Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B.

Glycopolymer monolith for affinity bioseparation of proteins in a continuous-flow system: Glycomonolith

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1. Synthesis of glycomonomer

p-Acrylamidophenyl-α-D-mannopyranoside (Man monomer) was synthesized using an established method (Y. Hoshino et al., *J. Am. Chem. Sci*., 2012, **134**, 15209-15212.), as shown in Figure S1.

***p*-Nitrophenyl-2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside (*p*NP-AcMan):** Acetic anhydride (340 mmol) was slowly added to a solution of *p*-nitrophenyl-α-D-mannopyranoside (*p*NP-Man, 20 mmol, Sigma-Aldrich Co., St. Louis, MO) in pyridine (30 mL). The reaction mixture was stirred for 24 h. The pyridine was removed first by evaporation and then azeotropic distillation with toluene (10 mL) several times. The residue was dissolved in ethyl acetate, and then washed twice with each of the following: 1 mol L^{-1} HCl, saturated $NaHCO_3$, and water. The organic layer was dried over $MgSO_4$, filtered, and the solvent was evaporated. The residue was purified by silica gel chromatography with ethyl acetate to yield a white solid.

***p*-Aminophenyl-2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside (*p*AP-AcMan):** Pd/C (450 mg) was added to a solution of *p*NP-AcMan (19.0 mmol) in dry methanol (40 mL). The reaction mixture was stirred at room temperature under hydrogen overnight. The resulting solution was filtered using glass fiber paper, and then concentrated *in vacuo*.

***p*-Acrylamidophenyl-2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside (AcMan monomer):** The crude *p*AP-AcMan was dissolved in DMF (40 mL), and then acryloyl chloride (22.8 mmol) and triethylamine (28.5 mmol) were added at 0°C. The solution was stirred at room temperature for 2 h, and then concentrated *in vacuo*. The residue was redissolved in chloroform, washed twice with water and twice with saturated NaCl, dried over $MgSO_4$, and then the solvent was evaporated. The residue was purified by silica gel chromatography with chloroform/methanol (10:1) and further recrystallization with ethyl acetate-diethyl ether to afford as a white solid.

***p*-Acrylamidophenyl-α-D-mannopyranoside (Man monomer):** Sodium methoxide (0.71 mmol) was added to a solution of AcMan monomer (2.7 mmol) in dry methanol (20 mL). The reaction mixture was stirred at room temperature for 1 h. The solution was neutralized using cationic exchange resin (Amberlyst 15DRY, Organo Co., Tokyo, Japan), filtered using filter paper, and then concentrated *in vacuo* to yield the Man monomer as a white solid.

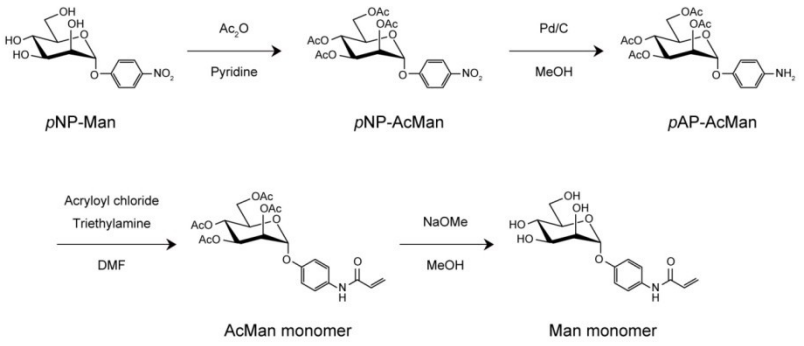

Figure S1. Synthesis of the Man monomer.

