

Selected Paper

Immobilization of Glycoconjugate Polymers on Cellulose Membrane for Affinity Separation

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Membranes with immobilized glycoconjugate polymers were prepared and lectin adsorption was evaluated. Glycoconjugate polymers having the carbohydrates, lactose, and mannose, the amino groups of the reaction point, and a fluorescence label were synthesized, while cellulose membranes were carboxymethylated. The content of the carboxyl group was evaluated by titration. Subsequently, the glycoconjugate polymers were immobilized on the cellulose membranes by amide linkages formed by condensation reaction. Analysis of the luminescence of the fluorescence labels revealed that the glycoconjugate polymers had been immobilized on the cellulose membranes. Examination of lectin adsorption by the glycoconjugate polymer on the membrane revealed that the membrane with the immobilized mannose-having polymer adsorbed 53% of the applied ConA and the membrane with the immobilized lactose-having polymer adsorbed 83% of the applied RCA_{120}. The membrane with immobilized glycoconjugate polymers selectively adsorbed each lectin. In addition, membranes with different types of immobilized glycoconjugate polymers were used in stacks, and in this case, the membranes selectively adsorbed lectin. Thus, membranes with immobilized glycoconjugate polymers efficiently and easily purify lectin.

Numerous techniques for the immobilization of enzymes and ligands that have an affinity for biomolecules have been developed.[1–4] In particular, modifications to cellulose have been developed extensively for practical use, such as the immobilization of enzymes and various proteins.[5–8] Modifications of cellulose by the introduction of functional groups such as amino groups, carboxyl groups, isocyanate groups, nitro groups, phosphate groups, epoxide, and imines have been reported.[6,8–13] In biotechnology, cellulose can be easily modified and utilized as filter paper or as a dialysis membrane. Furthermore, modified cellulose derivatives have also been utilized as ion-exchange adsorbents and separation membranes.[10]

Recent developments in membrane affinity chromatography have offered some advantages over typical column chromatography, which utilizes spherical beads and swelling gels. The advantages include higher flow rate, faster binding rate, lower pressure drop, easier preparation, higher productivity, and easier scale-up. Moreover, polymer membranes are suitable for modification due to their mechanical strength and progress in excellent film-forming techniques. However, synthetic polymer membranes have lower compatibility with biomolecules, and each of these membranes requires a different modification approach. In terms of compatibility with biomolecules, natural polymer membranes such as cellulose are superior.[13,14]

Carbohydrate–protein interactions occur in numerous biologically important events, such as immune defense response, viral replication, parasite infection, cell–cell adhesion, and inflammation. The interaction between carbohydrate ligand and protein usually presents with weak binding affinity i.e., K_d in the mM range. However, the multiple interaction presents with high affinity, i.e., K_d in the nM range, due to recognition of the multiple carbohydrate ligands that exist in nature. This effect is called the "glycoside cluster effect," and is so important that researchers have naturally tried to imitate nature. Consequently, artificial glycoconjugate polymers have been synthesized to mimic natural ligands.[15–17]

Here, we report an affinity membrane on which glycoconjugate polymers were immobilized. When a glycoconjugate polymer is immobilized on a membrane, it is expected that the polymer adsorbs more effectively than monovalent sugar due to the "glycoside cluster effect." Moreover, since the immobilized polymer is covalently bonded to the membrane, it is difficult for the polymer to be cleaved from the membrane. The isolated polymer and lectin are not separated by reason of similar molecular weight.

Two kinds of glycoconjugate polymers, having either lactose or mannose, were synthesized. D-Lactose possesses potency as a ligand for lectin, *Ricinus communis agglutinin* (RCA_{120}). RCA_{120} recognizes the terminal β-D-galactosyl residue of lactose.[18,19] α-D-Mannose possesses potency as a ligand for lectin isolated from jack bean seeds (*Canavalia ensiformis*), concanavalin A (ConA). ConA recognizes the mannosyl oligosaccharides in glycopeptides.[20,21] These glycoconjugate polymers, having immobilizing points and fluorescence labels, were immobilized onto a cellulose membrane. Finally, protein adsorption was evaluated using these modified membranes and lectins.

