Electronic Supplementary Material (ESI) for Polymer Chemistry.

Supporting information

Synthesis of well-defined cyclic glycopolymers and the relationship between their physical properties and their interaction with lectins

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Materials

The following chemical agents were purchased from commercial sources and were used as received except the notion: Diethyl ether was purchased from Kanto Chemical (Tokyo, Japan). Triethylamine (TEA, 99%), 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide hydrochloride (EDC·HCl, 98%), 2-(2-chloroethoxy)ethanol (98%), sodium ascorbate ($_L$-Asc-Na, 98%), sodium azide (99%), lithium bromide (LiBr, 99%) and *N*,*N*-dimethylacrylamide (DMA, 99%) were purchased from Tokyo Chemical Industry (Tokyo, Japan). 4-Dimethylaminopyridine (DMAP, 99%), 2,2'-azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride (AIPD, 98%), copper(II) sulfate pentahydrate ($CuSO_4$), and anhydrous sodium sulfate (Na_2SO_4, 99%) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Acetonitrile (MeCN) and propargyl acrylate (98%) was purchased from Sigma Aldrich (St. Louis, USA). Concanavalin A - fluorescein isothiocyanate (FITC-ConA) and blood cell suspension from a rabbit was purchased from Cosmo Bio Co. (Tokyo, Japan). Mannose acrylamide (Man)[1] and 2-{[(butylsulfanyl)carbonothioyl]sulfanyl}propanoic acid (BTPA)[2] were synthesized referring the previous reports. Commercial monomers including the radical inhibitor were purified by passing through an alumina column prior to use.

Characterization

Proton and carbon nuclear resonance (^{1}H NMR and ^{13}C NMR) spectra were recorded on a JEOL-ECP400 spectrometer (JEOL, Tokyo, Japan). Size exclusion chromatography (SEC) with organic solvent was performed on a HLC-8320 GPC Eco-SEC equipped with a TSKgel Super AW guard column and TSKgel Super AW (4000 and 2500) columns (TOSOH, Tokyo, Japan). The SEC analyses were performed at a flow rate of 0.5 mL/min by injecting 20 μL of a polymer solution (2 g/L) in DMF with 10 mM LiBr. The SEC system was calibrated with a polymethylmethacrylate standard (Shodex). All the samples for SEC analysis were previously filtered through a 0.45 μm filter. UV-vis spectra were recorded at room temperature using an Agilent 8453 spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). Fourier transform infrared (FTIR) spectra were recorded on a JASCO FT/IR-4700 (JASCO, Japan). Dynamic light scattering (DLS) measurements were performed on a ZETASIZER NANO-ZS (Malvern, UK) by using a 1 mL disposable cell of a polymer solution (3 mg/mL) in the buffer solution. All the samples for DLS were previously filtered through a 0.22 μm filter. The water used in this research was purified using a Direct-Q Ultrapure Water System (Merck, Ltd, Darmstadt, Germany). DSC measurements were performed using a Hitachi High Tech Science X-DSC7000. Scans were performed under a nitrogen atmosphere using a heating rate of 10 °C/min (from 35 to 180 °C). The glass transition temperature was determined from the second heating curve. Fluorescence quenching measurement was performed on a JASCO FP-6500 (JASCO, Japan). Peak-fitting analysis was conducted with PeakFit V4.12 (Systat Software, Inc., CA, USA).

Synthesis of 2-(2-azidoethoxy) ethanol. To the solution of 2-(2-chloroethoxy)ethanol (6.2 g, 50 mmol) in H_2O (25 mL), sodium azide (6.5 g, 100 mmol) was added. The mixture solution was heated at 80 °C with refluxed overnight. The solution was extracted with Et_2O (20 mL × 5). The organic phase was dried with Na_2SO_4 and was filtered. The filtrate was concentarted under reduced pressure (250 ~ 500 hPa). The product was obtained as colourless oil (5.79 g, 88%).

^{1}H NMR (400 MHz, $CDCl_3$) δ in ppm: 3.76 (m, C***H***$_2$−OH, 2H), 3.70 (t, *J* = 5.0 Hz, N_3−CH_2−C***H***$_2$−O, 2H), 3.62 (t, *J* = 4.6 Hz, −O−C***H***$_2$−CH_2−OH, 2H), 3.41 (t, *J* = 5.0 Hz, N_3−C***H***$_2$−CH_2−O, 2H), 2.11 (t, *J* = 6.4 Hz, −O***H***, 1H).

^{13}C NMR (100 MHz, $CDCl_3$) δ in ppm: 72.5 (−***C***H_2−OH), 70.2 (N_3−CH_2−***C***H_2−O), 61.9 (−***C***H_2−CH_2−OH), 50.8 (N_3−***C***H_2−).

Synthesis of 2-(2-azidoethoxy)ethyl 2-(((butylthio)carbonothioyl)thio)propanoate (AEBCP). To the solution of BTPA (950 mg, 4 mmol) in dry CH_2Cl_2 (30 mL), EDC·HCl (786 mg, 4.1 mmol) was added (color of the solution changed from yellow to red). 2-(2-Azidoethoxy) ethanol (511 mg, 3.9 mmol) and DMAP (98 mg, 0.8 mmol) were added. The mixture solution was stirred for 24 h at room temperature. The reaction procedure was confirmed by TLC (EtOAc: hex = 2: 3). The solution was washed with 1 wt% HCl(aq) (30 mL × 3), Milli-Q water (30 mL × 1), and brine (30 mL × 1). The organic phase was dried with Na_2SO_4 and was filtered. The filtrate was concentarted under reduced pressure, and purified by silica column chromatography (EtOAc: hex = 1: 5). The product was obtained as yellow oil (502 mg, 37%).

^{1}H NMR (400 MHz, $CDCl_3$) δ in ppm: 4.83 (q, *J* = 7.2 Hz, CH_3−C***H***(ester)−S, 1H), 4.29 (dd, *J* = 3.6 and 5.2 Hz, O−CH_2−C***H***$_2$−ester, 2H), 3.70 (ddd, O−C***H***$_2$−CH_2−ester, *J* = 3.6 and 5.2 Hz, 2H), 3.66 (t, *J* = 5.2 Hz, N_3−CH_2−C***H***$_2$, 2H), 3.37 (t, *J* = 5.2 Hz, N_3−C***H***$_2$−, 2H), 3.35 (t, *J* = 7.2 Hz, S−C***H***$_2$−, 2H), 1.67 (quin, *J* = 7.6 Hz, S−CH_2−C***H***$_2$−, 2H), 1.60 (d, *J* = 7.2 Hz, C***H***$_3$−CH(ester)−S, 3H), 1.41 (sext, *J* = 7.2 Hz, −C***H***$_2$−CH_3, 2H), 0.92 (t, *J* = 7.2 Hz, −CH_2−C***H***$_3$, 3H).

^{13}C NMR (100 MHz, $CDCl_3$) δ in ppm: 222.1 (S−***C***−S), 171.8 (−O−***C***=O), 70.2 (N_3−CH_2−***C***H_2−O), 69.0 (−O−***C***H_2−CH_2−ester), 64.9 (−O−CH_2−***C***H_2−ester), 50.8 (N_3−***C***H_2−), 48.0 (CH_3−***C***H(ester)−S), 37.0 (S−C***H***$_2$−C_3H_7), 30.0 (−CH_2−***C***H_2−C_2H_5), 22.1 (−C_2H_4−***C***H_2−CH_3), 16.7 (***C***H_3−CH(ester)−S), 13.7 (−C_3H_6−***C***H_3).

Preparation of glycopolymers by RAFT polymerization (P_{54} and P_{106}). DMA, glycomonomers (Man), RAFT agent (AEBCP), and AIPD (initiator) were dissolved in the solvent (DMF/water = 90: 10 vol %). The monomer concentration and the ratio of reactants are shown in Table S1. The ratio of [RAFT]/[initiator] was set at 1:0.1. The solution was prepared in a glass tube and degassed by freeze−thaw cycles (three times). The glass tube was sealed and put in an oil bath. The reaction

proceeded at 70 °C for 4 h. The reaction was stopped by exposing the solution to air. The monomer conversion was
determined by ^{1}H NMR. The polymer solutions were diluted with MeOH (2 mL) and reprecipitated in Et_2O (twice). The precipitated polymers were dried *in vacuo*.
^{1}H NMR (400 MHz, D_2O) δ in ppm: 7.94 (triazole), 5.97 (anomer H-1), 4.15 (O−CH_2−C***H***$_2$−ester), 4.01 (H-6a), 3.8−2.7 (H-3, 4, 5, 6b, CON(C***H***$_3$)$_2$, and amide−C_2***H***$_4$−triazole), 2.7−2.2 (−C***H***− main chain of DMA), 2.1−1.1 (−C***H***− main chain of Man, −C***H***$_2$− main chain), 1.03 (C***H***$_3$−CH(ester)−), 0.79 (−C_3H_6−C***H***$_3$).
^{1}H NMR (400 MHz, DMSO-d_6) δ in ppm: 8.10 (triazole), 5.86 (anomer H-1), 5.24 (OH-2), 5.03 (OH-3), 5.00 (OH-4), 4.59 (OH-6), 4.39 (H-2), 4.14 (O−CH_2−C***H***$_2$−ester), 3.84 (H-6a), 3.7−2.7 (H-3, 4, 5, 6b, CON(C***H***$_3$)$_2$, and amide−C_2***H***$_4$−triazole), 2.7−2.2 (−C***H***− main chain of DMA), 2.1−1.0 (−C***H***− main chain of Man, −C***H***$_2$− main chain, and C***H***$_3$−CH(ester)−), 0.88 (−C_3H_6−C***H***$_3$).