2. Preparation of the glycopolymer-grafted membrane

Thiol-terminated glycopolymer was prepared using a reversible addition fragmentation chain transfer (RAFT) polymerization following an established method (M. Takara et al., *Polym. Chem.*, 2014, **5**, 931-939), as shown in Figure S2 (a). A shirasu porous glass (SPG) membrane (SPG Technology Co. Ltd., Miyazaki, Japan) with similar pore size distribution to the glycomonolith prepared with butanol was used as a matrix. The thiol-terminated glycopolymer (polyMan-SH) was grafted onto SPG membrane by the thiol−ene click reaction (Michael addition reaction), as shown in Figure S2 (b). The maleimidated surface was prepared via an amide condensation reaction with an amine-containing silane coupling reagent (H. Seto et al., *ACS Appl. Mater. Interfaces*, 2012, **4**, 5125-5133). The membrane surfaces were washed by O_3 treatment for 30 min. The membranes were immersed in 3-aminopropyl trimethoxysilane (APTMS, 1 vol%, Azmax Co., Chiba, Japan) aqueous solution at 60°C for 3 h, and then at 110°C for 5 min. After washing with water, the aminated membranes were immersed in *N*-succinimidyl 3-maleimidopropionate (SMP, 15 mmol L^{-1}, Tokyo Chemical Industry Co. Ltd., Tokyo, Japan) solution in DMSO, and then incubated in the dark at 30°C for 3 h. The maleimidated membranes were washed with DMSO and water, and then quickly immersed in polyMan-SH aqueous solution (1.0 g L^{-1}) and incubated in the dark at 30°C for 12 h for preparation of the glycopolymer-grafted membranes. The membranes were washed with water. The amount of glycopolymer grafted on the membrane was determined from the absorbance at 246 nm before and after the grafting using an UV spectrometer (Agilent 8453, Agilent technologies Inc., Santa Clara, CA). The amount of grafted glycopolymer (160 μg cm^{-3}) corresponded to a Man density of 450 nmol cm^{-3}.

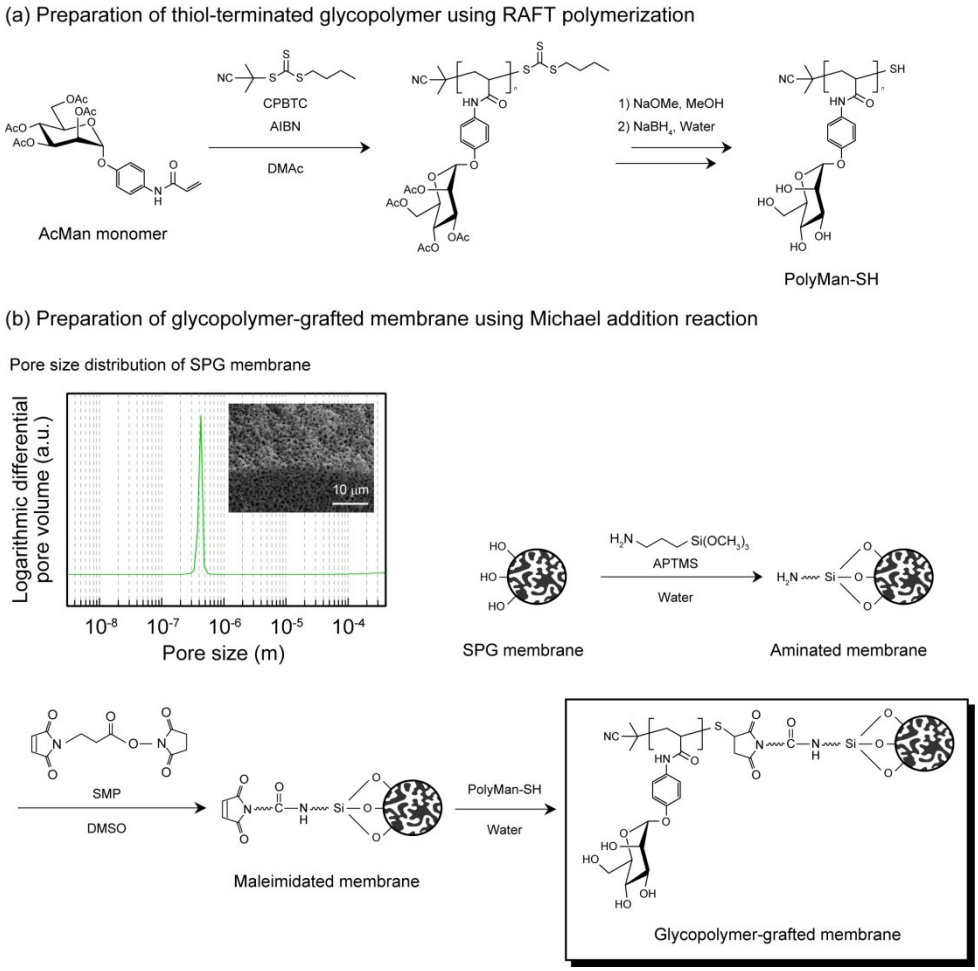

Figure S2. Preparation of (a) thiol-terminated glycopolymer using RAFT polymerization and (b) glycopolymer-grafted membrane using a Michael addition reaction.

