SEC-S3000 columns (Phenomenex), a fluorescence detector (Hitachi, L-7480) and a Gilson UV/vis-155 detector continuously measuring the relative intensity of the mobile phase. The system was eluted with 0.1 vol.% trifluoroacetic acid solution in water/acetonitrile (69/31, v/v) at a flow rate of 0.5 $mL \cdot min^{-1}$. The second one included a size exclusion column (Amersham, Tricorn, Superose 12, 10/300 GL) and a Gilson UV/vis-155 detector. The system was eluted with 10 mM phosphate buffer, pH = 7.0, at flow rate 0.5 $mL\ min^{-1}$.

SDS-PAGE was performed using a stacking polyacrylamide gel (5 % cross-linking), a resolving polyacrylamide gel (8 % cross-linking) and a running buffer consisting of 25 mM of Tris base, 250 mM of glycine and 0.1 % of SDS at pH 8.7.

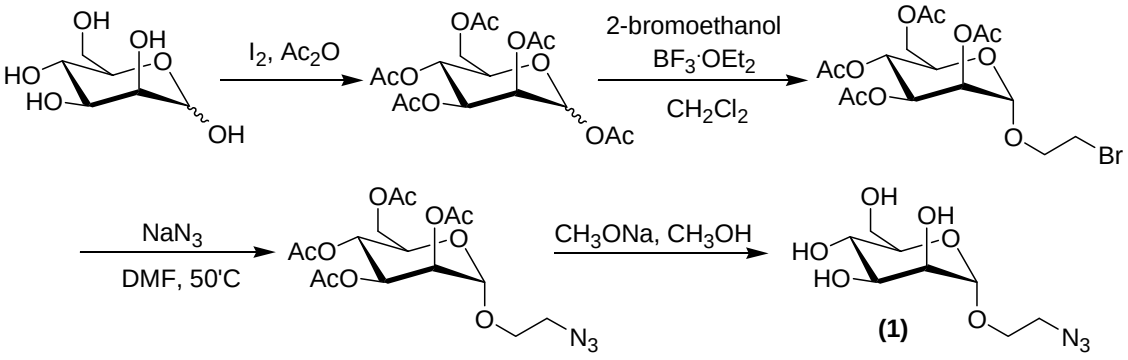
α-Helix and β-sheet content in the bioconjugates were determined by circular dichroism (CD) spectroscopy. Analysis was performed on a Jasco J715 spectropolarimeter with 0.1 cm quartz cuvette, wavelength: from 260 nm to 190 nm; Data pitch: 0.2 nm; Scanning mode: Continuous; Scanning speed: 100 nm / min; Response: 1sec; Band width: 1.0 nm; Scans: 16; at ambient temperature in phosphate buffer (50 mM, pH 8.5). Percentages of α-helices and β-sheets were determined by applying the protein CD structure fitting program, CDSSTR.[6] Pseudo-esterase assay was performed using an Varian Cary 50 Bio UV-Vis spectrometer, using 1 mL volume quartz cuvettes (1 cm pathlength).

The ELISA protocol was adapted from the WiesLab MBL Pathway Assay kit available from Eurodiagnostica.

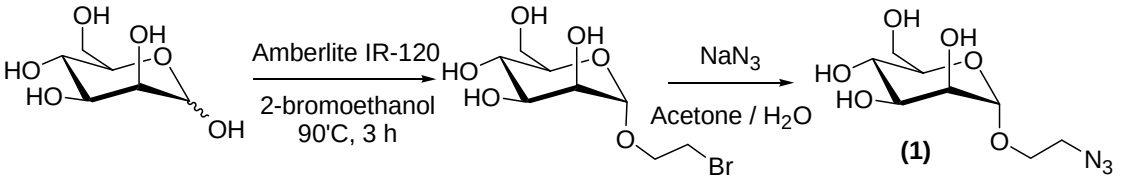
2.0 Synthesis of monomers and sugar azides

2.1 2'-Azidoethyl -α-D-mannopyranoside (1)

Method 1



Method 2



2.1.1 Synthesis of mannose azide (1): Method 1

1,2,3,4,6-Penta-O-acetyl- α-D-mannopyranoside

First reported by P. A. Levene,[7] prepared as described by Watt *et. al.*[8]

IR: 1737, 1431, 1367, 1201, 1145, 1086, 1052, 971, 930, 909, 835, 786, 701. ^{1}H NMR (400.03 MHz, $CDCl_3$, 298 K)[8] δ = 1.88 (s, 3H, CH_3); 1.94 (s, 3H, CH_3); 1.99 (s, 3H,

CH_3); 2.05 (s, 3H, CH_3); 2.12(s, 3H, CH_3); 3.71-3.892 (m, 1H, CH); 4.03-4.25 (m, 2H, CH_2OAc); 5.18-5.36 (m, 3H, 3×CH); 5.83 (m, 1H, CH) $^{13}C\{^1H\}$ NMR (100.59 MHz, $CDCl_3$, 298 K)[8] δ = 20.55 (1C, CH_3); 20.64 (1C, CH_3); 20.66 (1C, CH_3); 20.76 (1C, CH_3); 20.81 (1C, CH_3);62.10 (1C, CH_2OAc); 65.64 (1C, CH_2CH_2O); 68.44 (1C, CH); 68.83 (1C, CH); 70.71 (1C, CH); 70.01 (1C, CH); 90.51 ($C_{anomeric}$); 169.53 (1C, $CH_3C(O)O$); 169.66 (1C, $CH_3C(O)O$);169.71 (1C, $CH_3C(O)O$); 169.82 (1C, $CH_3C(O)O$); 170.40 (1C, $CH_3C(O)O$). Anal. Calcd. for $C_{16}H_{22}O_{11}$ C, 49.23; H, 5.68; Found: C, 49.33; H, 5.72; Mass Spectrometry (+ESI-MS) *m/z* (%):408(36), 409(6), 413 [M+Na] (100), 414(14), 429 [M+K] (18).

2'-Bromoethyl 2,3,4,6-tetra-O-acetyl-α-D-mannopyranoside

First reported by Dahmen *et. al*,[9] prepared as described in the same paper.

IR (neat): $\tilde{\nu}$ =2958, 1743, 1435, 1368, 1218, 1136, 1084, 1046, 979, 910 cm^{-1}. ^1H NMR(400.03 MHz, $CDCl_3$, 298 K)[10] δ = 1.99 (s, 3H, CH_3); 2.04 (s, 3H, CH_3); 2.10 (s, 3H, CH_3); 2.15 (s, 3H, CH_3); 3.45(m, 2H, CH_2Br); 3.75-3.89 (m, 2H, OCH2); 3.96-4.12 (m, 3H, $CHCH_2OAc$); 4.23 (m, 1H, CH); 4.81 (d, *J* = 1.75 Hz, 1H, CH); 5.21-5.35 (m, 3H, 3×CH). $^{13}C\{^1H\}$ NMR (100.59 MHz, $CDCl_3$, 298 K) δ = 20.82 (1C, CH_3); 20.89 (1C, CH_3); 21.02 (1C, CH_3); 21.11 (1C, CH_3); 29.74 (1C, CH_2Br); 62.56 (1C, CH_2OAc); 65.98 (1C, CH); 68.56 (1C, CH); 68.63 (1C, CH); 68.68 (1C, CH_2CH_2O);69.08 (1C, CH); 97.91 ($C_{anomeric}$); 169.88 (1C, $CH_3C(O)O$); 169.95 (1C, $CH_3C(O)O$); 169.97 (1C, $CH_3C(O)O$); 170.38 (1C, $CH_3C(O)O$). Anal. Calcd. for $C_{16}H_{23}BrO_{10}$ C, 42.21; H, 5.09; Found C, 42.16; H, 5.17.. Mass Spectrometry (+ESI-MS) *m/z* (%): 102 (80), 217 (38), 301 (58), 323 (100), 353 (45), 381 (79), 413 (44), 429 (36), 477 [M+Na] (57).

2'-Azidoethyl 2,3,4,6-tetra-O-acetyl-α-D-mannopyranoside

First reported by Chernyak *et al*[11], prepared as described by Kleinert *et al.*[12]

IR (neat): $\tilde{\nu}$ =2958, 1743, 1435, 1368, 1218, 1136, 1084, 1046, 979, 910 cm^{-1}. NMR(400.03 MHz, $CDCl_3$, 298 K)[10] δ = 1.93 (s, 3H, CH_3); 1.98 (s, 3H, CH_3); 2.04 (s, 3H, CH_3); 2.09 (s, 3H, CH_3); 3.45(m, 2H, CH_2N_3); 3.66-3.86 (m, 2H, CH_2CH_2O); 3.99-4.15 (m, 3H, $CHCH_2OAc$); 4.23 (1H, CH); 4.85 (d, J = 1.75 Hz, 1H, CH); 5.21-5.35 (m, 3H, 3×CH). $^{13}C\{^1H\}$ NMR (100.59 MHz, $CDCl_3$, 298 K)[10] δ = 21.08 (1C, CH_3); 21.13 (1C, CH_3); 21.16 (1C, CH_3); 21.30 (1C, CH_3); 50.79 (1C, CH_2N_3); 62.89 (1C, CH_2OAc); 66.43 (1C, CH); 67.48 (1C, CH); 68.68 (1C, CH_2CH_2O);69.28 (1C, CH); 69.08 (1C, CH); 98.18 ($C_{anomeric}$); 170.18 (1C, $CH_3C(O)O$); 170.23 (1C, $CH_3C(O)O$); 170.43 (1C, $CH_3C(O)O$); 171.04 (1C, $CH_3C(O)O$). Anal. Calcd. for $C_{16}H_{23}N_3O_{10}$ C, 46.04; H, 5.55; N 10.07; Found C, 46.23; H, 5.54; N 10.15. Mass Spectrometry (+ESI-MS) *m/z* (%): 102 (14), 129 (17), 301 (16), 323 (45), 353 (34), 381 (100), 382 (23), 413 (24), 440 [M+Na] (76).