Perparation of linear precursors by modifiyng alkyne groups to the polymer terminal (L-P_{54} and L-P_{106}). The synthesized glycopolymers (**P_{54}** and **P_{106}**, 180 mg) were dissolved in dry DMF (3 mL), and the polymer solutions were purged with nitrogen for 10 min. Propargyl acrylate (66 mg, 600 μmol), *n*-butylamine (22 mg, 300 μmol), and triethylamine (7.6 mg, 75 μmol) were added, and the mixtures were stirred for 16 h at room temperature under a nitrogen atmosphere. The polymer solutions were precipitated in Et_2O. The precipitation was dissolved in MeOH and was precipitated again. The polymers were dried *in vacuo*.
^{1}H NMR (400 MHz, D_2O) δ in ppm: 7.94 (triazole), 5.97 (anomer H-1), 4.15 (O−C***H***$_2$−CH_2−ester), 4.01 (H-6a), 3.8−2.7 (H-3, 4, 5, 6b, CON(C***H***$_3$)$_2$, and amide−C_2***H***$_4$−triazole), 2.7−2.2 (−C***H***− main chain of DMA), 2.1−1.1 (−C***H***− main chain of Man, −C***H***$_2$− main chain), 1.03 (C***H***$_3$−CH(ester)−).
^{1}H NMR (400 MHz, DMSO-d_6) δ in ppm: 8.10 (triazole), 5.86 (anomer H-1), 5.25 (OH-2), 5.04 (OH-3), 4.98 (OH-4), 4.69 (ester−C***H***$_2$−O−C≡CH), 4.60 (OH-6), 4.38 (H-2), 4.13 (O−CH_2−C***H***$_2$−ester), 3.84 (H-6a), 3.7−2.7 (H-3, 4, 5, 6b, CON(C***H***$_3$)$_2$, and amide−C_2***H***$_4$−triazole), 2.7−2.2 (−C***H***− main chain of DMA), 2.1−1.0 (−C***H***− main chain of Man, −C***H***$_2$− main chain, and C***H***$_3$−CH(ester)−).

Cyclization of the glycopolymers by CuAAC reaction. A mixture of H_2O (65 mL) and MeCN (30 mL) dissolving $CuSO_4$ (480 mg, 3.0 mmol) was degassed with nitrogen for 20 min (50 mL/min). The flow rate was decreased to 10 mL/min, and sodium-L-ascorbate (800 mg, 4.0 mmol) in H_2O (5 mL) was added. The mixtures of H_2O (7 mL) and MeCN (3 mL) dissolving the linear precursors (**L-P_{54}** and **L-P_{106}**, 40 mg) were added dropwise by syringe pump (0.5 mL/h). After the complete addition, the polymer solutions were kept stirring for 1 h at room temperature. The solution was concentrated by reduced pressure, and brown precipitates were removed by filtration. The filtrate was dialyzed against water, and the crude polymers were obtained after freeze-drying.

Purification of the synthesized cyclic glycopolymers by recycle SEC (C-P_{54} and C-P_{106}). Polymer purification was performed using a recycling SEC system (LaboACE LC-7080) equipped with JAIGEL-GS310/-P1. A mixture of MeOH: H_2O (4: 1) dissolving 10 mM LiBr was used as an eluent with a flow rate of 5.5 mL/min. The separation was conducted by injecting 3 mL of a polymer solution (10 g/L). Prior to injection, the solution was filtered through a 0.2 μm filter (PTFE). The SEC was performed under a recycling mode until the coinciding peaks were separated (detection: 220 nm). The desired fraction was collected using a fraction collector.

^{1}H NMR (400 MHz, D_2O) δ in ppm: 7.94 (s, triazole), 5.97 (s, anomer), 4.15 (s, O−C***H***$_2$−CH_2−ester), 4.01 (s, Man), 3.8−2.7 (mannnose, CON(C***H***$_3$)$_2$, and amide−C_2***H***$_4$−triazole), 2.7−2.2 (br, −C***H***− main chain of DMA), 2.1−1.1 (br, −C***H***− main chain of Man, −C***H***$_2$− main chain), 1.03 (C***H***$_3$−CH(ester)−, 3H), 0.79 (−C_3H_6−C***H***$_3$, 3H).

^{1}H NMR (400 MHz, DMSO-d_6) δ in ppm: 8.02 (triazole), 5.84 (anomer H-1), 5.21 (OH-2), 5.10 (ester−C***H***$_2$−O−triazole), 4.99 (OH-3), 4.96 (OH-4), 4.56 (OH-6), 4.37 (H-2), 4.07 (O−CH_2−C***H***$_2$−ester), 3.82 (H-6a), 3.7−2.6 (H-3, 4, 5, 6b, CON(C***H***$_3$)$_2$, and amide−C_2***H***$_4$−triazole), 2.6−2.2 (−C***H***− main chain of DMA), 2.1−1.0 (−C***H***− main chain of Man, −C***H***$_2$− main chain, and C***H***$_3$−CH(ester)−).

Hemagglutination inhibition (HI) assay.[3] Phosphate-buffered saline (PBS) solution with calcium and magnesium ions (PBS(+)) was added into a 96-well plate (25 μL/well) except the first lane. Con A in PBS(+) (1 g/L, 100 μL) was added into the first line of the 96-well plate, and the solution in the first lane was diluted by two steps (50 μL). Red blood cells (RBCs) in the purchased blood cell suspension were washed by centrifugation with PBS(+) three times. The concentrated RBCs were resuspended in PBS(+) (0.5 v/v %), and this was injected in each well (50 μL). The 96-well plate was incubated for 1 h at room temperature, and the lowest concentration of ConA required to aggregate the RBCs was determined. This process was triplicated, and the average value was defined as 1HAU.

Glycopolymer solution (2 mg/mL, 50 μL) was injected in the first lane. The solution in the first lane were twofold serially diluted (25 μL/well). ConA solution (4 HAU) was injected in each well (25 μL/well). The 96-well plate was incubated for 1 h at 25 °C. The RBC suspension was injected in each well (50 μL). The 96-well plate was incubated for 1 h at 25 °C. Precipitation of red blood cells was determined by visual inspection.

Fluorescence quenching measurement. A HEPES buffer solution (pH 7.4) was prepared using 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (10 mmol/L), NaCl (137 mmol/L), KCl (2.7 mmol/L), $CaCl_2$ (1.8 mmol/L), and $MgCl_2 \cdot 6H_2O$ (0.5mmol/L). FITC-ConA solution (MW: 100 kDa, 1.0 mg/mL) was diluted with the buffer solution to prepare a 0.033 μM lectin solution. The glycopolymer solutions (glycoepitope concentration: 1 mM) were prepared with the buffer solution. A total of 1 mL of the FITC-ConA solution was prepared in a disposable cell (1 mL). The fluorescence at 517 nm was recorded while injecting the polymer solution using an excitation wavelength at 490 nm. The fluorescence intensity of the FITC-ConA solution was first measured as the 0th measurement. An aliquot of glycopolymer solution was added every 5 min. The injection volumes were 1 μL (first round), 2 μL (second round), 7 μL (third round), 20 μL (fourth round), and 80 μL (fifth round). The fluorescence quenching without protein-sugar interaction was measured by the addition of the buffer solution as a blank. The difference of fluorescence intensity ($\Delta F_n = F_0 – F_n$) was calculated, where F_n indicates the fluorescence intensity after the "*n*" th sample injection (F_0 indicates the fluorescence intensity before sample injection). The value obtained after blank subtraction ($\Delta F_{n,sample} - \Delta F_{n,blank}$) was taken as the fluorescence intensity change $\Delta F_{n,net}$. The association constants were estimated by the following Langmuir isotherm (ΔF_{max}: maximum fluorescence intensity change, K_a: binding constant, C: glycoepitope concentration).

$$\frac{\Delta F}{\Delta F_{max}} = \frac{C}{\left(\frac{C}{K_a} + 1\right)}$$

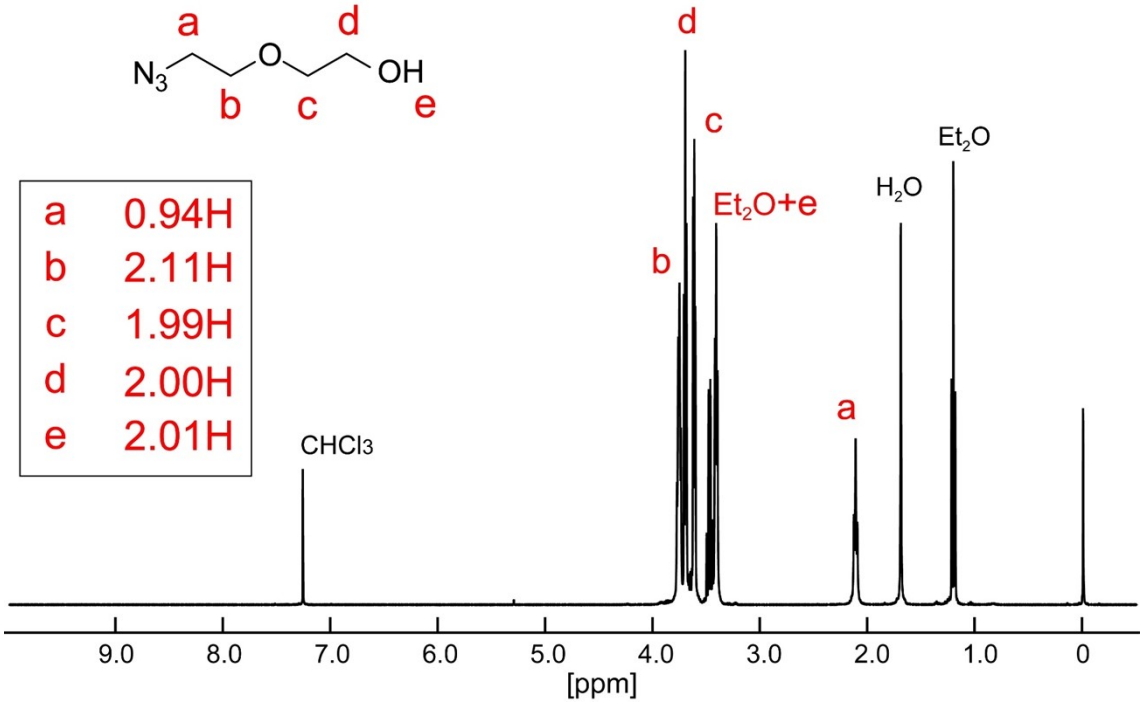

Figure S1. ^{1}H NMR spectrum of 2-(2-azidoethoxy) ethanol (400 MHz, $CDCl_3$).