3. Continuous-flow system

A continuous-flow system (Figure S3) was constructed for testing adsorption of protein by the prepared glycomonoliths. This system included a peristaltic pump (SJ-1211II-L, ATTO Co., Tokyo, Japan), pressure gauge (KDM-30, Krone Co., Tokyo, Japan), ultraviolet and visible (UV-Vis) spectrometer (USB2000+, Ocean Optics Inc., Dunedin, FL), deuterium halogen light source (DH-mini, Ocean Optics Inc.), and Z-type flow cell (FIA-Z-SMA-PLEX, Ocean Optics Inc.).

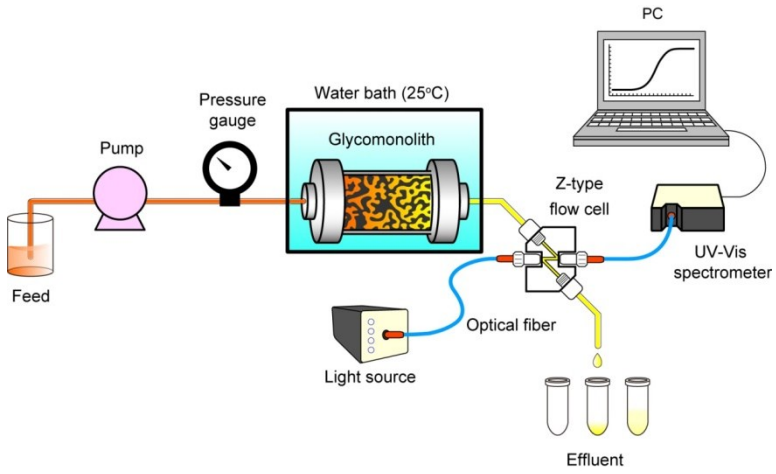

Figure S3. Schematic illustration of the continuous-flow system for adsorption and recovery of proteins using the glycomonoliths.

4. Determination of pore size distributions in glycomonolith

After lyophilization of the swollen monoliths, the pore size distributions and specific surface areas were determined using a mercury porosimeter (AutoPoreIV9520, Micromeritics Instrument Co., Norcross, GA). The macroporous monoliths had relatively narrow and unimodal pore size distributions (Figure S4). When butanol and octanol were used as the porogenic solvents, the macropore sizes in the lyophilized monoliths were between several hundred nanometers and several micrometers (Table S1). The specific surface areas of the monoliths prepared with butanol were higher than those prepared with octanol.

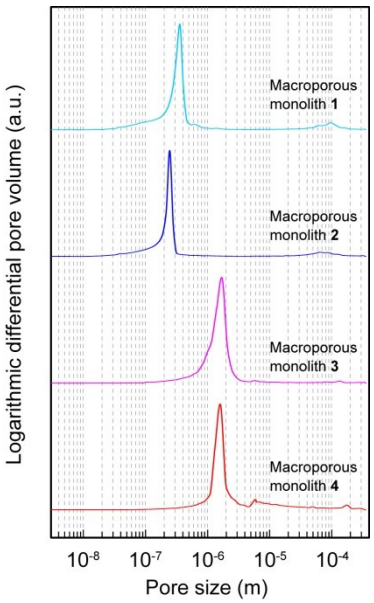

Figure S4. Pore size distributions for the macroporous monoliths. **1**: Man 0 wt% in butanol, **2**: Man 10 wt% in butanol, **3**: Man 0 wt% in octanol, **4**: Man 10 wt% in octanol.

Table S1. Median pore sizes and specific surface areas of the macroporous monoliths.