Results and Discussion

Synthesis of Saccharide Monomers. In the present study, saccharide monomers having an acrylamide group at the reducing end were synthesized. The 5-hexenyl group was selected as the aglycon. By radical addition to an olefin, the amino group was introduced to the reducing end. Conversion of the reducing end to the acrylamide group afforded the glycosyl monomer.[22]

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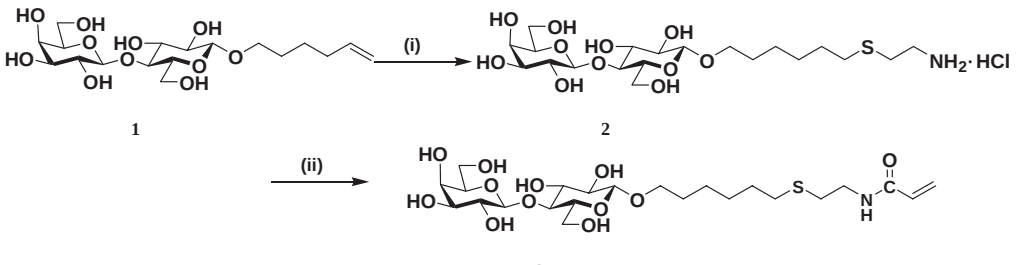
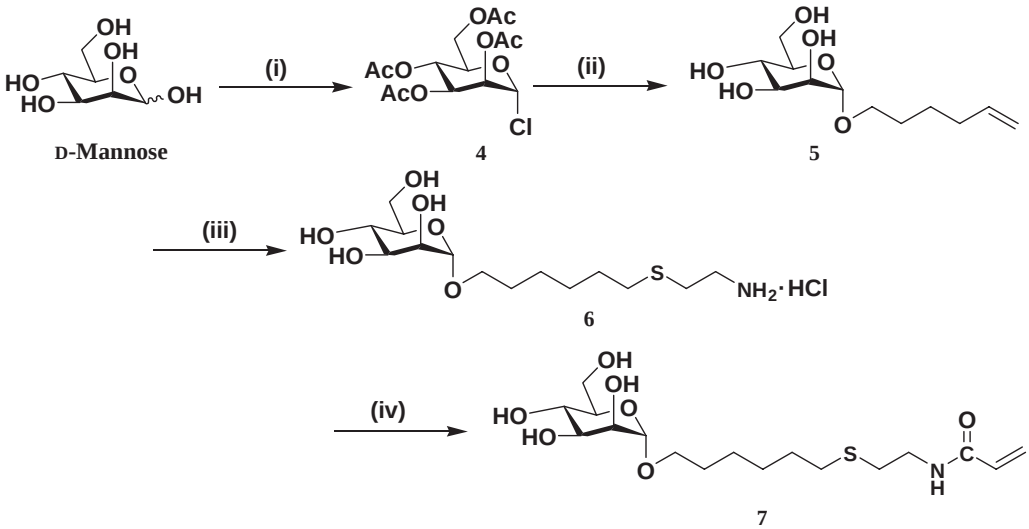


Scheme 1. *Reagents and conditions*: (i) $HSCH_2CH_2NH_2 \cdot HCl$, MeOH, $h\nu$ (254 nm), 0 °C, 5.5 h; (ii) 1) $CH_2{=}CHCOCl$, Na_2CO_3, MeOH, rt, 30 min, then Ac_2O, pyridine, rt, overnight, 2) NaOMe, MeOH, rt, 4.0 h, 83.5%.



Scheme 2. *Reagents and conditions*: (i) 1) Ac_2O, pyridine, rt, overnight, then $BnNH_2$, rt, 3.0 h, 2) $SOCl_2$, DMF, $ClCH_2CH_2Cl$, 0 °C, overnight, 95%; (ii) 1) 5-Hexen-1-ol, $Et_2O \cdot BF_3$, toluene, γ-collidine, AgOTf, −20 °C, 2.5 h, then rt, 1.0 h, 2) NaOMe, MeOH, rt, overnight, 42.6%; (iii) $HSCH_2CH_2NH_2 \cdot HCl$, MeOH, $h\nu$ (254 nm), rt, 4.0 h, quant.; (iv) 1) $CH_2{=}CHCOCl$, Na_2CO_3, MeOH, rt, 30 min, then Ac_2O, pyridine, rt, overnight, 2) NaOMe, MeOH, rt, 3.0 h, 69.6%.