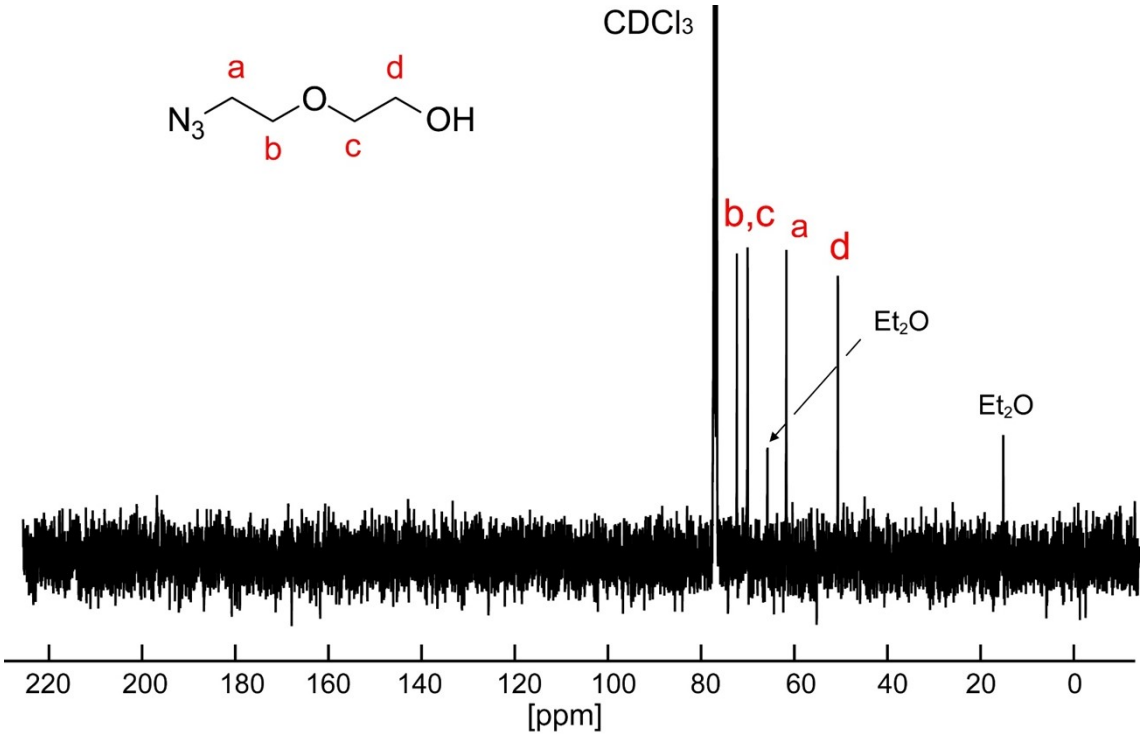

Figure S2. ^{13}C NMR spectrum of 2-(2-azidoethoxy) ethanol (100 MHz, $CDCl_3$).

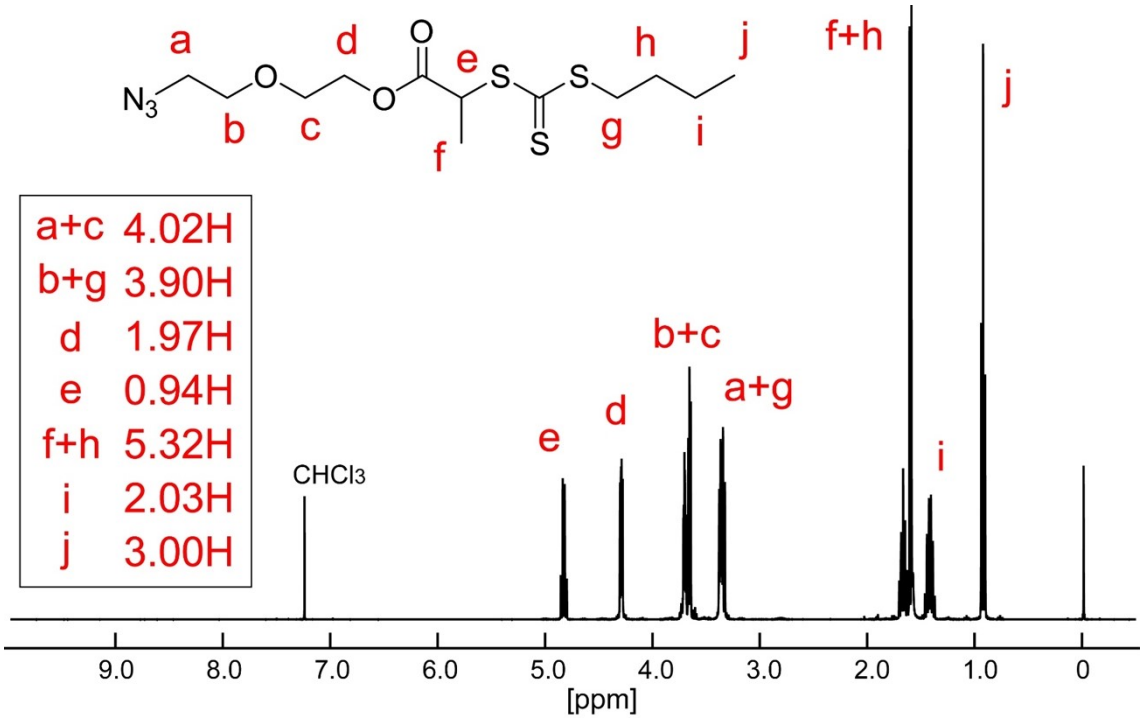

Figure S3. ^{1}H NMR spectrum of AEBCP (400 MHz, $CDCl_3$).

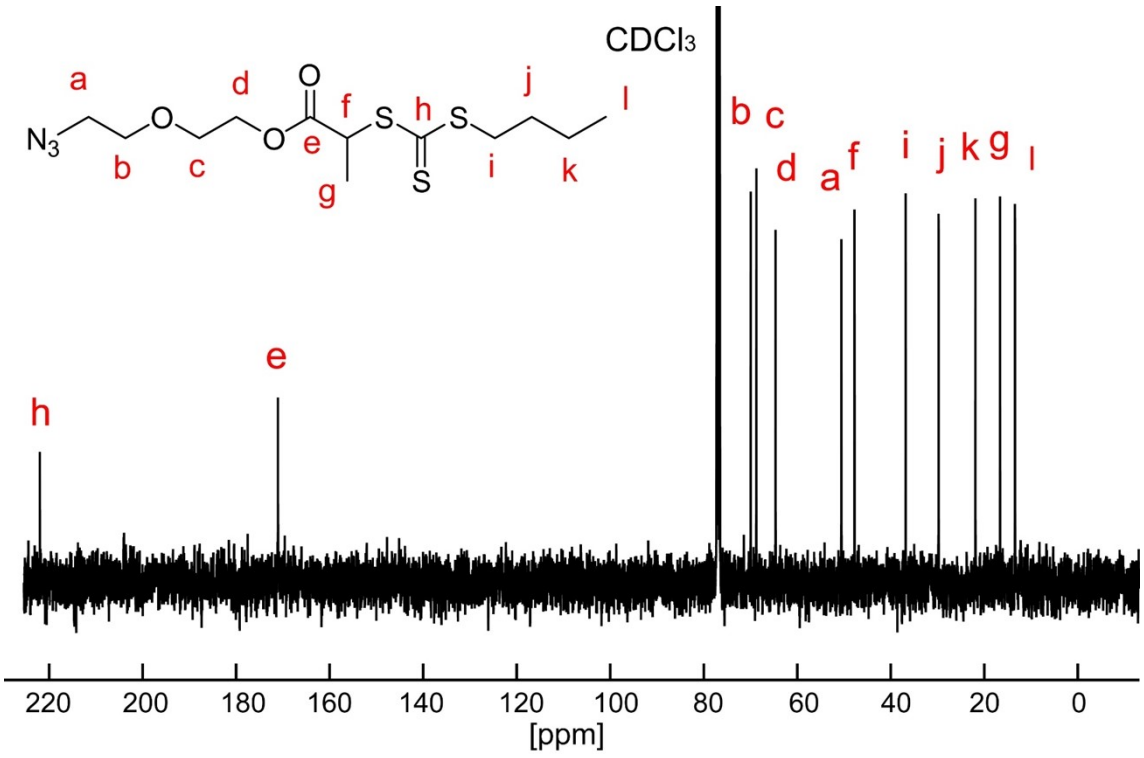

Figure S4. ^{13}C NMR spectrum of AEBCP (100 MHz, $CDCl_3$).

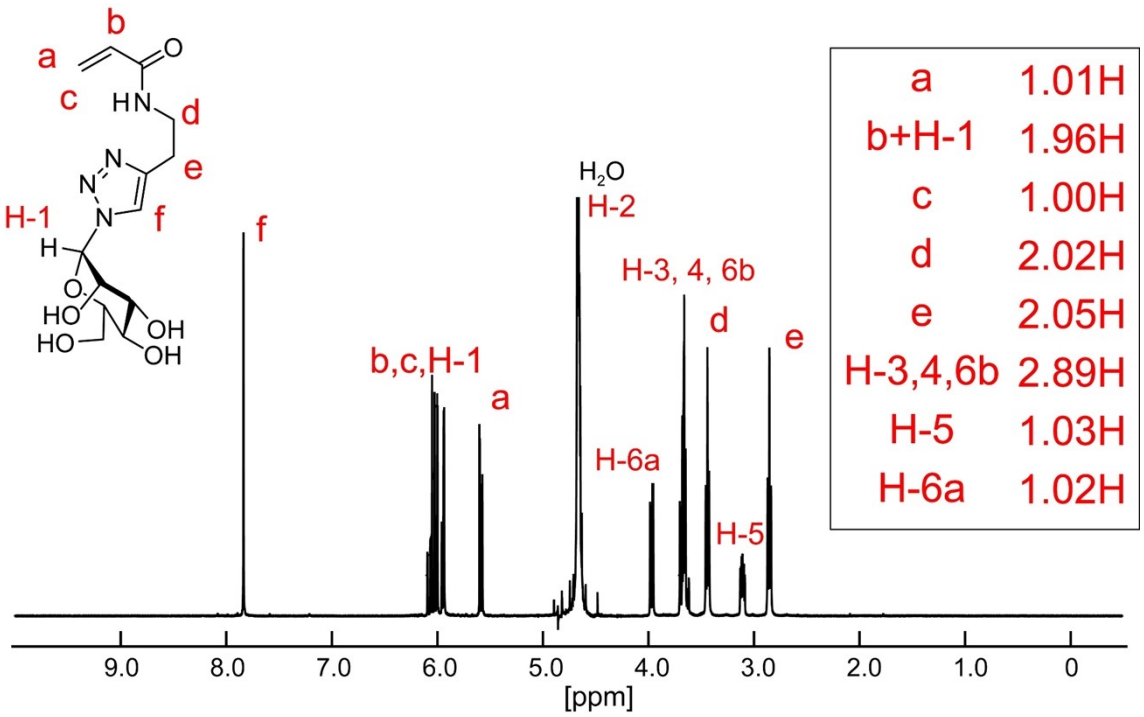

Figure S5. ^{1}H NMR spectrum of Mannose acrylamide (400 MHz, D_2O).