Macroporous monolith	Median pore size (nm)	Specific surface area (m^2 g^{-1})
1	350	44.2
2	250	53.9
3	1600	9.3
4	1690	7.1

5. Fourier transform infrared (FT-IR) spectra of the glycomonoliths

The presence of saccharide in the glycomonoliths was confirmed by FT-IR spectrometry (Figure S5). For comparison, a polyMan gel (Man content: 70 wt%) was also prepared in DMSO/butanol using the same method as the monoliths. All spectra showed peaks for the C=O stretching vibration (amide I) and N-H deformation vibration (amide II) of the polymer backbone at 1663 and 1529 cm^{-1}, respectively. The amide I peaks overlapped with the peaks corresponding to NH_2 of the primary amide in AAm. In the spectra of macroporous monoliths **1** and **3** (Man content: 0 wt%), peaks corresponding to aromatic C=C (1510 cm^{-1}) and ethereal/alcoholic C-O (970–1070 cm^{-1}) stretching vibrations were not observed. By contrast, these peaks did appear in the spectra of glycomonoliths **2** and **4** (Man content: 10 wt%) and polyMan gel (Man content: 70 wt%). The aromatic C=C peak was very prominent in the spectrum of the polyMan gel. The shoulder peak at 1510 cm^{-1} in the spectra of the glycomonoliths was identified as phenyl-α-D-mannopyranoside. Unfortunately, the peak corresponding to *p*-substituted aryl group (837 cm^{-1}) was not observed in the spectra of the glycomonoliths **2** and **4** (Man content: 10 wt%).

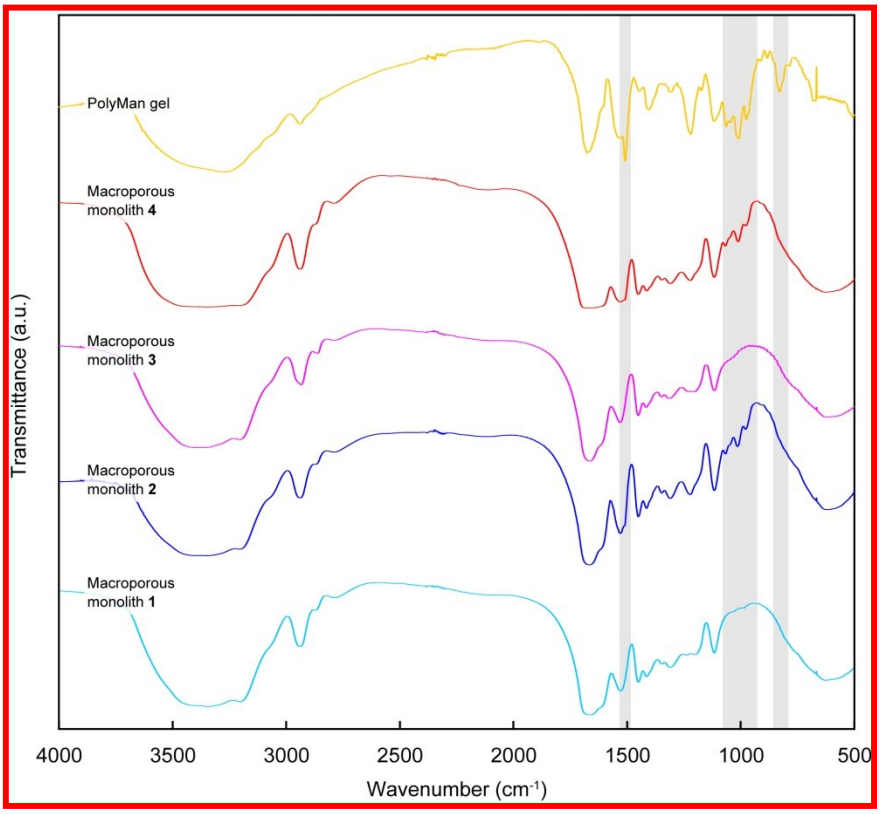

Figure S5. FT-IR spectra of polymer gels with the following Man contents: Man content: 0 wt% (macroporous monolith **1** and **3**), 10 wt% (glycomonolith **2** and **3**), and 70 wt% (polyMan gel).