2'- Azidoethyl -O-α-D-mannopyranoside (1)

First reported by Chernyak *et al*[11], prepared as described by Kleinert *et al.*[12]

IR (neat): $\tilde{\nu}$ =3358 (bs), 2927, 2097, 1644, 1301, 1262, 1132, 1056, 976, 913, 881, 812 cm^{-1} ^1H NMR (400.03 MHz, D_2O, 298 K) δ = 3.45 (m, 2H, CH_2N_3); 3.55-3.60 (m, 2H, $CH_2CH_2N_3$); 3.61-3.67 (m, 2H, CH_2OH); 3.67-3.91 (m, 4H, 4×CH); 4.92 (d , J = 1. 5 Hz, 1H, CH). $^{13}C\{^1H\}$ NMR (100.59 MHz, D_2O, 298 K) δ = 50.20 (1C, CH_2N_3); 60.91 (1C, CH_2OH); 66.30 (1C, CH_2CH_2O); 66.68 (1C, CH); 69.97 (1C, CH); 70.39 (1C, CH);

72.89 (1C, CH); 99.81 ($C_{anomeric}$); Anal. Calcd. for $C_8H_{15}N_3O_6$ C, 38.55; H, 6.07; N, 16.86; Found: C, 38.35; H, 6.11; N, 16.76; Mass Spectrometry (+ESI-MS) *m/z* (%): 102 (100), 118 (63), 172 (46), 217 (60), 272[M+Na] (39).

2.1.2 Synthesis of mannose azide (1): Method 2

2'-Bromoethyl-α-D-mannopyranoside

First reported by Davis *et. al.*[13]

Amberlite IR-120 (1.5 g) was suspended in 2-bromoethanol (20.3 g, 164 mmol) and the mixture was heated at 90 °C in a 50 mL round bottom flask equipped with a condenser. After 30 min D-mannose (1.5 g, 8.3 mmol) was added in a single portion. After stirring at 90 °C for 2.5 h the suspension was filtered through a very short cotton wool pad, washing the filtered solid with further 1 mL of 2-bromoethanol. An aliquot of the reaction was solution was diluted with DMSO-d_6 and analyzed by ^{13}C NMR. The spectrum revealed the absence of mannose starting material and the presence of one single sugar derivative with the anomeric carbon atom at ca. 101 ppm (see below). The excess of 2-bromoethanol was distilled off under reduced pressure, the resulting sticky residue was dissolved in methanol and SiO_2 (5 g) was added. The solvent was removed under reduced pressure and the silica-supported reaction mixture was loaded onto a column previously filled with SiO_2 and pre-eluted with ethyl acetate / methanol 19:1 vol/vol (R_f: 0.27). ** After elution with the same solvent mixture, appropriate fractions were collected and the solvents were removed under reduced pressure to give 2'-Bromoethyl-α-D-mannopyranoside (1.68 g, 5.9 mmol, 69 %) as a colourless oil that formed a rather thick foam when kept under high vacuum for 30 min.

IR (neat): $\tilde{\nu}$ =3336 (bs), 2926, 1421, 1278, 1133, 1089, 1055, 1024, 974, 880, 834, 805cm^{-1} ^{1}H NMR (400.03 MHz, CD_3OD, 298 K)[13] δ = 3.56 (t, 2H, CH_2Br); 3.63-3.73 (m, 2H, H-4 + H-5); 3.73-3.80 (m, 2H, H-3 + H-6"); 3.81-3.88 (m, 2H, H-6' + OCH*H*CH_2Br); 3.90 (dd, *J* = 3.1, 1.5 Hz, 1H, H-2); 3.99 (dt, *J* = 11.6, 5.8 Hz, 1H, OC*H*HCH_2Br); 4.84 (d, *J* = 1.5 Hz, 1H, H-1); $^{13}C\{^{1}H\}$ NMR (100.59 MHz, CD_3OD, 298 K) δ = 31.6 (1C, CH_2Br); 62.4 (1C, CH_2OH); 68.1 (1C, CH); 68.6 (1C, O*C*H_2CH_2Br); 71.7 (1C, CH); 72.2 (1C, CH); 74.5 (1C, CH); 101.4 (1C, $C_{anomeric}$); HRMS-ES Calcd. for $C_8H_{15}BrO_6$ Na(M-Na+): 308.9950: Found 308.9956.

** NOTE: particular care was taken in making sure that no residual 2-bromoethanol was present in the final 2'-bromoethyl-α-D-mannopyranoside product. This in order to avoid the formation of potentially explosive 2-azidoethanol in the next step.

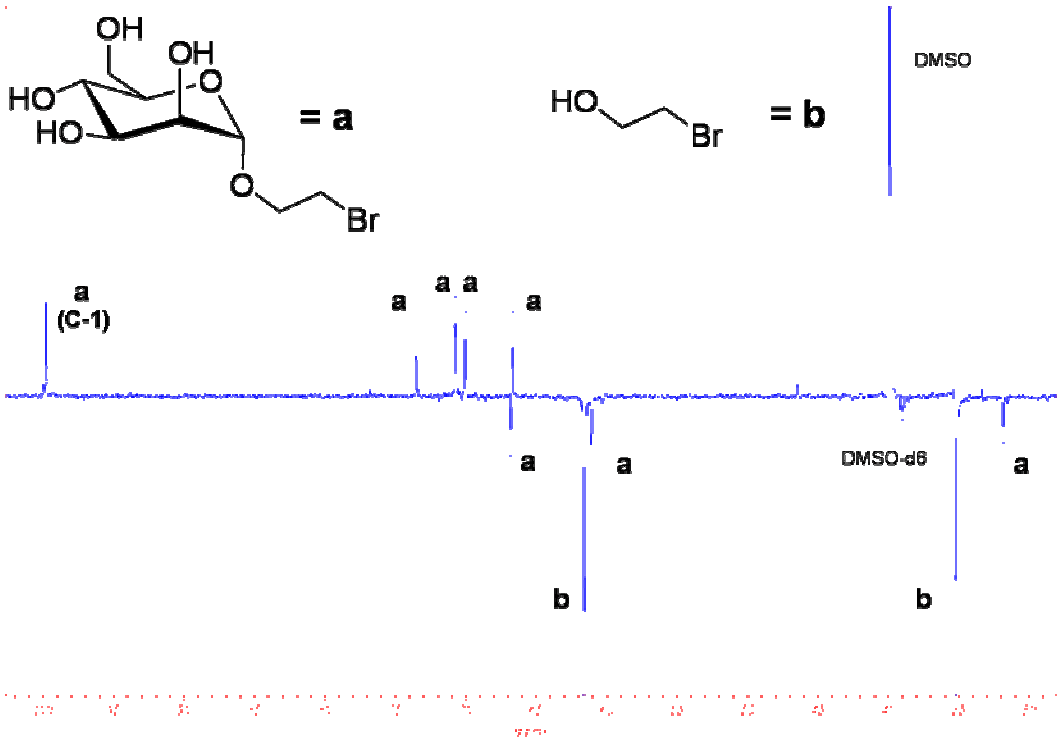


Figure S1. ^{13}C NMR in DMSO-d_6 of the reaction mixture after 2.5 h.

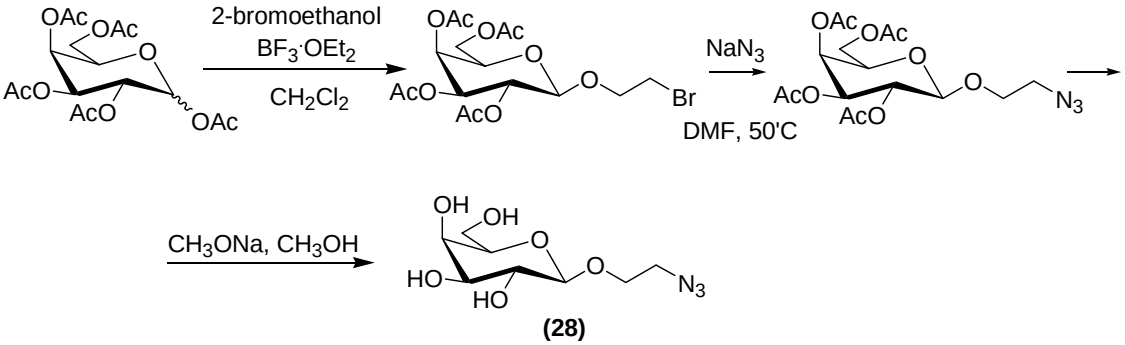
2'- Azidoethyl -O-α-D-mannopyranoside (1)

First reported by Chernyak *et al*[11]

Sodium azide (0.91 g, 14 mmol) and 2'-bromoethyl-α-D-mannopyranoside (2.0 g, 7.0 mmol) were dissolved in water (5 mL). Acetone (30 mL, the solution became slightly turbid) and the resulting mixture was heated up to reflux and stirred at this temperature for 20 h. A 1 mL aliquot was taken from the reaction mixture and acetone was removed under reduced pressure. ^{13}C NMR in D_2O of the resulting solution revealed the complete conversion of 2'-bromoethyl -O-α-D-mannopyranoside into the desired azide **(1)**. Acetone was then removed under reduced pressure and the resulting aqueous solution was freeze-dried. The resulting oily residue was dissolved in methanol, and SiO_2 (6 g)

was added. The solvent was removed under reduced pressure and the silica-supported reaction mixture was loaded onto a column previously filled with SiO_2 and pre-eluted with ethyl acetate / methanol 19:1 vol/vol (R_f **(1)**: 0.24). After elution with the same solvent mixture, appropriate fractions were collected and the solvents were removed under reduced pressure to give **(1)** (1.5 g, 6.0 mmol, 86 %) as a white solid[14] that gave ^1H and ^{13}C NMR, FT IR and MS spectra identical to those relative to the same product previously prepared following the Method 1. HRMS-ES Calcd. for $C_8H_{15}N_3O_6$ Na (M-Na+): 272.0859: Found 272.0865.