Scheme 1 describes the synthesis of the lactose monomer **3**. 5-Hexenyl β-lactoside (**1**) was synthesized by the glycosylation of 5-hexen-1-ol with acetyl β-lactoside and subsequent deacetylation. The mixture of **1** and 2-aminoethane-1-thiol hydrochloride was irradiated (254 nm) to give the amine hydrochloride derivative **2**. The amino group of **2** was *N*-acryloylated and the hydroxy groups were acetylated for purification. Deacetylation gave the lactose monomer **3** having an acrylamide group at the reducing end. The β-galactose unit at the nonreducing end of lactose is the ligand for RCA_{120}.

As shown in Scheme 2, the mannose monomer **7** was synthesized. D-Mannose was acetylated, followed by selective deacetylation[23] and chlorination[24] to afford **4**. Glycosylation of 5-hexen-1-ol with mannosyl chloride **4** in toluene using silver trifluoromethanesulfonate as the promoter[25] gave 5-hexenyl acetyl-α-mannoside, which was deacetylated to afford **5**. By the same procedure as for the lactose monomer, 5-hexenyl mannoside was converted to the mannose monomer **7**, which contains an acrylamide group at the reducing end, via radical addition, *N*-acryloylation and deacetylation. The α-mannosyl monomer **7** is the ligand for ConA.

Synthesis of Amine-Having and Fluorescent Monomers. The amine monomer **8** and fluorescent monomer **9**[26] were prepared by a simple process. The amine monomer **8** having an acrylamide group was synthesized by mono-*N*-acryloylation of hexamethylenediamine. The fluorescent monomer **9** having an acrylamide group was synthesized by sulfonamidation and *N*-acryloylation of hexamethylenediamine. Hexamethylenediamine was treated with dansyl chloride and the crude product was *N*-acryloylated to afford the fluorescent monomer **9**. The roles of these functionalized monomers (**8** and **9**) are illustrated in Fig. 1. The amine monomer **8** serves as the reactive point on the activated cellulose membrane. The amino group of the glycoconjugate polymer reacts with the carboxyl group on the cellulose membrane to immobilize glycoconjugate polymers to the membrane. The fluorescent monomer **9** serves as an indicator of the presence of the polymer immobilized on the cellulose membrane. Whether or not the polymer was immobilized to the cellulose membrane could be ascertained by the luminescence of the fluorescent monomer unit by irradiating the cellulose membrane using a UV lamp.

Radical Polymerization and Synthesis of Glycoconjugate Polymers. Glycosyl monomers **3** and **7** were copolymerized with acrylamide and functionalized monomers at room temperature using *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) and ammonium peroxodisulfate (APS) as initiators.[27] The copolymerization data are summarized in Table 1. Polymer A, which carries a fluorescence and immobilization point, was