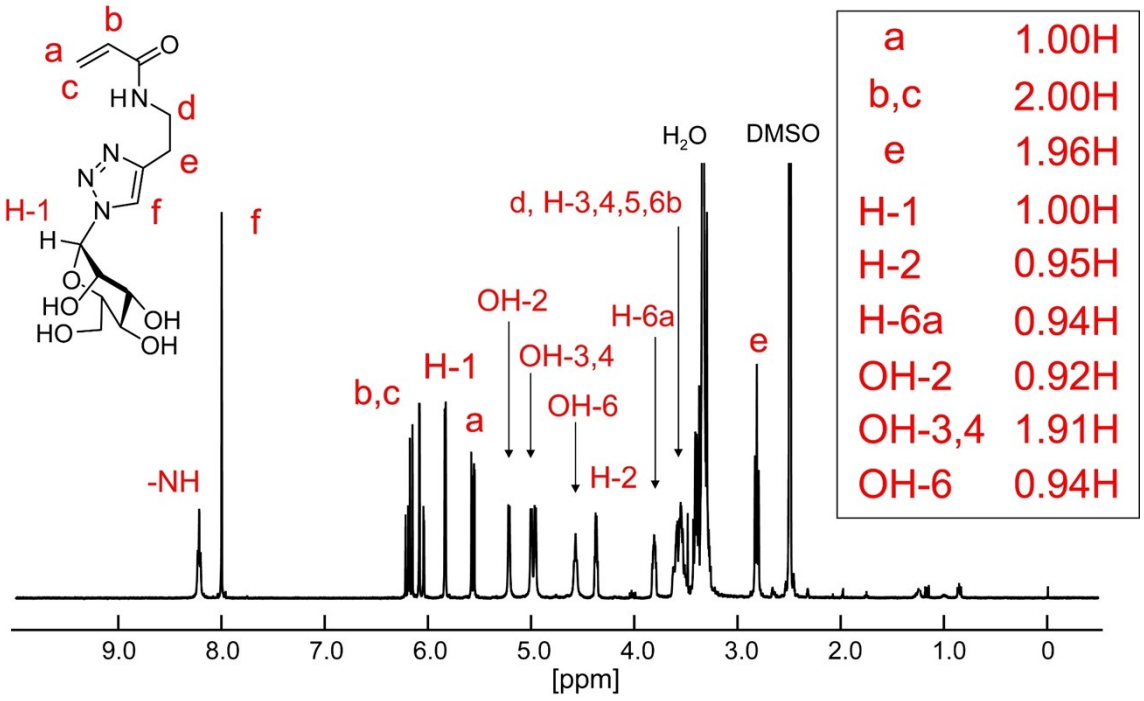

Figure S-6. ^{1}H NMR spectrum of Mannose acrylamide (400 MHz, DMSO-d_6).

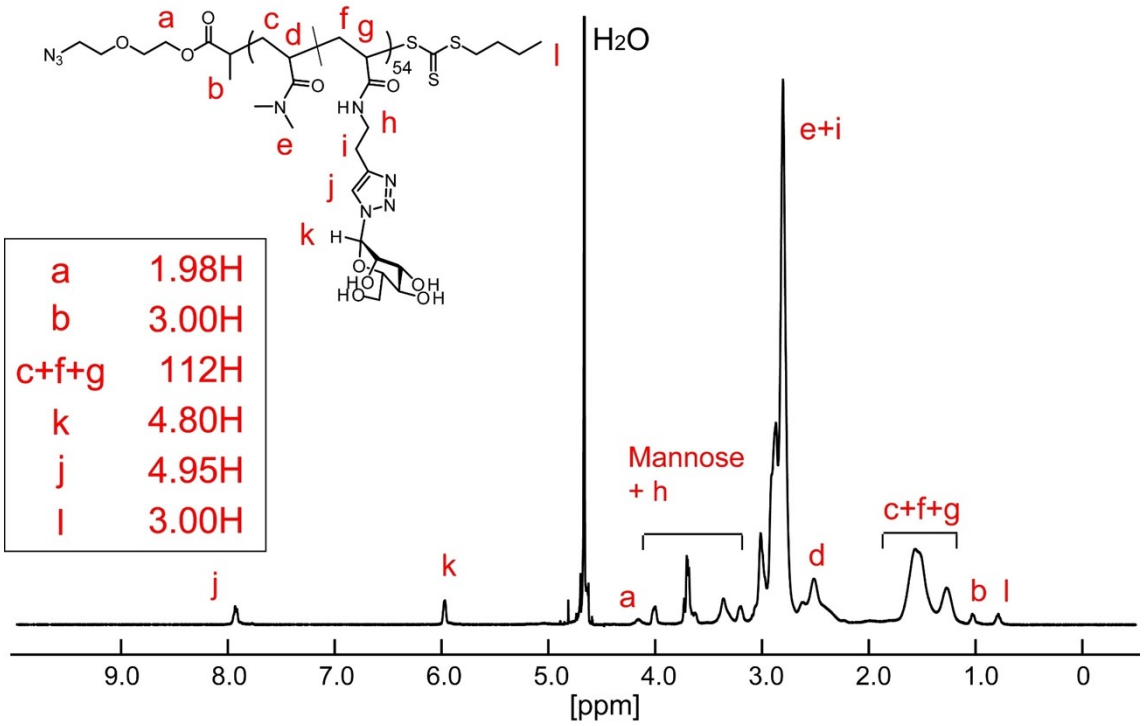

Figure S7. ^{1}H NMR spectrum of P_{54} (400 MHz, D_2O).

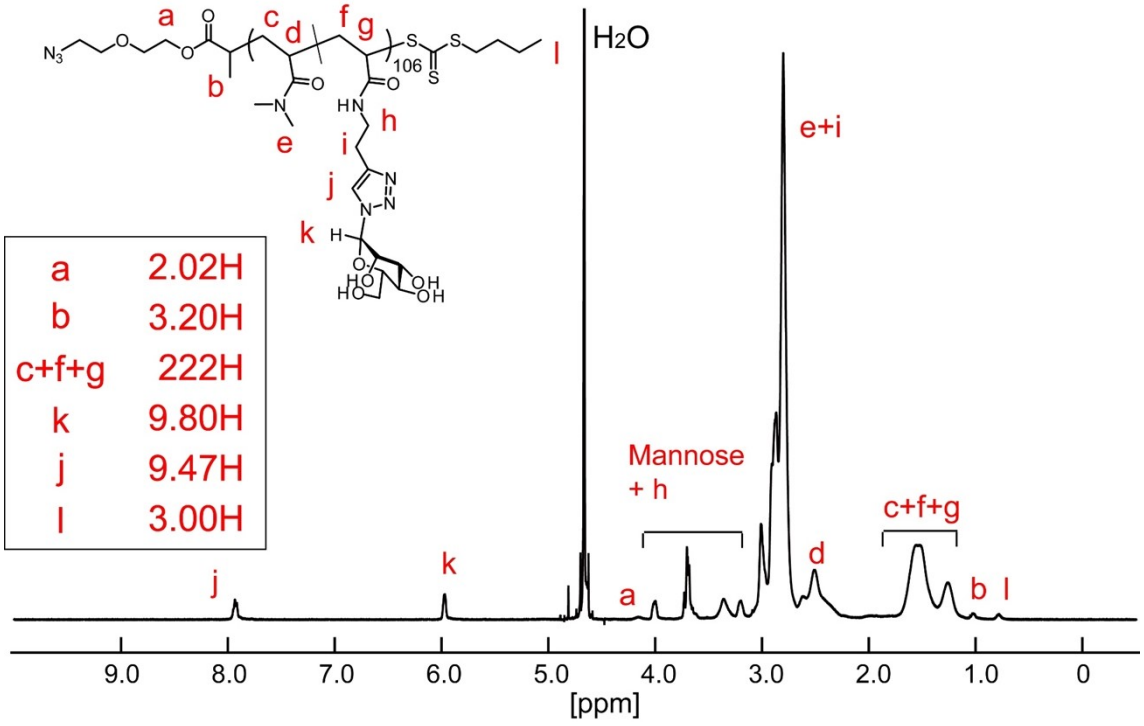

Figure S8. ^{1}H NMR spectrum of P_{106} (400 MHz, D_2O).