2.2 2'-Azidoethyl -α-D-Galactopyranoside (28)



2'-Bromoethyl 2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside

First reported by Coles *et. al.,*[15] prepared as described by Davis *et. al.*[13]

IR (neat): $\tilde{\nu}$ =2958, 1723, 1422, 1378, 1211, 1134, 1084, 1026, 966,cm^{-1}. ^1H NMR(400.03 MHz, $CDCl_3$, 298 K)[13] δ = 1.97 (s, 3H, CH_3); 2.03 (s, 3H, CH_3); 2.06 (s, 3H, CH_3); 2.13 (s, 3H, CH_3); 3.45(m, 2H, CH_2Br); 3.89-4.12 (m, 2H, OCH2); 4.10-4.16 (m, 3H, $CHCH_2OAc$); 4.36 (m, 1H, CH); 4.53 (d, *J* = 8.03 Hz, 1H, CH); 5.02 (m, 1H, CH); 5.18

(m, 1H, CH); 5.28 (m, 1H, CH). $^{13}C\{^{1}H\}$ NMR (100.59 MHz, $CDCl_3$, 298 K) δ = 20.70 (1C, CH_3); 20.80 (1C, CH_3); 21.99 (1C, CH_3); 21.11 (1C, CH_3); 30.06 (1C, *C*H_2Br); 62.93 (1C, CH_2OAc); 67.09 (1C, CH); 68.68 (1C, CH); 68.68 (1C, CH_2CH_2O);69.08 (1C, CH); 70.95 (1C, CH); 101.65 ($C_{anomeric}$); 169.66 (1C, $CH_3C(O)O$); 169.95 (1C, $CH_3C(O)O$); 170.27 (1C, $CH_3C(O)O$); 170.34 (1C, $CH_3C(O)O$). Anal. Calcd. for $C_{16}H_{23}BrO_{10}$ C, 42.21; H, 5.09; Found C, 42.16; H, 5.17.. Mass Spectrometry (+ESI-MS) *m/z* (%): 102 (80), 217 (38), 301 (58), 323 (100), 353 (45), 381 (79), 413 (44), 429 (36), 477 [M+Na] (57).

2'-Azidoethyl 2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside

First reported by Chernyak *et al.,*[16] prepared as described by D'Agata *et. al.*[17] except that the crude product was dissolved in ethyl acetate and washed extensively with water. The mobile phase employed for the flash chromatography was CH_2Cl_2 / methanol 10:1 vol/vol instead of CH_2Cl_2 / CH_3COCH_3 24:1 vol/vol:

IR (neat): $\tilde{\nu}$ =2922, 1712, 1465, 1345, 1221, 1123, 1087, 1065, 979, 910 cm^{-1}. NMR(400.03 MHz, $CDCl_3$, 298 K)[17] δ = 1.95 (s, 3H, CH_3); 2.01 (s, 3H, CH_3); 2.09 (s, 3H, CH_3); 2.15 (s, 3H, CH_3); 3.46(m, 2H, CH_2N_3); 3.65-3.79 (m, 2H, CH_2CH_2O); 3.99-4.15 (m, 3H, C*H*CH_2OAc); 4.23 (1H, CH); 4.54 (d, *J* = 8.03 Hz, 1H, CH); 4.99 (m, 1H, CH); 5.20 (m, 1H, CH); 5.35 (m, 1H, CH). $^{13}C\{^{1}H\}$ NMR (100.59 MHz, $CDCl_3$, 298 K)[17] δ = 20.57 (1C, CH_3); 20.67 (1C, CH_3); 20.77 (1C, CH_3); 21.10 (1C, CH_3); 50.58(1C, *C*H_2N_3); 61.27 (1C, CH_2OAc); 66.43 (1C, CH); 67.02 (1C, CH); 68.38 (1C, CH_2CH_2O);68.55 (1C, CH); 70.84 (1C, CH); 101.15 ($C_{anomeric}$); 169.49 (1C, $CH_3C(O)O$); 170.16 (1C, $CH_3C(O)O$); 170.24 (1C, $CH_3C(O)O$); 170.38 (1C, $CH_3C(O)O$). Anal. Calcd.

for $C_{16}H_{23}N_3O_{10}$ C, 46.04; H, 5.55; N 10.07; Found C, 46.12; H, 5.44; N 9.98. Mass Spectrometry (+ESI-MS) *m/z* (%): 102 (22), 129 (21), 301 (11), 323 (32), 353 (50), 381 (100), 382 (66), 413 (21), 440 [M+Na] (88).

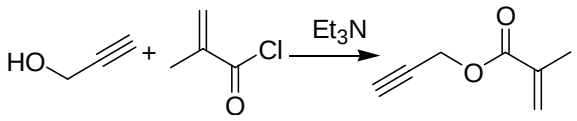
2'- Azidoethyl -O-β-D-galactopyranoside (28)

First reported by Susaki *et al* [18] prepared as described by Fazio *et. al.*[19]

IR (neat): $\tilde{\nu}$ =3322, 2953, 2098, 1644, 1303, 1265, 1121, 1061, 998, 910 cm^{-1} ^1H NMR (400.03 MHz, D_2O, 298 K) δ = 3.57 (m, 2H, CH_2N_3); 3.60 (m, 1H, CH) 3.64-3.72 (m, 2H, $CH_2CH_2N_3$); 3.76-3.80 (m, 2H, CH_2OH); 3.83 (m, 1H, CH); 3.93 (m, 1H, CH); 4.05 (m, 1H, CH); 4.46 (d , *J* = 7.78Hz, 1H, CH). $^{13}C\{^1H\}$ NMR (100.59 MHz, D_2O, 298 K) δ = 50.55 (1C, CH_2N_3); 60.95 (1C, CH_2OH); 68.38 (1C, CH_2CH_2O); 68.63 (1C, CH); 70.69 (1C, CH); 72.71 (1C, CH); 75.18 (1C, CH); 102.89 ($C_{anomeric}$); Anal. Calcd. for $C_8H_{15}N_3O_6$ C, 38.55; H, 6.07; N, 16.86; Found: C, 38.62; H, 6.00; N, 16.73; Mass Spectrometry (+ESI-MS) *m/z* (%): 102 (100), 118 (34), 172 (42), 217 (11), 272[M+Na] (65).

2.3 Synthesis of the monomer (2)

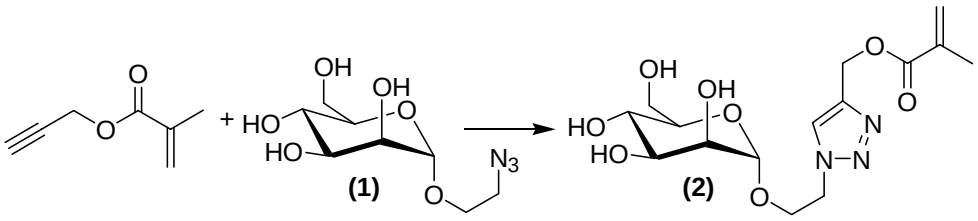
2-Methyl-acrylic acid prop-2-ynyl ester (propargyl methacrylate)



A solution of 2-propyn-1-ol (15.0 mL, 0.257 mol) and Et_3N (46.6 mL, 0.333 mol) in Et_2O (100 mL) was cooled to -20 ˚C and a solution of methacryloyl chloride (22.1 mL, 0.283 mol) in Et_2O (100 mL) was added dropwise over ca. 1 h. The mixture was then stirred at ambient temperature overnight. The triethylammonium chloride salt was filtered off and the volatiles removed under reduced pressure. The crude product was purified by distillation under reduced pressure, to give the propargyl methacrylate monomer (26.1 g, 0.210 mol, 81%) as a colourless liquid.

IR (neat): $\tilde{\nu}$ = 3295, 2957, 2128, 1732, 1637, 1453, 1293, 1152, 1013, 943, 813, 740. ^{1}H NMR (400.03 MHz, $CDCl_3$, 298 K) δ =1.93-1.94 (m, 3H, C*H*$_3$C=CH$_2$); 2.4-2.5 (m, 1H *H*C≡C), 4.73 (s, 2H, OCH_2), 5.58 (m, 1H, C=C*H*H); 6.14 (m, 1H, C=CH*H*) ^{13}C{^{1}H} NMR (100.59 MHz, $CDCl_3$, 298 K) δ = 18.2 (1C, C*H*$_3$C=CH$_2$); 52.2 (1C, OCH_2); 74.8 (1C, H*C*≡C); 77.8 (1C, HC≡*C*); 126.5 (1C, CH_3C=*C*H$_2$); 135.6 (1C, CH_3*C*=CH$_2$); 166.5 (1C, CO_{ester}). Anal. Calcd. for $C_7H_8O_2$ C, 67.73; H, 6.50;Found: C,67.17; H, 6.49. Mass Spectrometry (+ESI-MS) *m/z* (%): 102 (100), 118 (30), 125 [M+H] (5).

2-Methyl-acrylic acid 1-[2-(3,4,5-trihydroxy-6-hydroxymethyl-tetrahydro-pyran-2-yloxy)-ethyl]-1H-[1,2,3]triazol-4-ylmethyl ester (2)



To a mixture of propargyl mathacrylate (5.86 g, 47.0 mmol), the azido-sugar **(1)** (10.7 g, 43.0 mmol) in methanol/water (1:1 vol/vol, 150 mL) sodium ascorbate (0.85 g, 4 mmol)

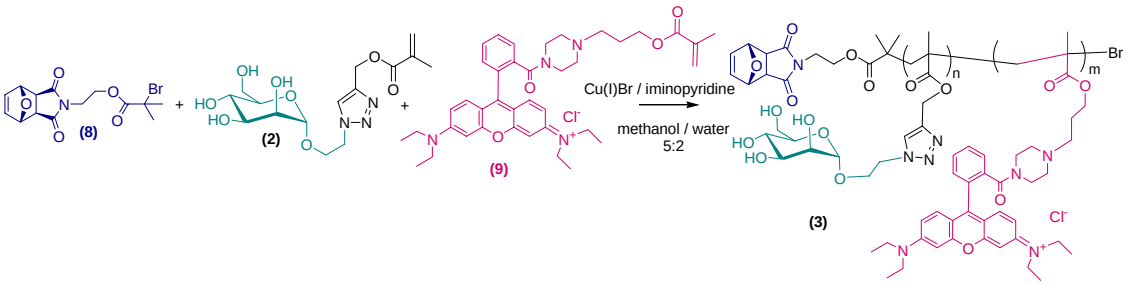
and Cu(II)SO_4 (0.54 g, 2 mmol) were sequentially added. The mixture was stirred at ambient temperature overnight, then the methanol was removed under reduced pressure and finally the mixture was freeze-dried. The residue was purified by flash chromatography (CC, SiO_2, methanol/CH_2Cl_2 1:10 vol/vol). The relevant fractions were collected and after removal of the solvent pure **(2)** (12.0 g, 32.1 mmol, 68 %) was isolated as a white solid.