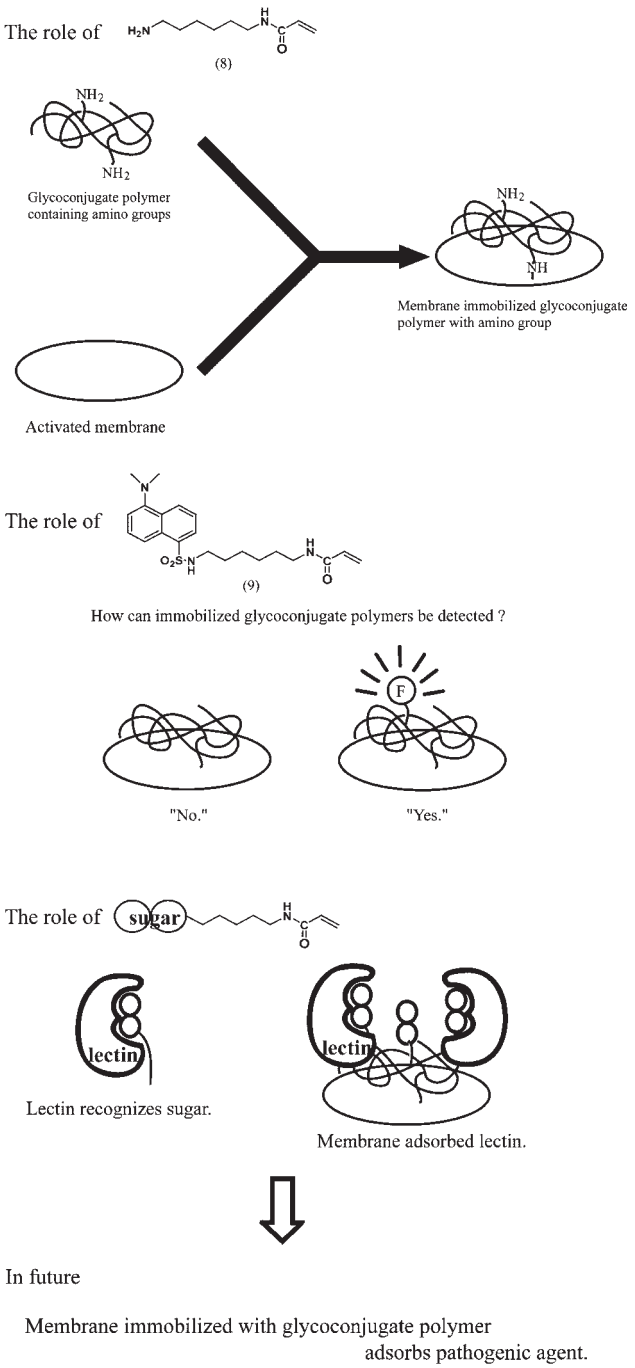


Fig. 1. The roles of functional monomers.

utilized to detect whether the glycoconjugate polymer was immobilized onto the cellulose membrane. These glycoconjugate polymers were immobilized to the cellulose membrane and were utilized as multivalent affinity ligands. To confirm whether the amino group (and not the hydroxy group) was the reactive point, Polymer D not having amino group and E not having hydroxy group were utilized for the immobilization reaction.

Modification of the Cellulose Membrane. The cellulose membrane (Whatman, No. 41, $\phi = 55$ mm) was activated to immobilize glycoconjugate polymers. Various methods for modifying cellulose have been developed. Initially, methods introducing carboxyl groups[10] and epoxy groups[13] to cellulose membranes were applied in the present study. Since the glycoconjugate polymers have hydroxy groups and amino groups, it is important that only the amino group selectively reacts with the modified cellulose membranes. However, cellulose membranes with epoxy groups reacted not only with the amino groups, which the glycoconjugate polymers have, but also with the hydroxy groups that cellulose membranes have. Consequently, carboxymethylation of cellulose membranes was adopted. Cellulose membranes were soaked in 1, 3, or 6 M NaOH and saturated NaCl solution to activate the hydroxy groups of cellulose. Addition of bromoacetic acid led to carboxymethylation of cellulose membranes. This procedure is both classical and industrial; hence, the method is facile. The content of the carboxyl group introduced to the cellulose membrane was determined by titration. The results are shown in Table 2.

Immobilization of Glycoconjugate Polymers to Cellulose Membranes. The glycoconjugate polymers were immobilized to the carboxymethylated cellulose membranes via the condensation reaction. Initially, Polymer A (having an amino group and a fluorescence label) was immobilized to the cellulose membranes, and activated by bromoacetic acid in 3 concentrations of NaOH solution. A comparison of the observed luminescence of the membranes by irradiating with a handheld UV lamp showed that the prepared carboxymethylated membrane in 6 M NaOH solution had a fairly weak intensity of luminescence when compared to the other prepared carboxymethylated membranes (Fig. 2).

Next, Polymer A was immobilized onto the cellulose membranes with different carboxyl group contents. The membranes that immobilized the polymer showed a similar intensity of luminescence (Fig. 3).

These results suggest that the preferred concentration of NaOH solution used for the activation of the membranes was the mild condition (1 M NaOH) over the hard condition (6 M NaOH), and that the content of carboxyl group did not affect the amount of immobilized polymers during the immobilizing reaction. Then, this immobilization was achieved by condensation between the amino groups of glycoconjugate polymers and the carboxyl groups of the modified cellulose membranes. Immobilization of the polymer having lactose and a fluorescence label (Polymer D) was performed on the activated cellulose membranes. Immobilization of the polymer having amino groups and a fluorescence label (Polymer E) was also performed. As shown in Fig. 4, only Polymer E (having amino groups) was immobilized. Thus, the carboxyl groups on the membranes did not react with the hydroxy groups, but reacted with the amino groups of the polymer.

Finally, the synthesized glycoconjugate polymers (Polymers B and C) were immobilized on activated cellulose membranes under optimized conditions. For immobilization, 2.5 mg of glycoconjugate polymer per membrane and water-soluble condensing reagent were used. Consequently, about 1.9 mg of Polymer C (having mannose) per membrane and 2.5 mg of Polymer B (having lactose) per membrane were immobilized to the cellulose membrane, respectively.