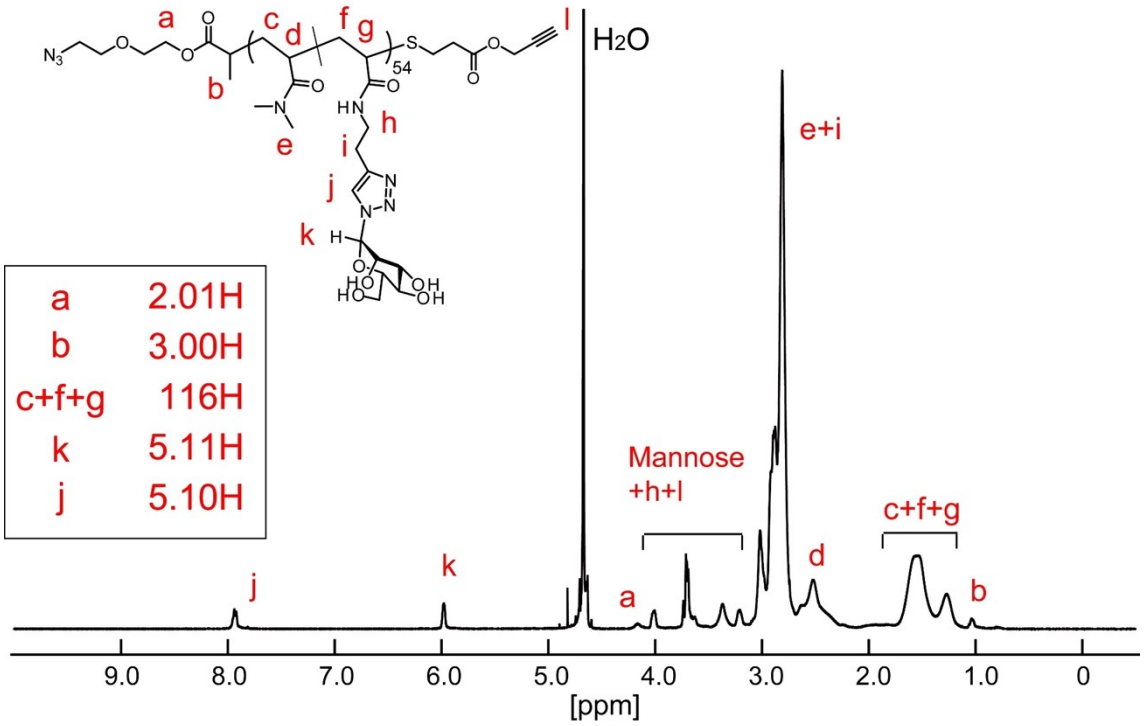

Figure S9. ^{1}H NMR spectrum of L-P_{54} (400 MHz, D_2O).

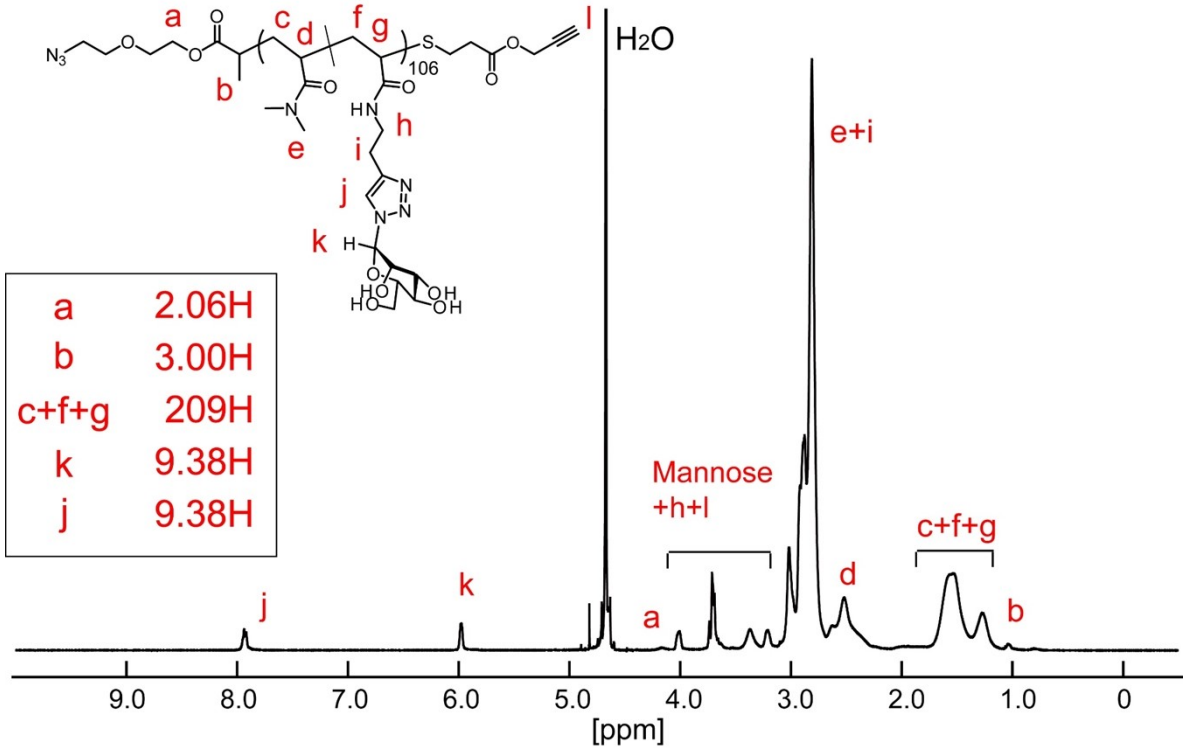

Figure S10. ^{1}H NMR spectra of spectrum of L-P_{106} (400 MHz, D_2O).

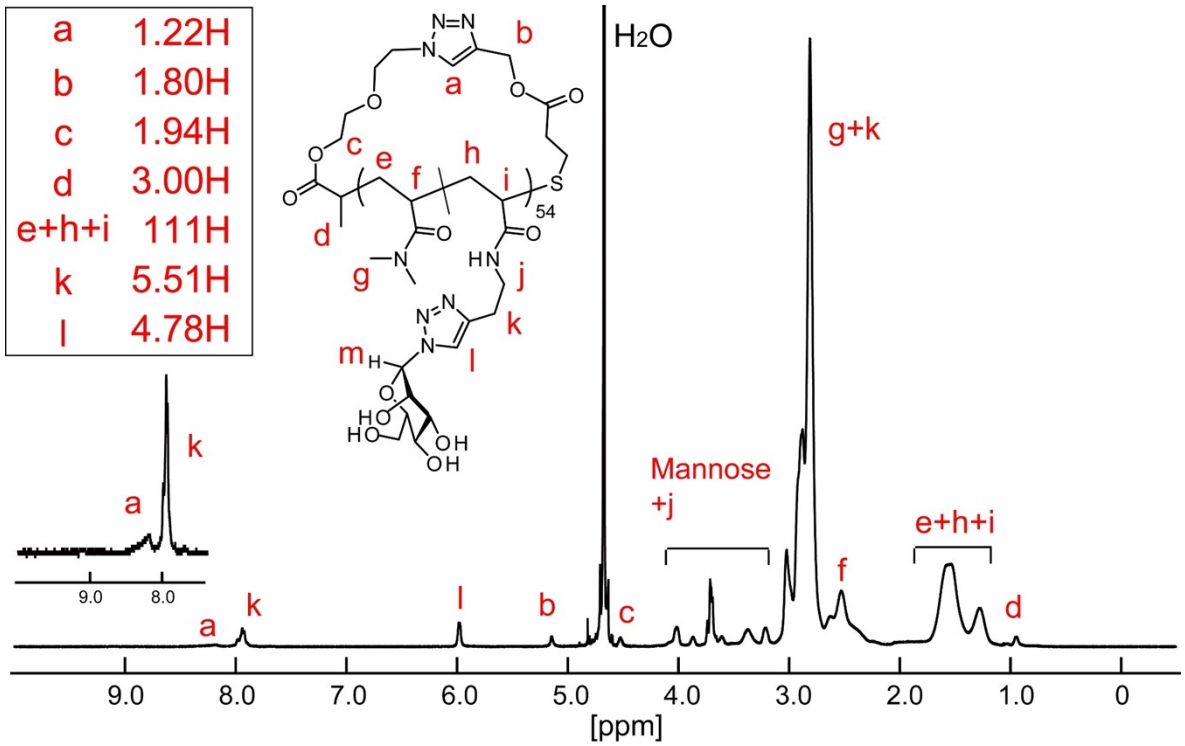

Figure S11. ^{1}H NMR spectrum of C-P_{54} (400 MHz, D_2O).

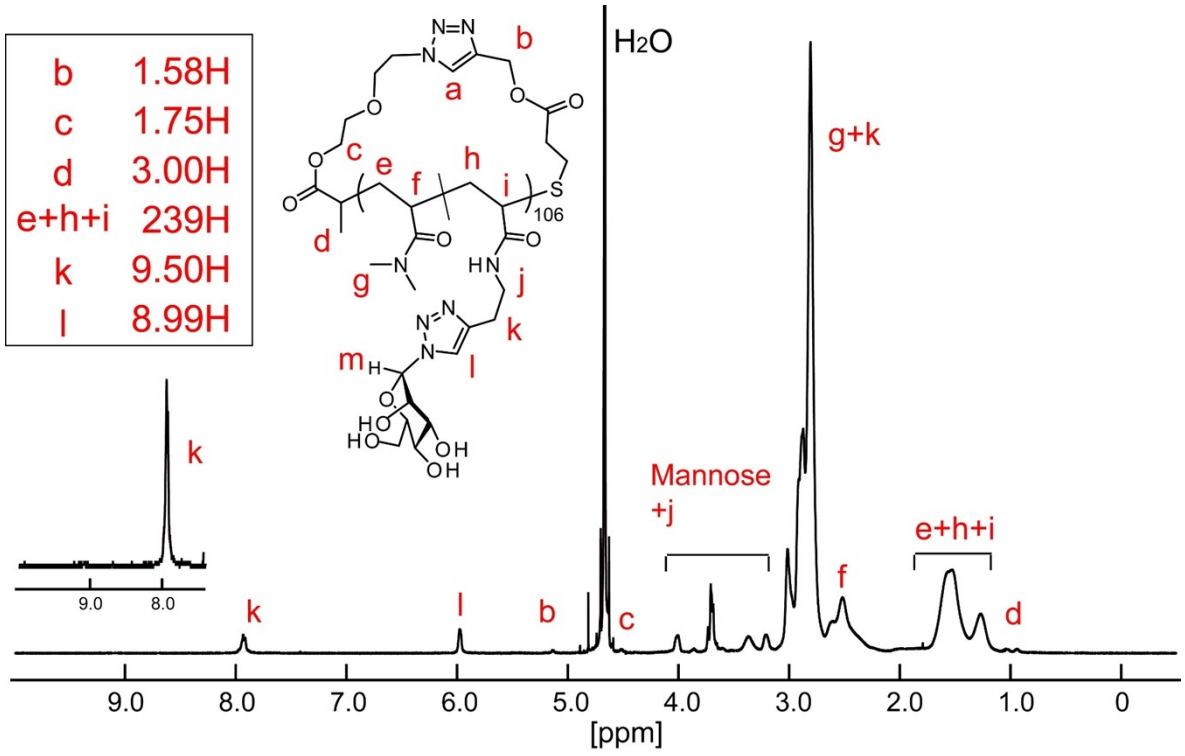

Figure S12. ^{1}H NMR spectrum of C-P_{106} (400 MHz, D_2O).