^{1}H NMR (400.03 MHz, CD_3OD, 298 K) δ =1.93-1.94 (m, 3H, C*H*$_3$C=CH_2); δ = 3.48-3.53 (m, 2H, OCH*H*CH_2 + H-5); 3.62 (apparent t, *J* = 9.4 Hz, 1H, H-4); 3.69 (dd, *J* = 9.3, 3.3 Hz, 1H, H-3); 3.72 (dd, *J* = 11.9, 5.6 Hz, 1H, H'-6); 3.79-3.85 (m, 3H, H-2, H-6", H-7"); 3.92 (m, 2H, CH_2C*H*$_2$N); 4.13 (td, *J* = 6.8, 2.8 Hz, 2H, C*H*$_2$$CH_2$N); 4.42 (d, *J* = 1.5 Hz,1H, H-1); 5.34 (s, 2H, OCH_2), 5.74 (m, 1H, C=C*H*H); 6.16 (m, 1H, C=CH*H*) ^{13}C{^{1}H} NMR (100.59 MHz, CD_3OD, 298 K) δ = 17.30 (1C, C*H*$_3$C=CH_2); 50.11 (1C, *C*H_2N); 57.55 (1C, CH*C*H_2O); 60.58 (1C, *C*H_2OH); 65.47 (1C, O*C*$H_2$$CH_2$); 66.27 (1C, CH); 69.86 (1C, CH); 70.37 (1C, CH); 72.72 (1C, CH); 99.51($C_{anomeric}$); 120.12 (1C, N*C*H=C); 124.20 (1C, CH_3C=*C*H_2); 135.6 (1C, CH_3*C*=CH_2); 142.78 (1C, NCH=*C*); (166.5 (1C, CO_{ester}). IR (neat): $\tilde{\nu}$ = 3354, 2926, 1989, 1712, 1635, 1453, 1316, 1295, 1055, 974, 880, 814. Anal. Calcd. for $C_{16}H_{27}N_3O_8$ C, 48.25; H, 6.21; N, 11.25; Found: C, 47.85; H, 6.65; N, 11.79; Mass Spectrometry (+ESI-MS) *m/z* (%):374[M+H] (30), 375(5), 396[M+Na] (100) 397(18), 412[M+K] (7).

3.0 Polymerization.

3.1 Polymers (4) via *Path A*

3.1.1 Synthesis of polymers (3)



N-(Ethyl)-2-pyridylmethanimine ligand (6 µl, 0.04 mmol) the initiator **(8)** (3.7 mg 0.014 mmol), the glycomonomer **(1)** (0.51 g, 1.4 mmol) and rhodamine B monomer **(9)** (9.2 mg, 0.014 mmol) were charged to a dry Schlenk tube along with methanol/water mixture (7 mL, 5:2 vol/vol) as the solvent. The tube was sealed with a rubber septum and subjected to five freeze-pump-thaw cycles. This solution was then cannulated under nitrogen into another Schlenk tube, previously evacuated and filled with nitrogen, containing Cu(I)Br and a magnetic follower. The solution was stirred at ambient temperature under nitrogen atmosphere with constant stirring (t = 0). Samples were removed periodically using a degassed syringe for molecular weight and conversion analysis. At the end of the polymerization, the mixture was diluted with 10 mL of methanol/water mixture (5:2 vol / vol), and air was bubbled for at least 12 h. Then, the catalyst was removed by passing the reaction mixture through a column packed with basic alumina. The solvents were removed under vacuum and the residue was dissolved into the minimum amount of water and freeze-dried to give the polymers **(3)** as white powders.

The molecular weight of the polymer was calculated by ^{1}H NMR comparing the integral of the chain-end vinyl signal at 6.5 ppm and that of the triazole repeating unit, at 8.2 ppm.

Conversions were calculated by comparison between the signal relative to the C(O)OC*H*$_2$ protons of the glycomonomer **(1)** (triplet, 5.6 ppm, decreasing with time) and those of the analogous C(O)OC*H*$_2$ protons of the polymer repeating unit (broad singlet, 5.0 ppm, increasing with time).

(3a) [Cu(I)Br]:[ligand]:[**(8)**]:[**(1)**]:[**(9)**] = 1:2:1:100:1; T =20°C. DP(NMR) = 105, M_n(NMR)=32.2 kDa; M_w/M_n (SEC)=1.20.

(3b) [Cu(I)Br]:[ligand]:[**(8)**]:[**(1)**]:[**(9)**] = 1:2:1:20:0.2; T =20°C. M_n(NMR)=7.5 kDa; M_w/M_n (SEC)=1.22.

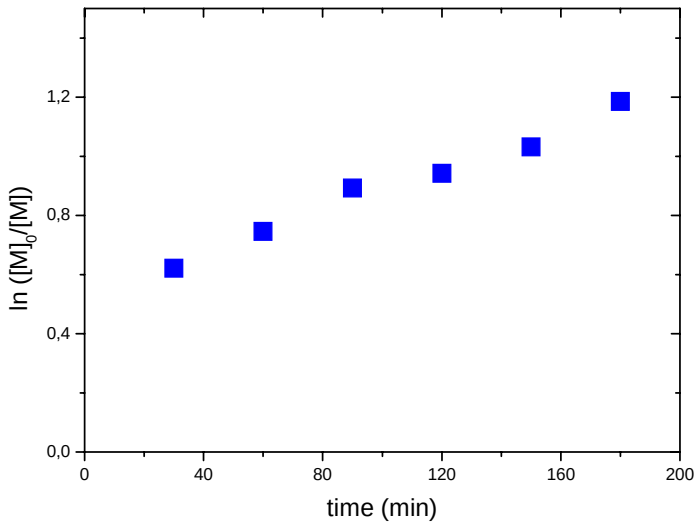


Figure S2. Polymer **(3a)** First order kinetic plot.

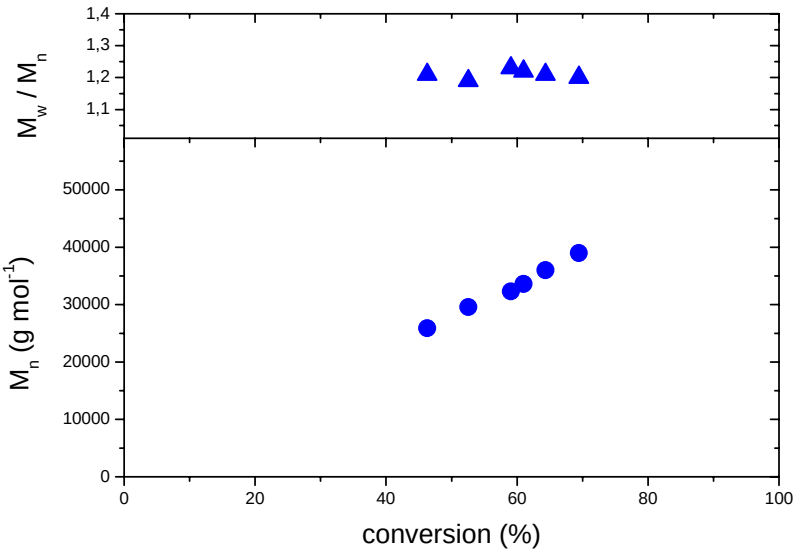


Figure S3: Polymer **(3a)**. Evolution of M_n (SEC) and M_w/M_n with conversion.