Protein Adsorption on Membranes Having Glycoconjugate Polymers. Cellulose membranes having glycoconjugate

Table 1. Results of Copolymerization

Polymer	Monomer ratio				Total yield /%	Polymer composition[a)]	Mw[b)] /kDa
	Glycosyl monomer **3** or **7**	Amine monomer **8**	Fluorescent monomer **9**	Acrylamide			
Polymer A	1 (**3**)	0.1	0.05	4	80.7	1:0.11:0.06:4	320
Polymer B	1 (**3**)	0.05	—	—	93.1	1:0.08	301
Polymer C	1 (**7**)	0.05	—	—	69.0	1:0.04	n.d.
Polymer D	1 (**3**)	—	0.05	4	75.7	1:0.04:4.7	147
Polymer E	—	0.05	0.05	1	91.7	0.03:0.02:6	n.d.

a) The polymer composition was determined from the integration value of ^{1}H NMR. b) Mws were estimated by the SEC method with the TOSOH TSKgel G-Oligo-PW column, TSKgel G2500PWXL column, TSKgel G3000PWXL column, and TSKgel G4000PWXL column [pullulans (5.8, 12.2, 23.7, 48.0, 100, 186, and 380 kDa, Shodex Standard P-82) were used as standards].

Table 2. The Carboxyl Group Content (μmol/paper) Introduced to the Cellulose Membrane

	0 μmol[b)] (control)	36 μmol[b)]	180 μmol[b)]	360 μmol[b)]	720 μmol[b)]
1 M NaOH (aq)[a)]	0	3.9	8.3	14	25
3 M NaOH (aq)[a)]	0	4.1	12	14	25
6 M NaOH (aq)[a)]	0	n.d.[c)]	n.d.[c)]	n.d.[c)]	26

a) The concentration of applied NaOH solution in activating the cellulose membrane. b) The amount of $BrCH_2COOH$ used in activating the cellulose membrane. c) Not determined.

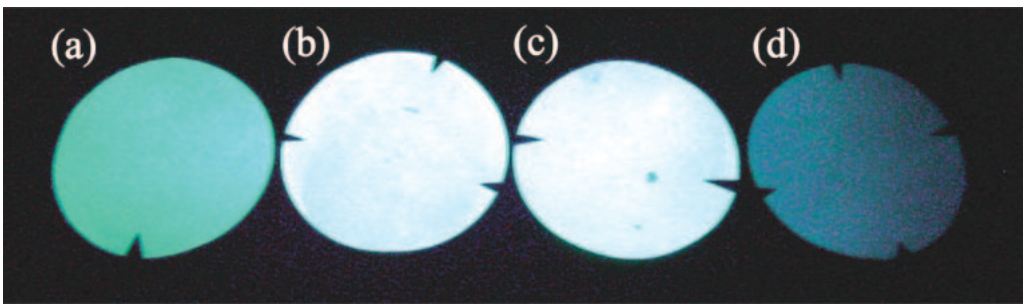


Fig. 2. Luminescence of membranes with immobilized Polymer A (having fluorescence label, amino group, and lactose) during irradiation with a UV lamp. (a) 6 M NaOH solution, (b) 3 M NaOH solution, (c) 1 M NaOH solution, and (d) non-modified cellulose membrane.

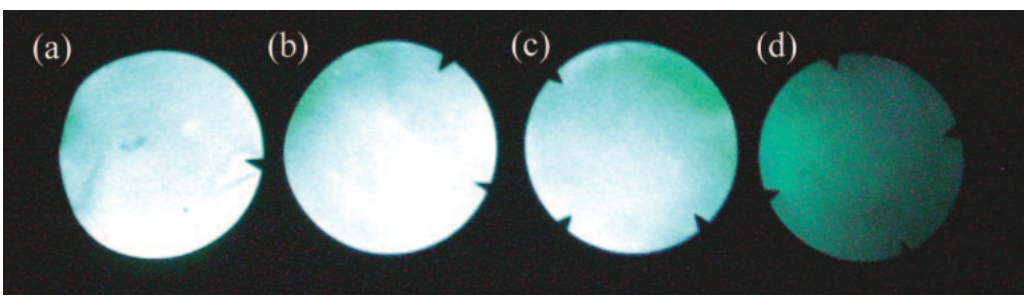


Fig. 3. Luminescence of membranes with immobilized Polymer A (having fluorescence label, amino group, and lactose) during irradiation with a UV lamp. (a) 26 μmol carboxyl group content, (b) 14 μmol carboxyl group content, (c) 3.6 μmol carboxyl group content, and (d) non-modified cellulose membrane.