6. Permeabilities of the glycomonoliths

Permeability of water through each glycomonolith was confirmed to evaluate their suitability for application in the continuous-flow system. Water was permeated through the glycomonoliths at a flow rate of 6 mL h^{-1}, and the pressure loss at steady state was determined. The water permeabilities were calculated using Darcy's law as follows:

$$\Delta P = \frac{1}{k_D} \frac{\mu L}{A} Q$$

where ΔP, k_D, μ, L, A, and Q are the pressure loss, permeation coefficient, water viscosity, thickness, base area, and flow rate, respectively. The permeation coefficients of glycomonoliths **2**, **4**, and the glycopolymer-grafted membrane are shown in Figure S6. These results were compared to the permeation coefficients of commercially available hydrophilic polytetrafluoroethylene membrane filters (Omnipore, Millipore Corporation, Billerica, MA) with various pore sizes, which were estimated from the manufacturer's values of flow times (http://www.emdmillipore.com). The permeation coefficient of glycomonolith **2**, which was prepared using butanol, was less than that of the glycomonolith **4**, which was prepared using octanol. The permeabilities of the glycomonoliths agreed with the porous properties of the lyophilized monoliths (Figure S4). The permeation coefficient of the glycopolymer-grafted membrane with a mean pore size of 430 nm was 10^{-15} m^2. The glycomonoliths **2** and **4** were comparable to those of the Omnipore membrane filters with pore size ranges of 0.1–1 and 1–10 μm, respectively.

After the continuous-flow adsorption of concanavalin A (Con A, J-Oil Mills Inc., Tokyo, Japan), the pressure losses during permeation of water under the same conditions were determined to evaluate the effect of protein adsorption on the permeation coefficients. The permeation coefficients of glycomonoliths **2**, **4**, and the glycopolymer-grafted membrane did not show large changes, indicating that fouling with Con A was negligible. Therefore, the Con A is not separated by size exclusion in the glycomonoliths.

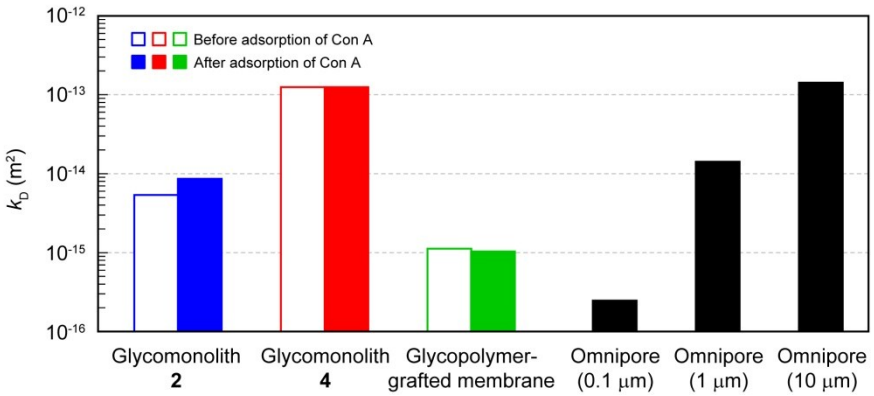

Figure S6. Comparison of the permeation coefficients of Omnipore membrane filters with various pore sizes, glycomonoliths **2** and **4**, and the glycopolymer-grafted membrane before and after continuous-flow adsorption of Con A.

7. Selectivity of the glycomonolith for the target protein

The selectivity of the glycomonolith was investigated in both batch and the continuous-flow adsorption experiments. The dried monoliths were used for batch adsorption of protein. Con A, peanut agglutinin (PNA, Vector Laboratories Inc., Burlingame, CA), and bovine serum albumin (BSA, Sigma Co., St. Louis, MO) were used as protein probes. Con A (104 kDa as a tetramer) is a lectin that specifically binds with glucoside and mannoside. PNA (110 kDa as a tetramer) is also a lectin, and does not bind to mannoside but does specifically bind with galactoside. BSA (approximately 66 kDa) does not have interaction with saccharides. The dried monoliths were crushed, and portions of the monoliths were immersed in buffered solutions (pH 7.4) of each protein (1 g L^{-1}). The solid/liquid ratio was 50 mg/20 mL. After shaking at 25°C for 20 h, the monoliths were removed using membrane filters and the protein concentrations in the filtrates were determined by UV-Vis spectrometry. The amount of protein adsorbed in each macroporous monolith (q) was estimated as follows:

$$q\ (\mathrm{mg\ g^{-1}}) = \frac{C_i - C}{\beta}$$

where C_i and C are the protein concentrations before and after adsorption, and β is the solid/liquid ratio. The amounts of Con A, PNA, and BSA adsorbed in the macroporous monoliths are shown in Figure S7. Macroporous monoliths **1** and **3**, which contained no saccharide, did not adsorb any of the proteins because of the hydrophilicity and neutrality of polyAAm. Glycomonoliths **2** and **4** did not adsorb PNA as well as BSA. The amounts of PNA adsorbed in the glycomonoliths were all less than 3 mg g^{-1}. Glycomonoliths **2** and **4**, which had Man units, showed the adsorption of Con A. These results confirm that the glycomonoliths are specific for the target biomolecule.

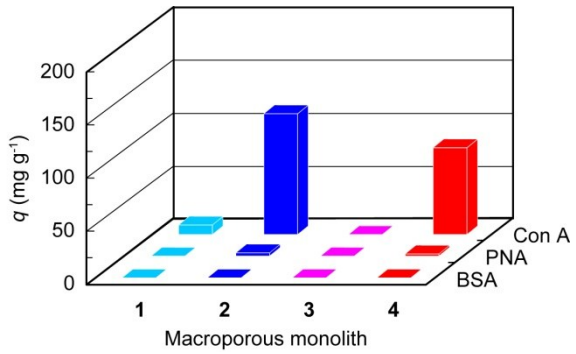

Figure S7. Amounts of Con A, PNA, and BSA adsorbed in the macroporous monoliths.

The reusability of the glycomonolith was also investigated in batch adsorption experiment. After the adsorption of Con A using the glycomonolith **2**, the monolith was immersed in acetic acid solution (pH 2.0) to desorb Con A. The glycomonolith was washed with the buffer solution, and then reused at above Con A adsorption condition. The relative amount of Con A adsorbed in the glycomonolith **2** was 0.76 ± 0.12.

The wet glycomonolith **2** was then applied to continuous-flow adsorption of protein. The void volumes of the monoliths were determined by permeation of blue dextran (200 mg L^{-1}, 2000 kDa, Sigma-Aldrich Co.) in the buffered solution at 1 mL h^{-1}. The concentrations of blue dextran were monitored continuously by UV-Vis spectrometry using the absorbance at 620 nm. The dimensionless void volume of glycomonolith **2** was 4.4, and those of monolith **1** and **4** were 4.3 and 3.7, respectively. For the continuous-flow experiment, the buffered feed solution of Con A or BSA (1 g L^{-1}) was permeated through the glycomonolith **2** at 1 mL h^{-1}. The concentrations of protein in the effluent were monitored continuously by UV-Vis spectrometry using the absorbance at 280 nm. The breakthrough curves of Con A and BSA for glycomonolith **2** are shown in Figure S8. Most of Con A in the feed solution was rejected by the glycomonolith until a bed volume of 30. The breakthrough for BSA occurred just after permeation began, and the curve agreed with that of the void marker. This overlap indicates that pathway of BSA through the monolith was the same as that of blue dextran, which has a much higher molecular weight. This suggests that these molecules passed through the macropores of the monolith. Therefore, the monolith does not separate the protein by size fractionation, as occurs in gel permeation chromatography. Instead, affinity adsorption is the dominant mechanism for Con A separation.

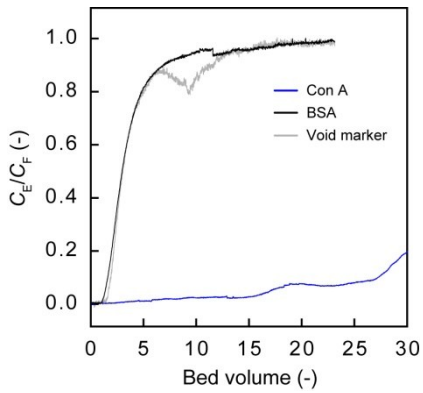

Figure S8. Breakthrough curves for Con A and BSA permeation through glycomonolith **2**. The space velocity for the proteins in the monoliths was 0.61 h^{-1}.