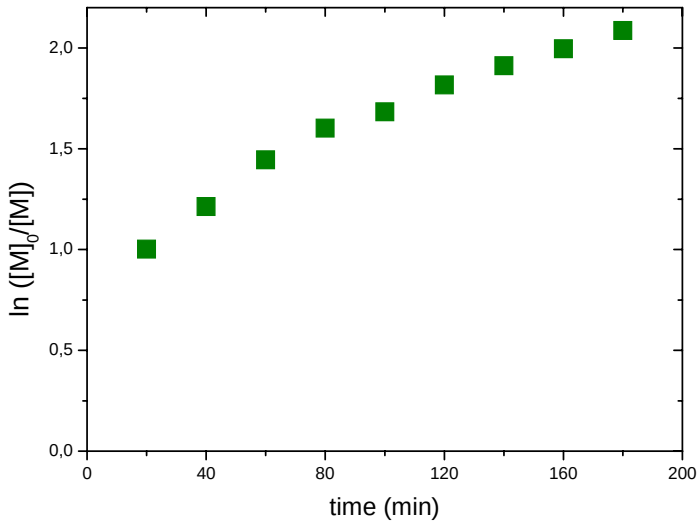


Figure S4: Polymer **(3b)**, first order plot

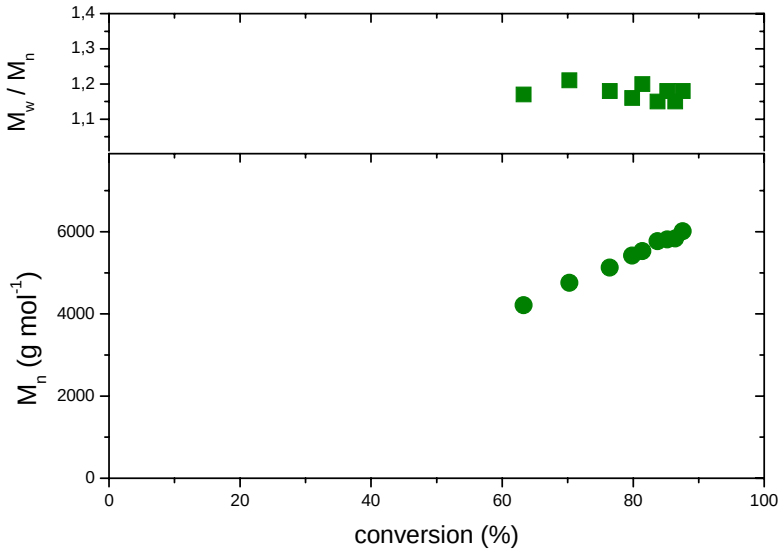


Figure S5: Polymer **(3b)**: Evolution of M_n (SEC) and M_w/M_n with conversion

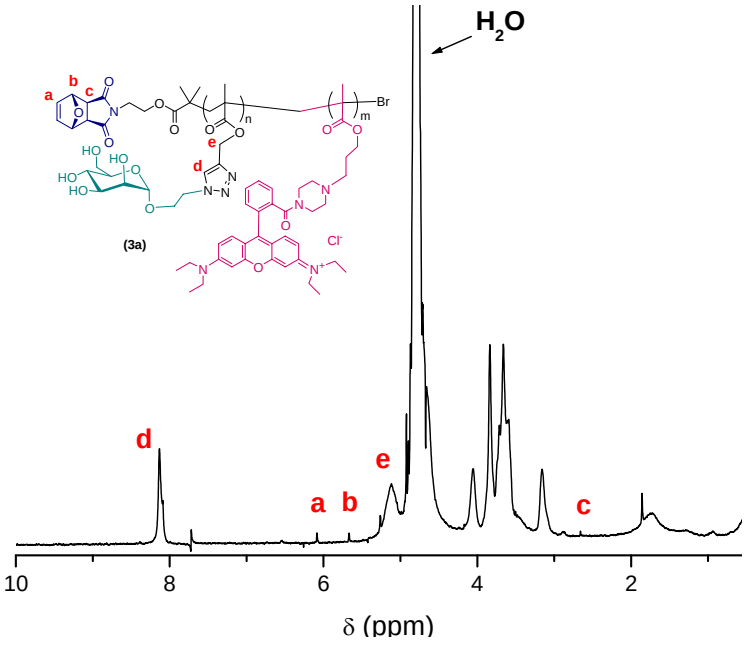
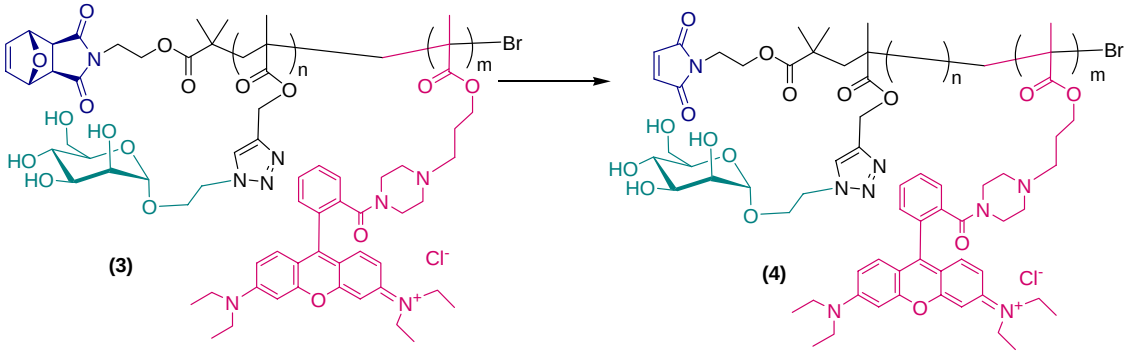


Figure S6: ^{1}H NMR spectrum of polymer **(3a)** in D_2O

3.1.2 Polymer deprotection (Retro Diels-Alder reactions)



Method 1: deprotection in toluene

Polymer **(3a)** (50 mg, 1.3 10^{-3}) was suspended in toluene (5 mL) and the mixture was refluxed for 12 h. The suspension was then allowed to cool down to ambient temperature and filtered. The collected solid was then washed with dichloromethane to give **(4a)** as a pink powder.

Method 2: solvent-free deprotection in the vacuum oven (4 mmHg) at 80 °C

Polymer **(3a)** (50 mg, 1.3 10^{-3} mmol) was placed in a vacuum oven (reduced pressure, 4 mmHg) at 80°C and left at this temperature overnight (12 h) to give **(4a)** as a pink powder.

The molecular weight of the polymers was calculated by ^{1}H NMR, by comparison of the integrals of the maleimide vinyl signal at 6.6 ppm and that of the triazole repeating unit, at 8.2 ppm and agreed well with the one expected one, basing on the molecular weight of the precursor **(3a)**.

Polymer **(4a)**: M_n(NMR)=29.8 kDa; M_w/M_n (SEC)=1.20

Polymer **(4b)**: M_n(NMR)=8.2 kDa; M_w/M_n (SEC)=1.23

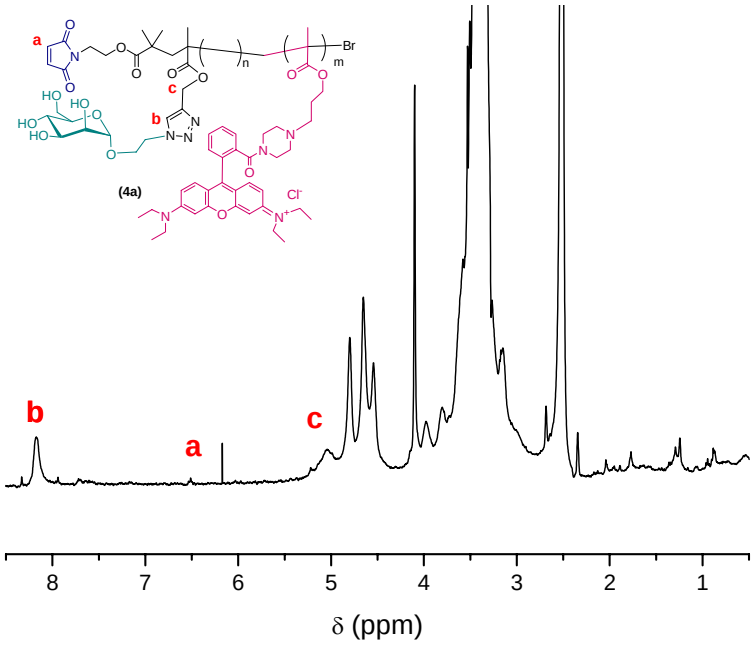
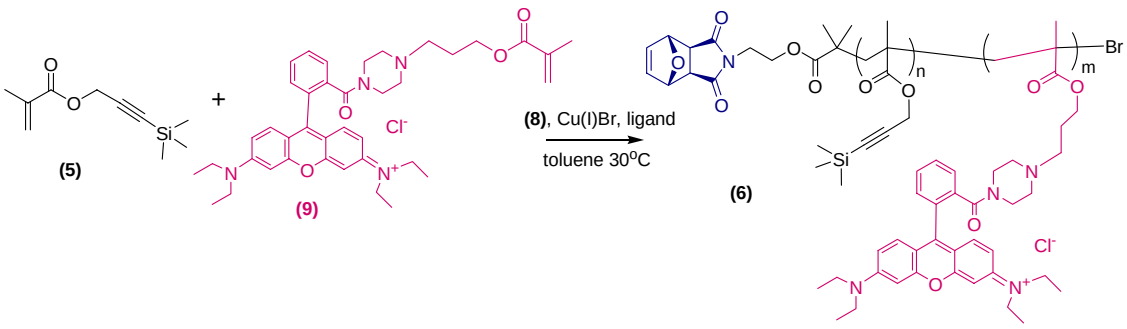


Figure S7: ^{1}H NMR spectrum of polymer **(4a)** in DMSO-d_6

3.2 Polymer (4c) via *Path B*

3.2.1 Synthesis of polymer (6)



(5) (2.02 g, 10.3 mmol), *N*-(ethyl)-2-pyridylmethanimine ligand (0.129, 0.826 mmol), initiator **(8)** (0.0566 g, 0.206 mmol) and mesitylene (internal NMR standard, 1.0 mL) were charged into a dry Schlenk tube along with toluene (12.0 mL). A solution of Rhodamine B monomer (0.0693 g, 0.103 mmol) in methanol (4 mL) was subsequently added; the tube was sealed with a rubber septum and subjected to five freeze-pump-thaw cycles. This solution was then cannulated under nitrogen into a second Schlenk tube, previously evacuated and filled with nitrogen, containing Cu(I)Br (0.119 g, 0.823 mmol) and a magnetic follower. The temperature was adjusted to 30 ˚C with constant stirring (t = 0). Samples were removed periodically using a degassed syringe for molecular weight and conversion analysis. At the end of the polymerization the mixture was diluted with 20 mL of toluene and air was bubbled through for 4 h. The reaction mixture was passed through a short neutral alumina column and subsequently washed with toluene. The volatiles were removed under reduced pressure and the residues dissolved in THF (ca. 10

mL) prior to precipitation into methanol/water (10:2 vol/vol) mixture (ca. 200 mL). The white solid was isolated by filtration, washed with additional methanol/water mixture and volatiles removed under reduced pressure.

M_n(NMR) = 8.0 kDa; DP(NMR) = 41; M_w/M_n(SEC) = 1.22.

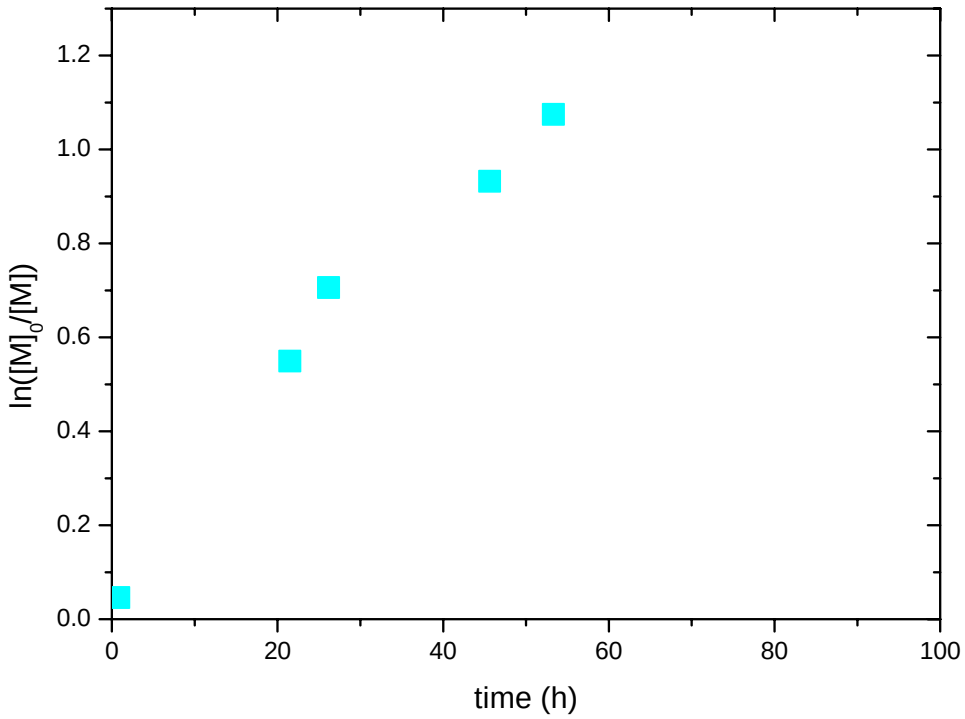


Figure S8. Polymer **(6),** first order kinetic plot.