polymers were cut into small disks 25 mm in diameter. The small cellulose membranes (5 pieces) were set in a filter holder. The sugar content of the polymers, which were immobilized onto the set membranes, was 300 times the content of the charged lectin and was sufficient for adsorbing the lectin. PBS(−) (phosphate buffered saline) solution was allowed to flow through the holder containing the membranes with immobilized Polymer C (having mannose). ConA-PBS(−) solution was then allowed to flow through the holder, and the holder was washed with PBS(−) solution. Next, a 0.2 M mannose-PBS(−) solution was allowed to flow through the holder in order to elute ConA. Finally, each fraction was evaluated using

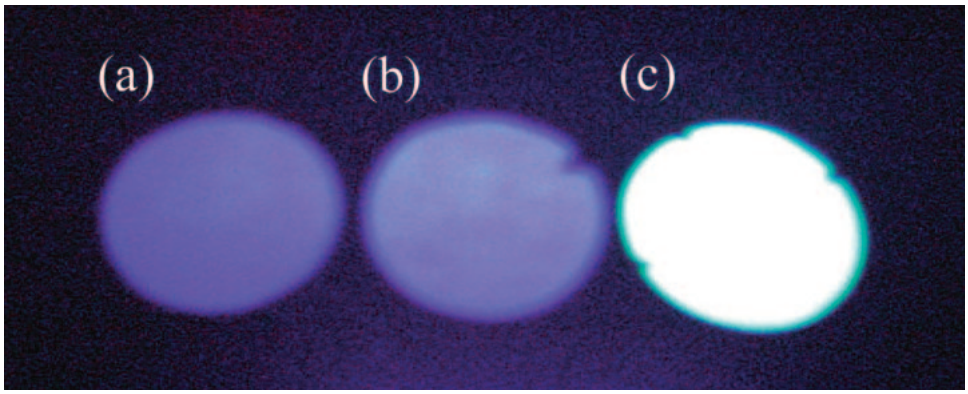


Fig. 4. Luminescence of membranes with immobilized glycoconjugate polymers during irradiation with a UV lamp. (a) Non-modified cellulose membrane, (b) Polymer D (having lactose and fluorescence label), and (c) Polymer E (having amino group and fluorescence label).

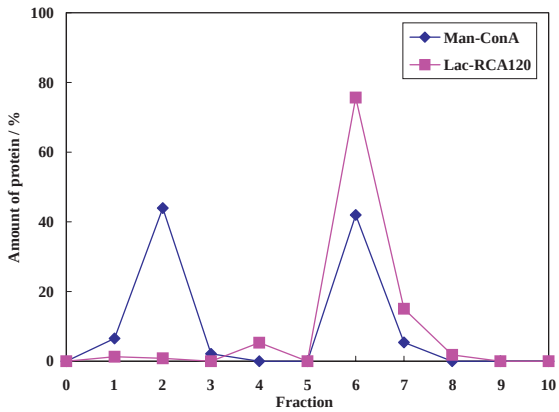


Fig. 5. Adsorption of lectins on the membranes with immobilized glycoconjugate polymers (the membrane with immobilized Polymer C having mannose for ConA and the membrane with immobilized Polymer B having lactose for RCA_{120}). Horizontal axis: The volume of each fraction is 1.0 mL. Fractions 1 and 2: 200 μg mL^{-1} lectin-PBS(−) solution, Fractions 3–5: PBS(−) solution, Fractions 6–10: 0.2 M sugar-PBS(−) solution. Vertical axis: Amount of protein shows percentage of the total amount of applied protein.

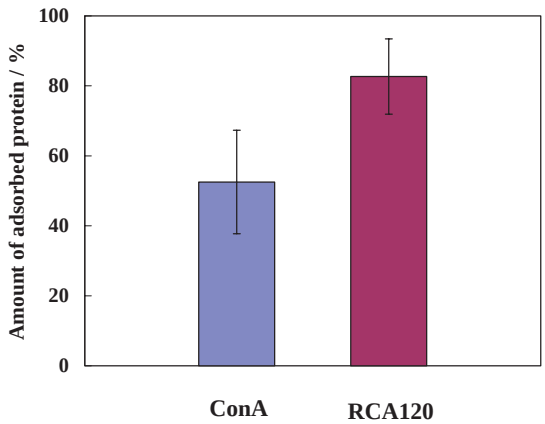


Fig. 6. Amount of adsorbed ConA and RCA_{120} on the membranes with immobilized glycoconjugate polymers (the membrane with immobilized Polymer C having mannose for ConA and the membrane with immobilized Polymer B having lactose for RCA_{120}). Data represent the mean of 3 determinations. Vertical axis: Amount of protein shows percentage of the total amount of applied protein.