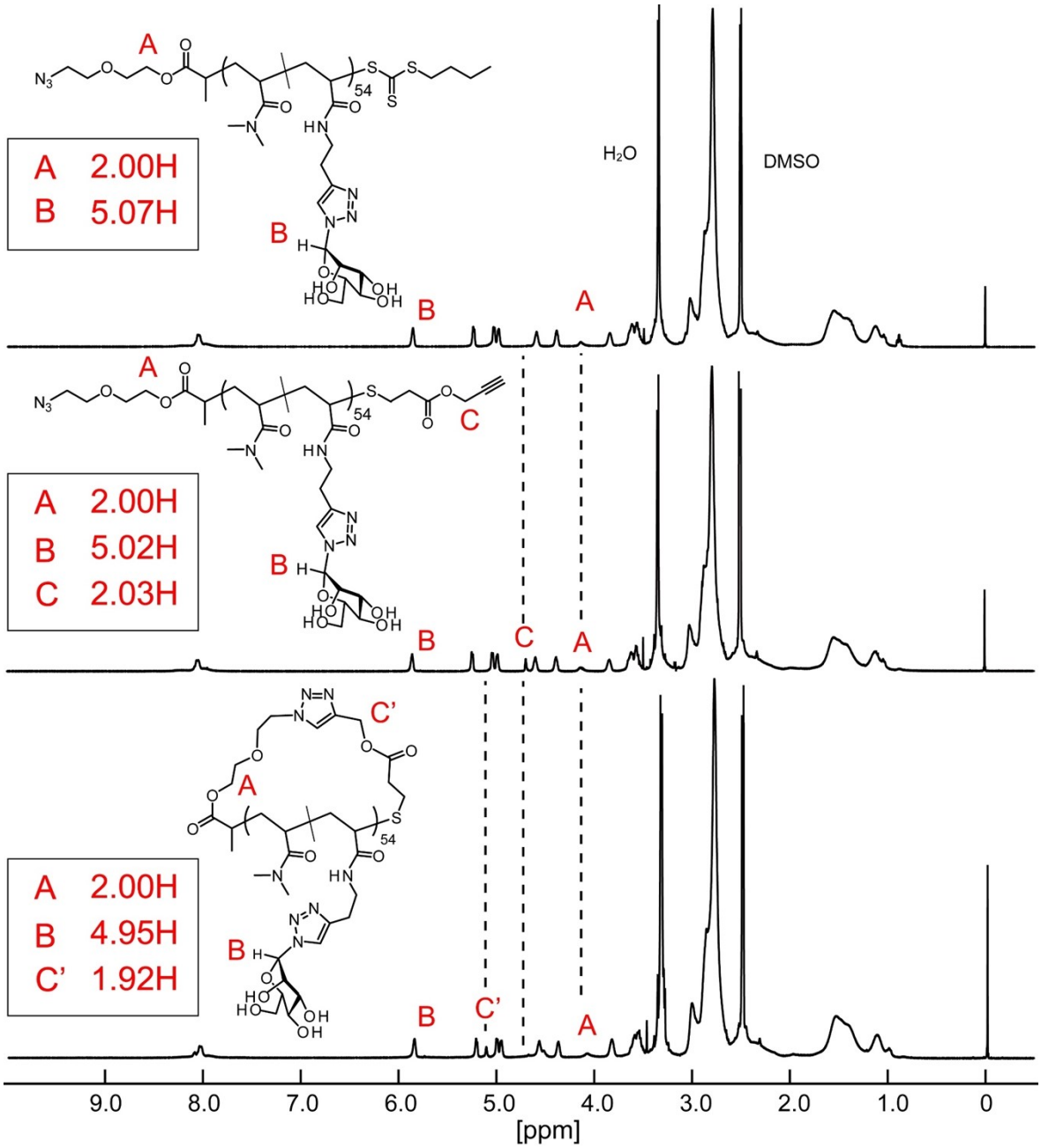

Figure S13. ^{1}H NMR spectra of P_{54} series (400 MHz, DMSO-d_6).

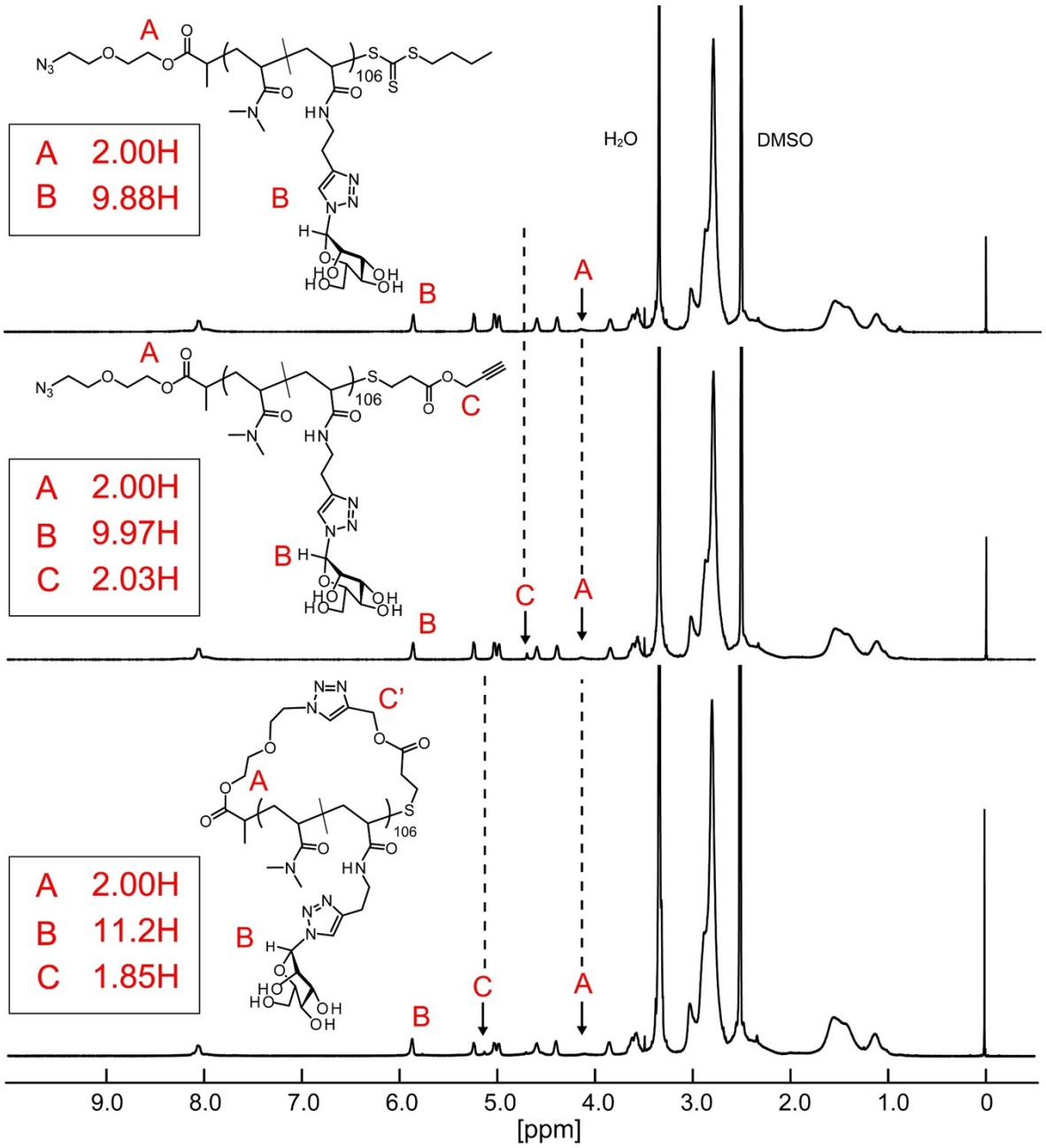

Figure S14. ^{1}H NMR spectra of P_{106} series (400 MHz, DMSO-d_6).

Table S1. Detailed conditions of the RAFT polymerization for the glycopolymers.

Polymer	Feed ratio										Polymer composition[a]		
	DMA (mmol)	Man (mmol)	AEBCP (μmol)	AIPD (μmol)	[M] (mol/L)	H_2O (mL)	DMF (mL)	Time (min)	Conv.[a] (%)	DP[a]	DMA	Man	$M_{n,th}$[b] (g/mol)
P_{54}	4.5 (446 mg)	0.5 (164 mg)	100 (35.3 mg)	10 (3.2 mg)	2	0.25	2.25	180	94	54	49	5	6 800
P_{106}	4.5 (446 mg)	0.5 (164 mg)	50 (17.7 mg)	10 (3.2 mg)	4	0.13	1.13	180	98	106	96	10	13 100

[a]Conversion rate (Conv.), degree of polymerization (DP), and composition in the polymer were determined by ^{1}H NMR.