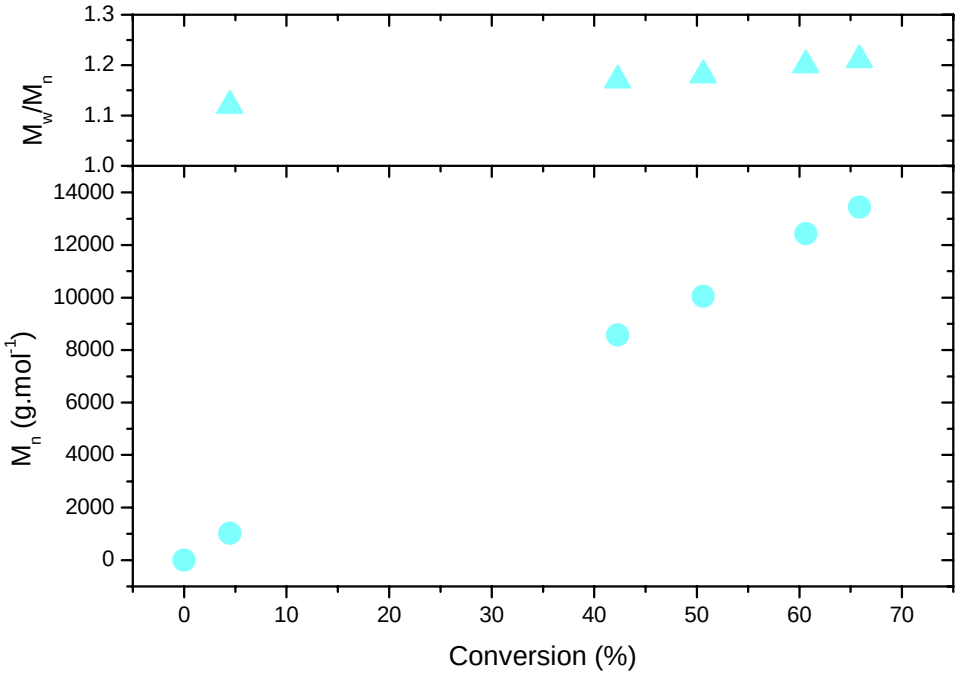
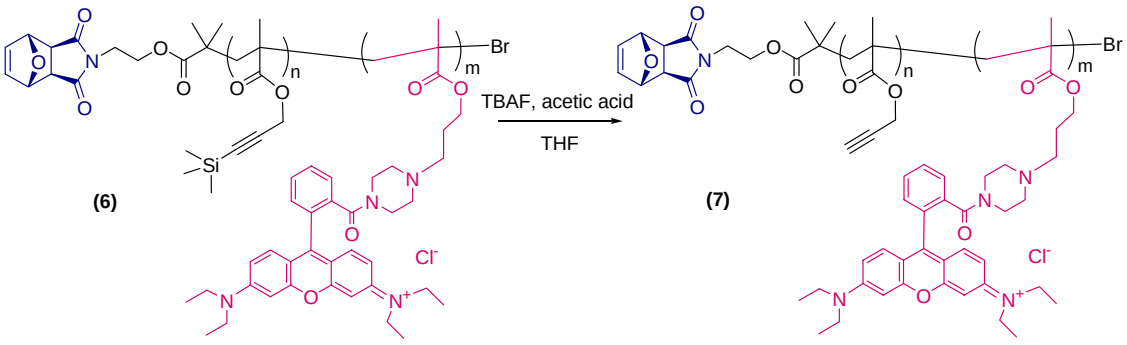


Figure S9: Polymer **(6)**: Evolution of M_n (SEC) and M_w/M_n with conversion

3.2.2 Removal of $Si(CH_3)_3$ protecting group: synthesis of polymer (7)

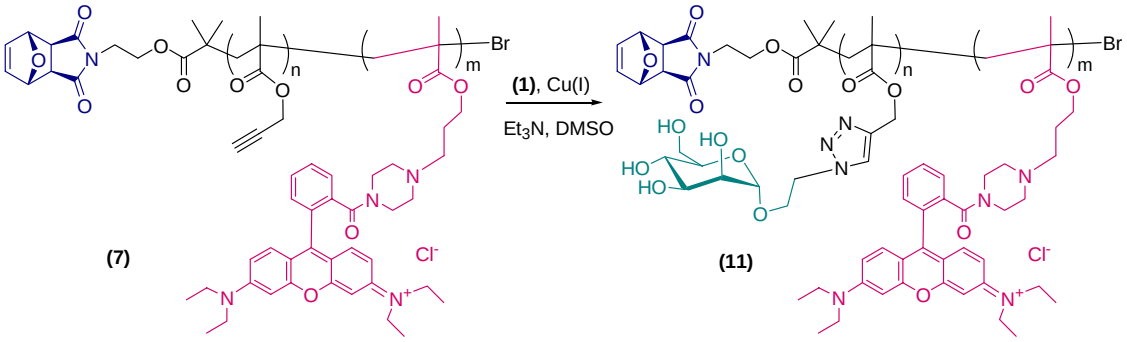


Polymer **(6)** (500 mg, 2.55 mmol of alkyne-trimethylsilyl groups) and acetic acid (0.22 ml, 3.8 mmol, 1.5 eq. with respect to the alkyne-trimethylsilyl groups) were dissolved in THF (10 mL). Nitrogen was bubbled (ca. 10 min) and the solution was cooled to -20 °C. A 0.20 M solution of TBAF·$3H_2O$ in THF (1.2 g, 3.2 mmol, 1.5 eq to the alkyne-

trimethylsilyl groups) was added dropwise in ca. 2 min. The resulting mixture was stirred at this temperature for 30 min and then allowed to warm up to ambient temperature. After stirring overnight, Amberlite IR-120 (PLUS) ion-exchange resin was added and the resulting slurry stirred for 30 min. The resin was then filtered off, the resulting solution was concentrated under reduced pressure and the polymer was precipitated into petroleum ether.

M_n(NMR) = 5.0 kDa; DP(NMR) = 40; M_w/M_n(SEC) = 1.25.

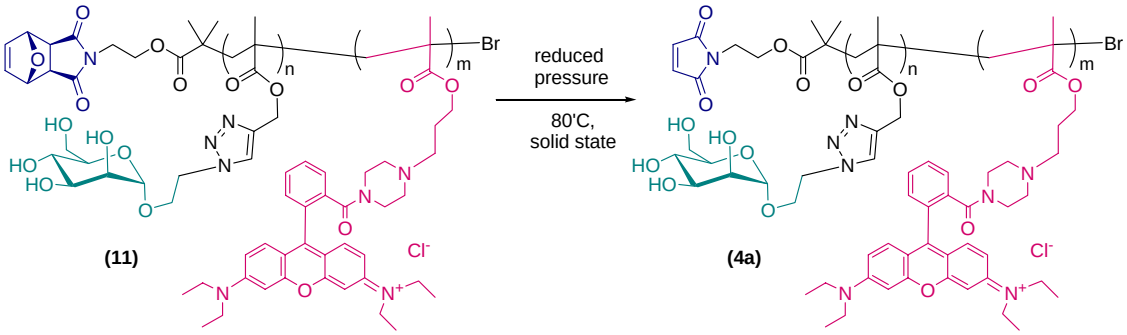
3.2.3 Glycopolymer (11): sugar "clicking"



A solution of polymer **(7)** (50 mg, 0.40·mmol of clickablealkyne units), the azido-sugar **(1)** (0.1g, 0.40 mmol) and triethylamine (26 mg, 35 µL, 0.20 mmol) in DMSO (10 mL) was degassed by bubbling nitrogen for 10 min. [$(PPh_3)_3CuBr$] (75.0 mg, 0.08 mmol) was then added and nitrogen was bubbled into the resulting solution for further 5 minutes. The very pale yellow clear solution was stirred at ambient temperature for 3 days and then ionic-exchange resin (Smopex) was added and the suspension gently stirred overnight. After filtration, the solution was added dropwise to a 2:1 CH_2Cl_2/methanol mixture and the polymer was separated by centrifugation. The precipitated polymer was

dissolved in 10 ml water and dialyzed against water in order to remove residual traces of mannose azide **(1)**. The resulting solution was then freeze-dried, to give polymer **(11)** as a pink powder that was used for the next step without further purification.

3.2.4 Glycopolymer (4c): retro Diels-Alder maleimide deprotection



Polymer **(11)** (10 mg, 6.5× 10^{-4}mmol) was left in a vacuum oven at 80°C and ca. 4 mmHg for 18 h to give **(4c)** as a pink powder.

M_n(NMR) = 16.4 kDa; DP(NMR) = 44; M_w/M_n(SEC) = 1.28.