Bio-Rad Protein assay reagents.

Similarly, the membranes with immobilized Polymer B (having lactose) were evaluated for RCA_{120}. As shown in Fig. 5, the lectins were adsorbed by the membranes and eluted. Fractions 1–5 show that about 50% of ConA passed through the membrane without stopping, while most of the RCA_{120} was adsorbed. Fractions 6–10 show that both lectins were eluted by eluents containing their ligand sugars. Even when half the amount of ConA was used, about 50% of ConA passed through the membrane without stopping. These results indicate that ConA has a low affinity for simple α-mannose while RCA_{120} has a high affinity for terminal galactose.[21] In the literature,[28,29] the K_a value of ConA for a glycoconjugate polymer having mannose is about $10^4\ M^{-1}$ and the K_a value of RCA_{120} for a glycoconjugate polymer having terminal galactose is about $10^7\ M^{-1}$. The difference of the amounts of adsorbed lectins corresponds to the difference of the K_a values of lectins. The amounts of adsorbed proteins are summarized in Fig. 6.

Non-specific adsorption of both ConA and RCA_{120} was next examined using a non-modified membrane. As shown in Fig. 7, about 20% of the charged lectins were non-specifically adsorbed on the non-modified membrane.

Moreover, the specific adsorption was investigated to be sugar-specific. The holder containing the membranes with immobilized Polymer C (having mannose) was examined for the adsorption of RCA_{120}. Correspondingly, the holder in which the membranes with immobilized Polymer B (having lactose) were set was likewise examined for the adsorption of ConA. Each solution flowed into the holder in the order of PBS(−) solution, followed by lectin-PBS(−) solution, PBS(−) solution, and then the eluting solution. The results are shown in Fig. 8. All proteins were eluted by a washing buffer, indicating the absence of non-specific adsorption. Thus, the interactions between the mannose-having membrane and ConA, and the lactose-having membrane and RCA_{120} were proved to be specific

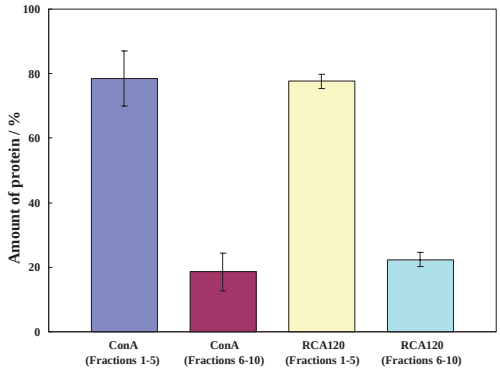


Fig. 7. Amount of adsorbed (non-specific) ConA and RCA_{120} on non-modified membranes. Data represent the mean of 3 determinations. Vertical axis: Amount of protein shows percentage of the total amount of applied protein.

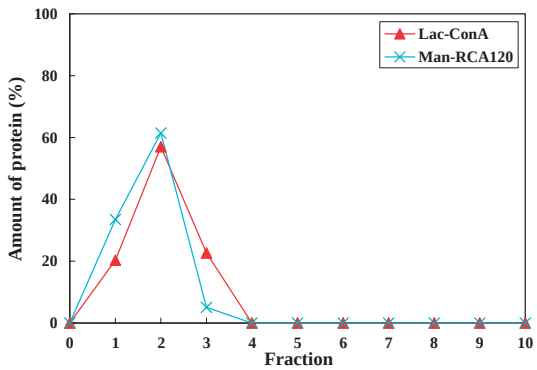


Fig. 8. Evaluation of sugar-specific adsorption of lectins on the membranes with immobilized glycoconjugate polymers (the membrane with immobilized Polymer B having lactose for ConA and the membrane with immobilized Polymer C having mannose for RCA_{120}). Horizontal axis: The volume of each fraction is 1.0 mL. Fractions 1 and 2: 200 μg mL^{-1} lectin-PBS(−) solution (mannose for RCA_{120} and lactose for ConA); Fractions 3–5: PBS(−) solution; Fractions 6–10: 0.2 M sugar-PBS(−) solution. Vertical axis: Amount of protein shows percentage of the total amount of applied protein.