[b]Theoretical molecular weight was calculated by the following formula: $M_{n,th}$ = MW_{Man} × Man composition + MW_{DMA} × DMA composition + MW_{RAFT}

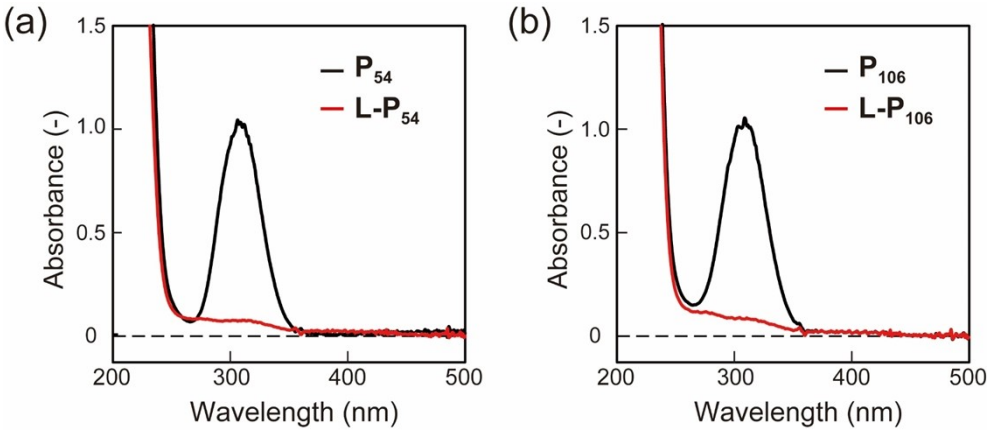

Figure S15. UV spectra of glycopolymers either with trithiocarbonate (black line) or with alkyne terminals (red line). The polymer concentration was (a) 0.5 and (b) 1.0 g/L in water.

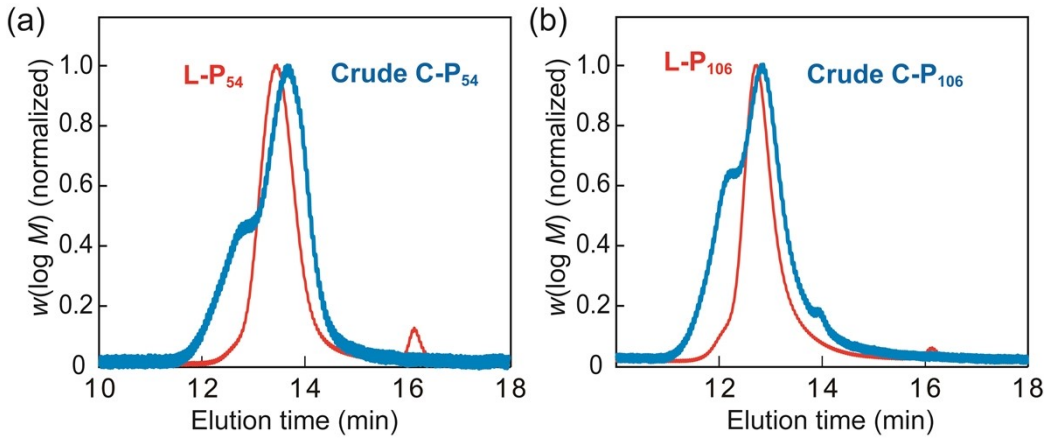

Figure S16. SEC chromatographs of the two series of glycopolymers. The eluent was DMF with 10 mM LiBr. The red and blue lines indicate the glycopolymers with alkynyl terminals and cyclic structures. The cyclic polymers were crude (before purification by cycled SEC system) and included the by-products. The system was calibrated with polymethylmethacrylate standards.

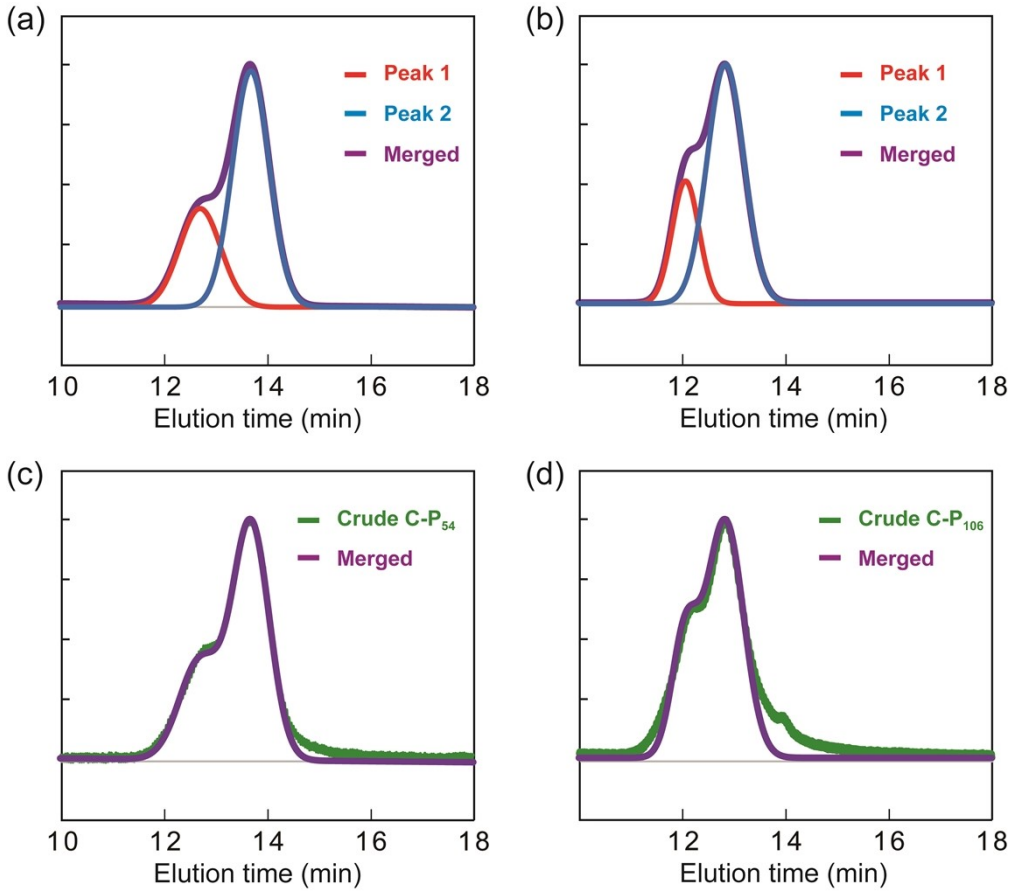

Figure S17. Images of peak-fitting analysis for SEC chromatographs of the two series of glycopolymers. The degree of polymerization was 54 (a and c) and 106 (b and d). The red and blue lines indicate the glycopolymers with higher molecular weight (peak 1), lower molecular weight (peak 2). The purple lines indicate the merged ones, which corresponds to the original SEC chromatographs of the crude cyclic polymers (c and d). When calculating the percentage of each product, three assumptions were utilized according to the paper.[4] The first assumption made is that there is no leftover starting material. The second assumption is that the cyclic peak is Gaussian in nature. The third is that the RI increment, dn/dc, is the same or similar for each component of the polymer mix.

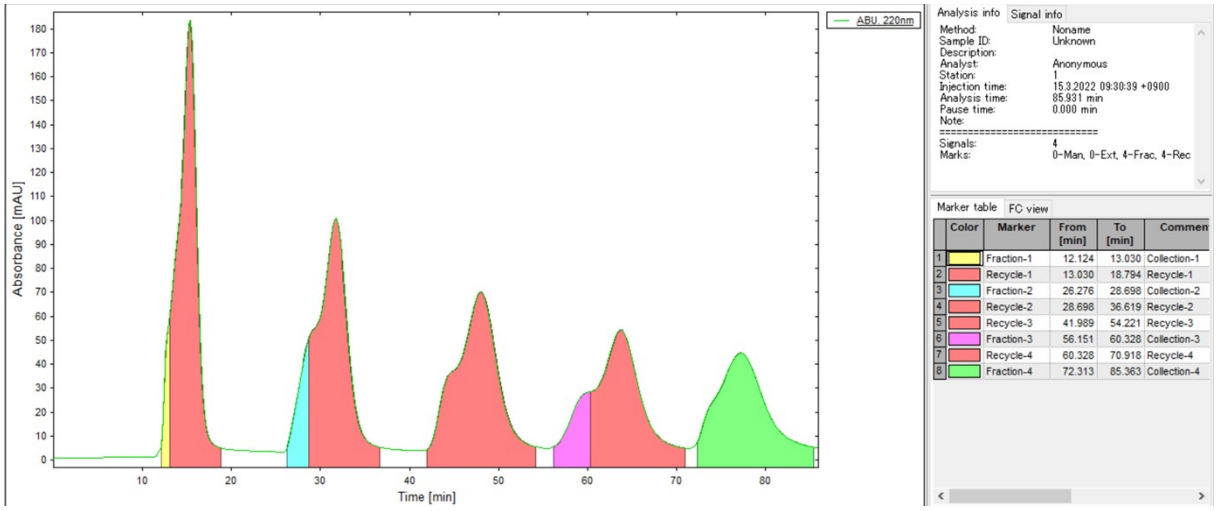

Figure S18. SEC traces of the crude mixture (**C-P$_{54}$**) during the recycling separation. The fraction-4 was collected.