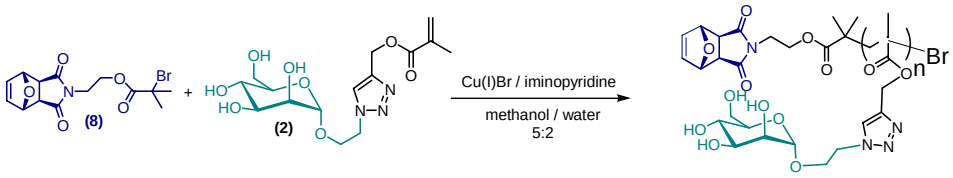
3.2.5 Glycopolymers with different mannose epitope density: (23)-(27)

The reaction conditions were analogous to those described for the synthesis of the maleimide glycopolymer **(4c)** (Path B)

General procedure: synthesis of **(24)**. A solution of clickable scaffold **(12)** (17.4 kDa, 90 mg, 0.73 mmol), mannose azide **(1)** (63 mg, 0.65 mmol), galactose azide **(28)** (54 mg, 0.22 mmol) and triethylamine (51 μL, 0.37 mmol) in DMSO (8 mL) was deoxygenated by N_2 bubbling, for 15 minutes. $[(PPh_3)_3Cu(I)Br]$ (135 mg, 0.15 mmol) was added and

dinitrogen was bubbled for further 5 minutes. The solution was stirred at room temperature for 2 days, Smopex 112 (ca. 300 mg) was added and the resulting suspension stirred at ambient temperature overnight. After filtering, the polymer was precipitated in CH_2Cl_2 / methanol (150 mL, 2:1 vol / vol), the polymer isolated by centrifugation, redissolved in the minimum amount of water and finally reprecipitated in THF. The polymer was isolated by centrifugation, redissolved in the minimum amount of water and finally freeze-dried to give the glycopolymer **(24)** as light pink solid.

3.2.6 Non-fluorescent glycopolymer (28)



N-(Ethyl)-2-pyridylmethanimine ligand (9 µL, 0.06 mmol) the initiator **(8)** (5.3 mg 0.020 mmol), the glycomonomer **(2)** (0.73 g, 2.0 mmol) and were poured into a dry Schlenk tube along with a methanol/water mixture (7 mL, 5:2 vol/vol). The tube was sealed with a rubber septum and subjected to five freeze-pump-thaw cycles. This solution was then cannulated under nitrogen into another Schlenk tube, previously evacuated and filled with nitrogen, containing Cu(I)Br and a magnetic follower. The solution was stirred at ambient temperature under nitrogen atmosphere with constant stirring (t = 0). Samples were removed periodically using a degassed syringe for molecular weight and conversion analysis. At the end of the polymerization, the mixture was diluted with 10 mL of methanol/water mixture (5:2 vol/vol), and air was bubbled for at least 12 h. The copper salts were removed by passing the reaction mixture through a short basic alumina pad.

The solvents were removed under vacuum and the residue was dissolved into the minimum amount of water and freeze-dried to give the polymer **(29)** as a white powder.

Polymer **(29)**: DP(NMR): 136; M_n(NMR)=50.7 kDa; M_w/M_n (SEC)=1.28

4.0 BSA-glycoprotein mimic (10)

4.1 Conjugation method 1: excess of conjugating polymer (4).

BSA (2 mg, 0.03 μmol) and polymer **(4a)** (16 mg, 0.30 μmol) were dissolved in 1.5 mL of 50 mM phosphate buffer (pH = 7.0). The mixture was gently stirred at ambient temperature and reaction was followed by SEC-HPLC. After three hours no appreciable changing was detected by SEC-HPLC analysis (SEC-HPLC system fitted with two BioSep-SEC-S3000 columns and fluorescence detector with λ_{exit} = 566 nm, λ_{em} = 596 nm). DMSO (1.5 mL) was then added to the conjugation solution via syringe. Aliquots taken after 10 minutes and 1.5 hours were analysed by SEC-HPLC and new peak, increasing with time, corresponding to a higher molecular weight fluorescent species, identified as the BSA-glycopolymer conjugate **(10)**, was detected (Figure S8). After 1.5 h no further detectable increases in the new peak size was detected. The conjugate was separated from unreacted glycopolymer **(4)** by SEC (Figure S9). Unreacted BSA and BSA dimer were removed by passing a solution of the conjugate in through a column packed with sepharose-immobilized Concanavalin A** eluting with phosphate buffer (50 mM, pH = 7.0) as the mobile phase. The conjugate **(10)** was fully retained by the column and formed a visible bright pink layer at the top of it. After the non-unreacted BSA and BSA dimer were eluted, D-mannose (500 mM) was added to the mobile phase and a quick elution of the conjugate was observed. The fraction containing **(10)** was the

dialyzed against water and the resulting solution of **(10)** was directly employed for the bioassays.

** Con A-Sepharose was prepared using 2 ml of a 5 mg ml^{-1} solution of purified Con A (Sigma Chemical Company) dissolved in phosphate-buffered saline incubated with 2 ml packed volume of cyanogen-bromide activated Sepharose 6B for 2 hours at room temperature. The Sepharose was washed in cold saline and then incubated in 10 ml of 1M ethanolamine hydrochloride pH 8.0 for 1 hour at room temperature to derivatize non-reacted active groups. Protein coupling efficiency was determined as 96% by comparison of A280 readings of solution phases taken before and after incubation with activated resin. The Con A-Sepharose was washed in 30 ml of phosphate-buffered saline prior to loading into a 2 mL column case and stored in the same buffer at 4 ºC.

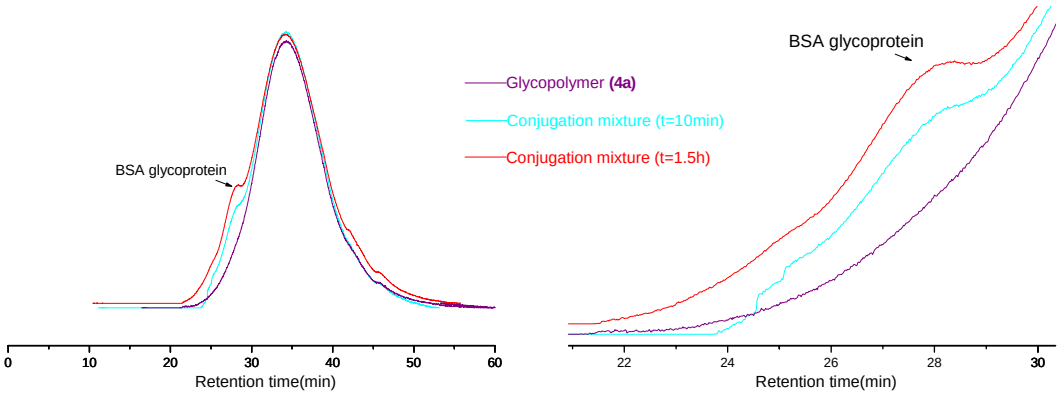


Figure S10: Conjugation of BSA with maleimide-terminated glycopolymer **(4a)** in 1:1 DMSO/phosphate buffer (pH 7, 50mM): SEC-HPLC chromatography traces using fluorescence detection (λ_{exit} = 566 nm, λ_{em} = 596nm).

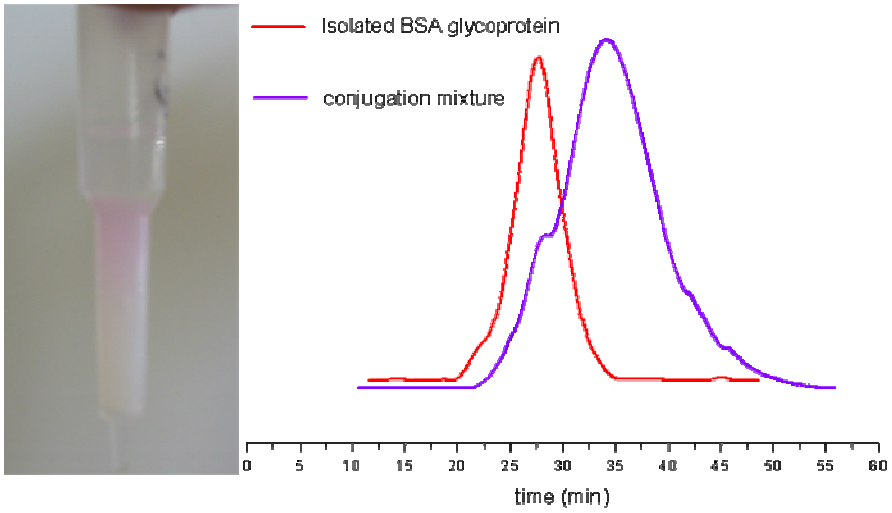


Figure S11: Left: immobilized Concanavalin A column purification of conjugate **(10)** before elution with 1.0 M mannose solution. Right: SEC analysis of the conjugation reaction mixture (purple) and the final purified biohybrid **(10)**.

4.2 Conjugation method 2: excess of BSA protein

General procedure: synthesis of **(10)**. To a solution of BSA (100 mg, 1.5 μmol) in phosphate buffer (1.5 mL, 50 mM, pH = 7.0) glycopolymer **(4a)** (10 mg, 0.18 μmol dissolved in 1.5 ml DMSO) was added. The mixture was gently stirred at ambient temperature for 5 hours, then the crude product was purified by anion-exchange chromatography using a source Q column(10 mL, Amersham) (Figure S9).

Separation conditions. Buffer A: 20mM TRIS-HCl buffer pH 9.0; Buffer B: 20 mM TRIS-HCl, 1 M NaCl. Solvent gradient program: 100 % A for 3column volumes (CV), from 0 to 50 % B in 18 CV, 50 % B constant for 4 CV, 100 % A for 3 CV. The flow rate:

5 mL / min, T : 4 ^{o}C. Program time: 28 CV, sample concentration was 2 mg mL^{-1} and injected volume was 2 mL.

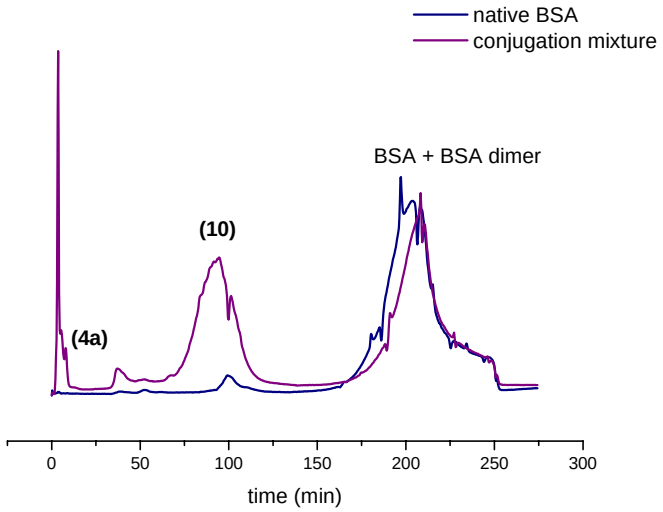


Figure S12. Anion-exchange chromatography purification of the conjugate **(10)**

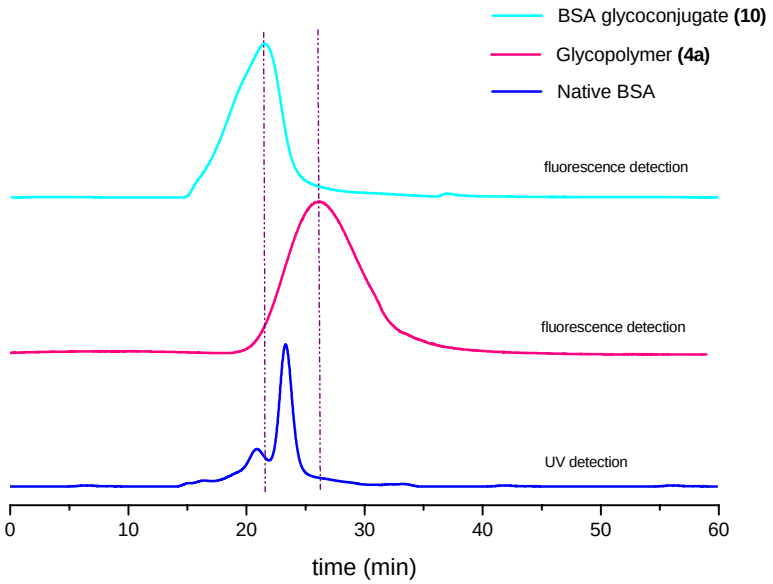


Figure S13. SEC analysis of the conjugate **(10)** previously purified by anion-exchange chromatography (top), glycopolymer **(4a)** (middle), commercially available BSA (bottom). UV detection: 280 nm; Fluorescence detection λ_{exit} = 566 nm, λ_{em} = 596nm.

The fractions containing the bioconjugate **(10)** obtained after anion exchange FPLC purification, collected, were dialysed against 25 mM pH 7.4 HEPES buffer containing NaCl (150 mM), $CaCl_2$ (2 mM) and $MgCl_2$ (2 mM) and employed directly for the *in vitro* bioassays. The concentration of **(10)** in this solution was determined by UV-vis spectroscopy, measuring its absorbance at 280 nm and at 572 nm (rhodamine B chromophore) and comparing these experimental results with two calibration curves obtained using native BSA and polymer **(3a)** starting material at various concentrations. At 572 nm only the synthetic polymer part of the conjugate **(10)** was responsible for the observed absorbance, while at 280 nm both the protein and the synthetic polymer contributed to it. For the latter case, it was assumed that at a given concentration **(10)** had the same absorbance of a solution of the two unreacted starting material, BSA and glycoploymer **(3a)**. As virtually the same bioconjugate **(10)** concentration was obtained in the two cases, $1.63 \cdot 10^{-6}$ mmol mL^{-1} (280 nm) and $1.62 \cdot 10^{-6}$ mmol mL^{-1} (572 nm) it was concluded that this assumption was more than reasonable.

4.3 Circular dichroism analysis

Table S1: BSA-glycopolymer conjugates analysed by circular dichroism and their concentration in the CD analysis

Protein conjugates	Concentration (mmol / mL)[a]	Mannose (%)	Galactose (%)
(23)	5.5×10^{-7}	100	0
(24)	6.6×10^{-7}	75	25
(25)	4.5×10^{-7}	50	50
(26)	5.1×10^{-7}	25	75
(27)	5.1×10^{-7}	0	100

[a] samples dissolved in phosphate buffer (50 mM, pH 8.5)

Table S2. Circular dichroism (CD) analysis of the conjugates **(23)-(27)**

Glycoprotein mimics	α-helix (%)	β-sheet (%)
(23)	65	1
(24)	59	0
(25)	57	0
(26)	53	1
(27)	51	2
Native BSA	54	1

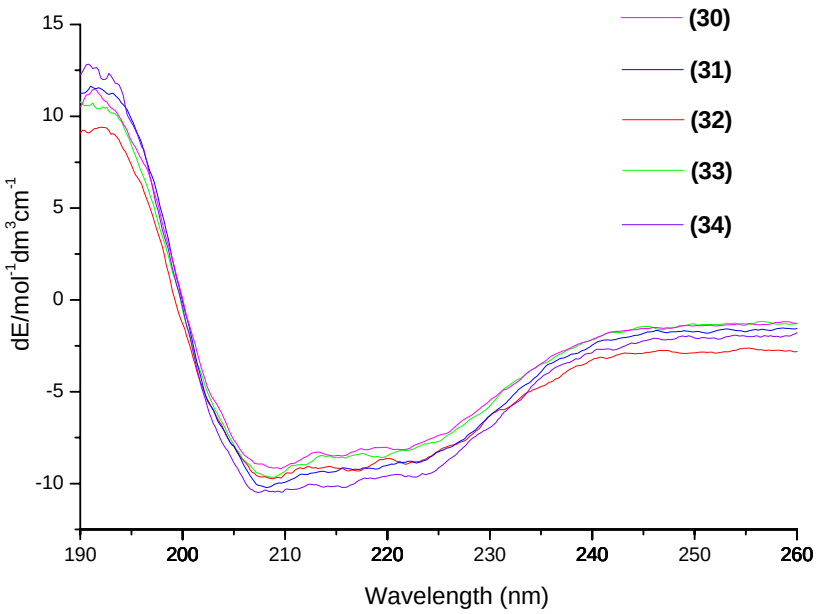


Figure S14. CD spectra of the conjugates **(30)-(34)**

CD spectra of the conjugating polymers did not give an appreciable signal under the conditions (concentration) employed for the analysis of the conjugates and native BSA.

5.0 Bioassays

5.1 Surface plasmon resonance analysis

Sensorgrams were recorded at 25 ºC at a flow rate of 5 µL per minute on a BIAcore 2000 surface plasmon resonance instrument (GE Healthcare). Protein-coated CM5 sensor flow cells were prepared at 1000-2000 response units per cell at pH 4.0 (RU) using the amine coupling kit provided by the manufacturer. Negative control flow cells were coated with albumin-free bovine haemoglobin (Sigma Chemical Company). Samples were run in

HEPES-buffered saline pH 7.4 supplemented with 5 mM $CaCl_2$ and 0.001% surfactant P-20, and flow cells regenerated using the same buffer containing 5 mM EDTA instead of $CaCl_2$. Data were processed using the accompanying BIA evaluation software.

5.2 Complement activation assay

Assays were carried using a modified protocol for the Lectin Pathway kits described by Seelen *et al*[20] using diluted human serum as a source of complement activity. Target materials including glycopolymer-BSA conjugate, BSA, mannan and haemoglobin, were dissolved in 100 mM $NaHCO_3$ pH 9.0 buffer and incubated on Nunc Maxisorp Lock-Well microtitre plates overnight at 4 degrees for optimal immobilization. Plates were washed and human serum, diluted 1 in 100 in commercially available Lectin Pathway Dilution Buffer (Eurodiagnostica), was incubated on the wells for 60 minutes at 37 degrees. Diluted serum was discarded, and plates washed three times in Complement Assay Wash Buffer (Eurodiagnostica). Plates were then probed by immunoassay using an alkaline phosphatase-linked monoclonal antibody recognizing a neo-epitope of activated complement C9 (Eurodiagnostica). Incubation of antibody conjugate was carried out for 45 minutes at ambient temperature. Following another washing step as described above, plates were developed with p-nitrophenyl-phosphate and positive signal indicated by yellow colour measured by optical density at 405 nm. The reaction was stopped when the mannan-coated wells, as positive controls, reached an A405 value greater than 1.0., Values were corrected for blank background and negative control background provided by the haemoglobin-coated microwells, typically giving an A(405nm) of 0.07. In accordance with the established protocol, values less than 0.2 were considered negative.

5.3 Esterase activity assay[21-23]

Fractions obtained after FPLC anionic-exchange purification of bioconjugate **(31)** were concentrated by using 0.5 mL Vivaspin concentrators (30 kDa MWCO), exchanging the buffer to PBS (50 mM, pH 8.5). The concentration of the conjugate was adjusted to $9.1 \cdot 10^{-4}$ mM by dilution with PBS (50 mM, pH 8.5) (concentration determined spectroscopically by UV, at λ = 280 nm, using a previously built calibration curve). 1.0 mL of this solution was poured into a 1 mL quartz cuvette, which was then placed in a UV-vis spectrometer sample holder. 10 µL of a 10 mM solution of *p*-nitrophenyl acetate in acetonitrile was added and the evolution of the absorbance of this sample over time was recorded. The test was then repeated using native BSA ($9.1 \cdot 10^{-4}$ mM in 50 mM, pH 8.5) instead of conjugate **(31)** (Figure S15).

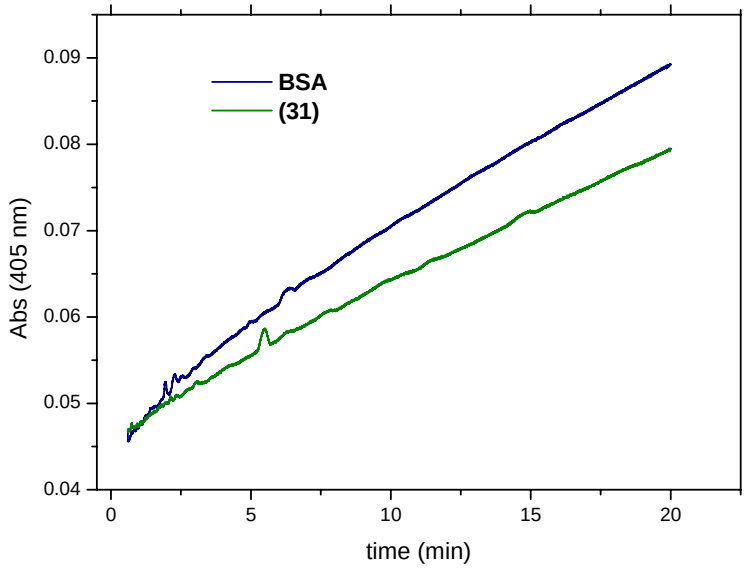


Figure S15. BSA (blue trace) and BSA-conjugate **(31)** (green trace) – catalyzed hydrolysis of *p*-nitrophenyl acetate.

6.0 References

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