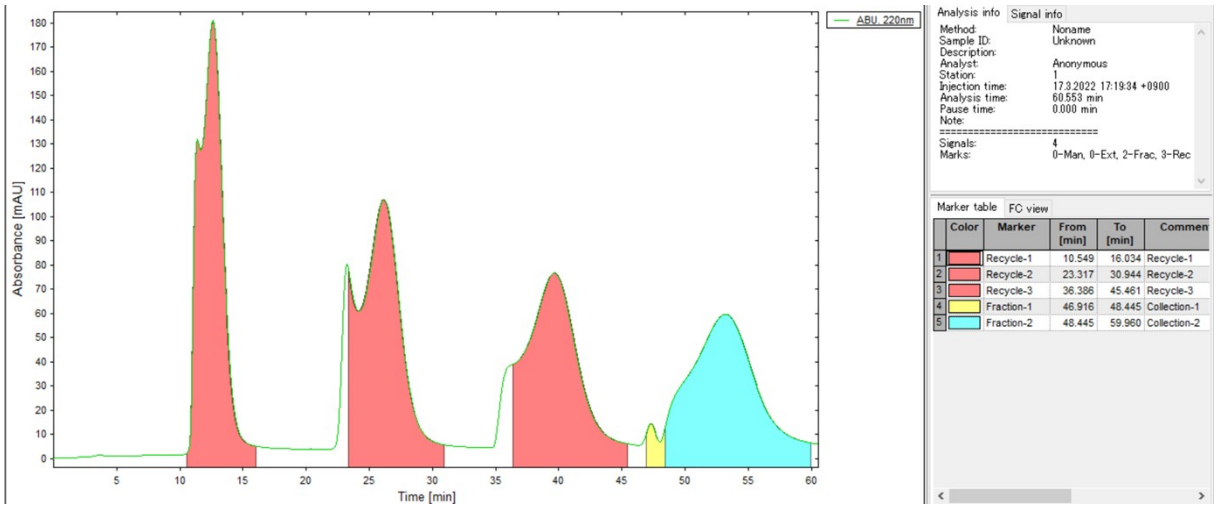

Figure S19. SEC traces of the crude mixture (**C-P$_{106}$**) during the recycling separation. The fraction-2 was collected.

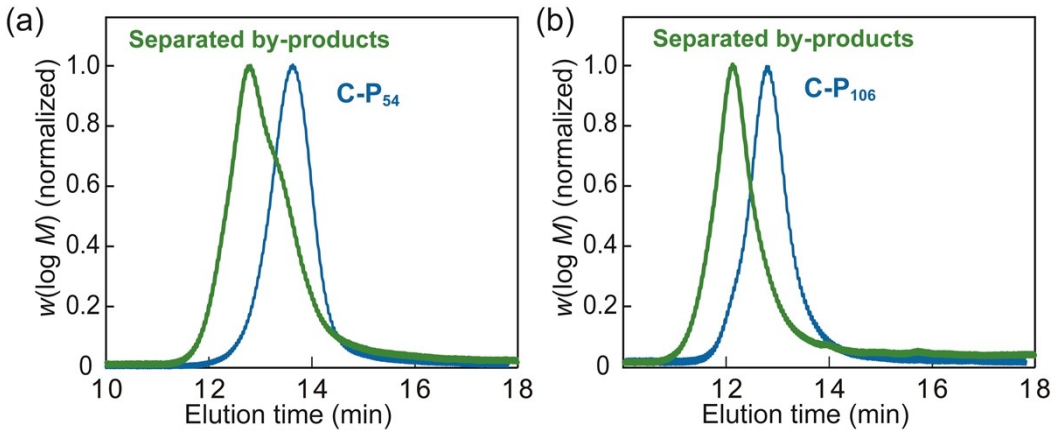

Figure S20. SEC chromatographs of the two series of glycopolymers. The eluent was DMF with 10 mM LiBr. The green and blue lines indicate the oligomeric by-products of the intramolecular cyclization and the objective cyclic glycopolymers. The system was calibrated with polymethylmethacrylate standards.

Table S2. Percentages and molecular weights of each component in the crude samples.

	Percentage in the crude samples	M_n (g/mol)*	M_w (g/mol)*
Linear precursor (L- P_{54})	-	4,100	52,00
By-products with C-P_{54}	32%	6,300	10,100
Objective cyclic polymers (C-P_{54})	68%	3,000	4,000
Linear precursor (L- P_{106})	-	8,100	11,000
By-products with C-P_{106}	27%	17,800	23,700
Objective cyclic polymers (C-P_{106})	73%	7,200	9,800
*The molecular weights of each component were obtained by SEC analysis (solvent: DMF with 10 mM LiBr) after the cycled SEC separation.			

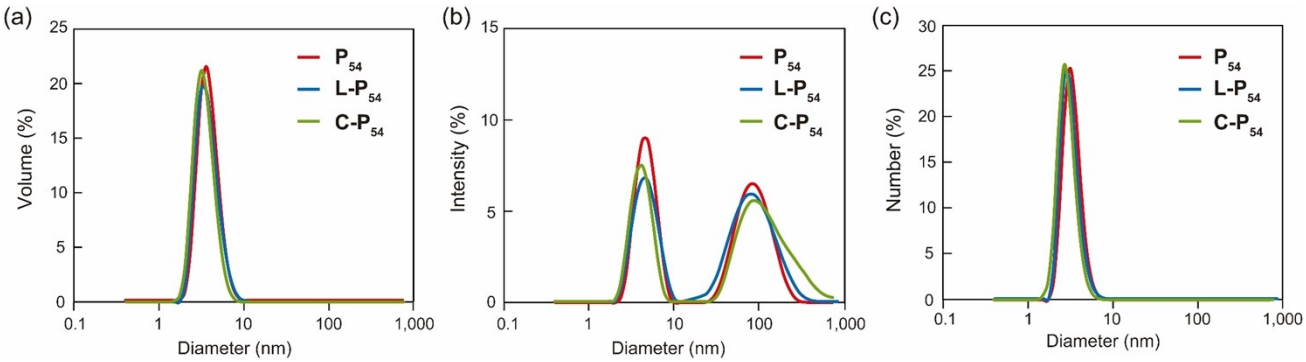

Figure S21. Dynamic light scattering distributions of the glycopolymers in (a) volume percentage, (b) intensity percentage, and (c) number percentage. Red, blue, and green lines indicate **P_{54}**, **L-P_{54}**, and **C-P_{54}**, respectively.

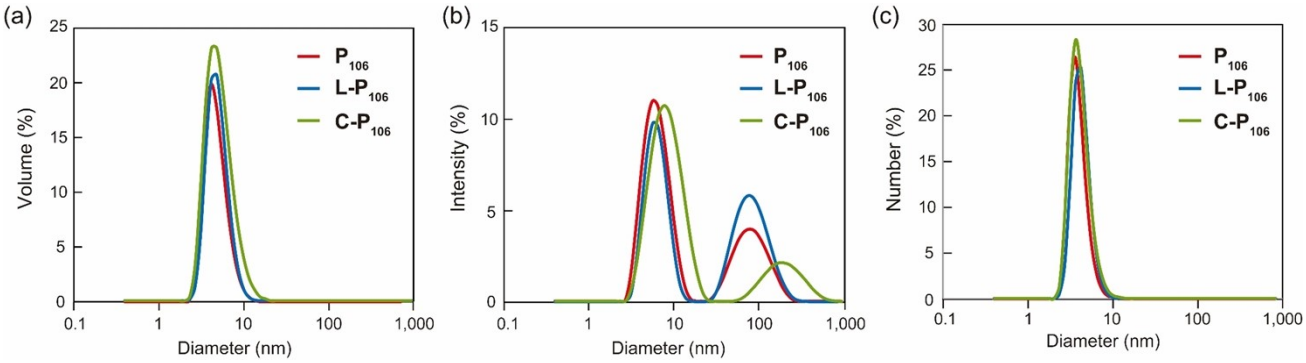

Figure S22. Dynamic light scattering distributions of the glycopolymers in (a) volume percentage, (b) intensity percentage, and (c) number percentage. Red, blue, and green lines indicate **P_{106}**, **L-P_{106}**, and **C-P_{106}**, respectively.

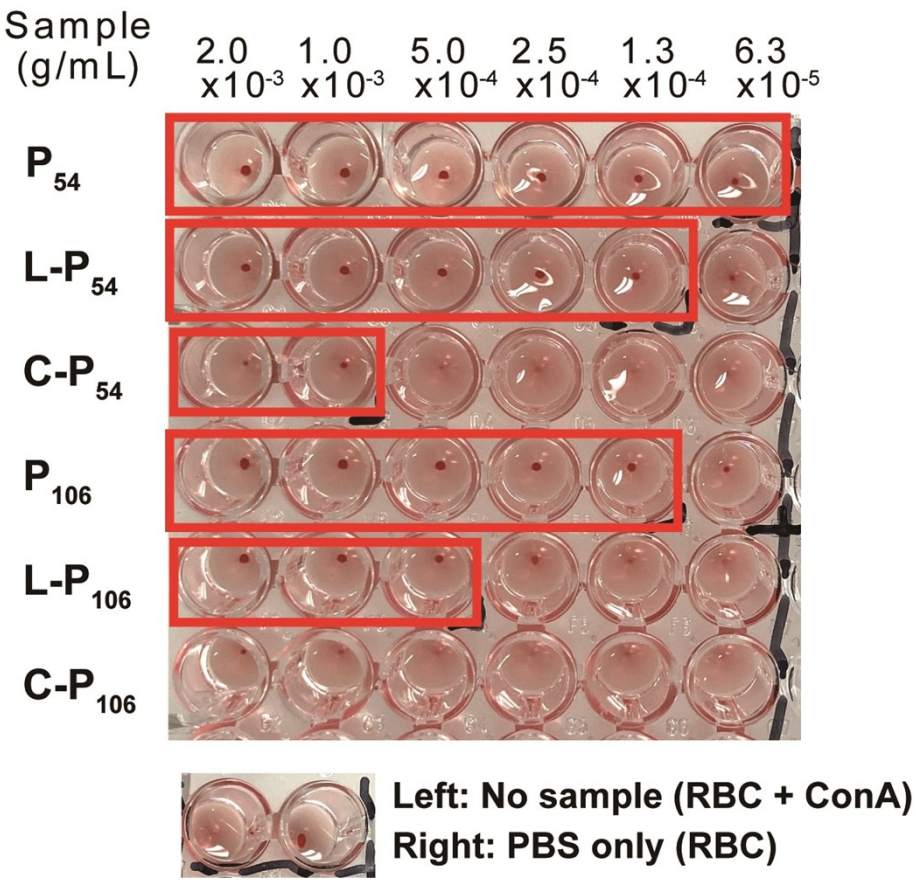

Figure S23. HI assays of the glycopolymers (**P$_{54}$** and **P$_{106}$**), linear precursors (**L-P$_{54}$** and **L-P$_{106}$**), and cyclic glycopolymers (**C-P$_{54}$** and **C-P$_{106}$**) against ConA (4HAU). The images of the 96-well plates after incubation. The red squares show the concentration required for HI activity.

Reference:

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