carbohydrate–protein interactions. Finally, the membranes with different types of immobilized glycoconjugate polymers were set in the filter holder in the following order: immobilized Polymer C (having mannose), immobilized Polymer B (having lactose), and non-modified membrane. Then, the solution of ConA-FITC in PBS(−) was allowed to flow through the holder, and the holder was washed with PBS(−) solution. Then, each membrane in the holder was observed under a fluorescence microscope. Similarly, RCA_{120}-FITC solution was allowed to flow through the holder and the results were examined. As shown in Fig. 9, only the membrane with the immobilized glycoconjugate polymer having a specific sugar for lectin showed adsorption of lectin, as determined by fluorescence of FITC (Figs. 9b and 9f). Consequently, these membranes can be used by superposing them, and each membrane will adsorb a specific lectin.

Conclusion

The functionalized glycoconjugate polymers with a fluorescence label and reactive point were prepared by copolymerizations of the monomers that were synthesized simply by *N*-acryloylation. Cellulose membranes were modified by carboxymethylation and the content of the introduced carboxyl groups was detected. The glycoconjugate polymers were immobilized to the modified cellulose membranes. The luminescence of the cellulose membranes indicated that the glycoconjugate polymers were immobilized on the membranes. The polymers having sugar moieties, a fluorescence label, and reactive point were adapted to a variety of immobilization reactions. The membranes with immobilized glycoconjugate polymers were used as affinity membranes, and found to retain 53% of ConA and 83% of RCA_{120}. Thus, membranes with immobilized glycoconjugate polymers can be used to purify proteins, just as affinity column chromatography does, and are useful for medical diagnostic techniques, such as sugar chips. Affinity membranes are easier to prepare than affinity gels, since only setting of the filter paper on the holder is required. Further development of affinity membranes with immobilized multivalent ligands is currently being conducted.

Experimental

General. Unless otherwise stated, all commercially available solvents and reagents were used without further purification. *N*,*N*-Dimethylformamide (DMF), tetrahydrofuran (THF), 1,2-dichloroethane, dichloromethane, and pyridine were stored over molecular sieves 4 Å. Methanol was stored over molecular sieves 3 Å. Powdered molecular sieves were dried in vacuo at ca. 180 °C for at least 2 h. Acrylamide was recrystallized from benzene. Acetic acid solution (0.1 M) was purchased from Wako Pure Chemical Industries Co., Ltd. Whatman filter paper (No. 41, $\phi = 55$ mm), which was used as cellulose membranes was purchased from Whatman International Ltd., and a SWINNEX® filter holder was purchased from MILLIPORE.

^{1}H NMR spectra were recorded at 400 or 600 MHz using a JEOL JNM-AL400 or JEOL ECP-600 spectrometer in chloroform-*d* or deuterium oxide. ^{13}C NMR spectra were recorded at 100.6 or 150.9 MHz with the same instruments. Tetramethylsilane (TMS) was used as the internal standard. Proton assignments in the NMR spectra were made by first-order analysis of spectra, and supported by correlation spectroscopy and heteronuclear chemical shift correlation. The average molecular weights of the polymers were estimated by size exclusion chromatography (SEC) with a TOSOH TSKgel G-Oligo-PW column, TSKgel G2500PWXL column, TSKgel G3000PWXL column, and TSKgel G4000PWXL column with pullulans (5.8, 12.2, 23.7, 48.0, 100, 186, and 380 kDa, Shodex Standard P-82) used as standards. Reactions were monitored by thin-layer chromatography (TLC) on a precoated plate of silica gel 60 F_{254} (layer thickness, 0.25 mm; E. Merck, Darmstadt, Germany). For detection of intermediates, TLC sheets were dipped in (a) a solution of 85:10:5 (v/v/v) methanol–*p*-anisaldehyde–concentrated sulfuric acid and heated for a few minutes (for carbohydrates), or (b) an aqueous solution of 5 wt % potassium permanganate and heated similarly (for double bond). Column chromatography was performed on silica gel (Silica Gel 60; 40–63 μm, E. Merck, or Silica Gel 60, spherical neutral; 40–100 μm, E. Merck). Fluorescence was observed using an Olympus IX70 fluorescence microscope. Protein adsorption was determined

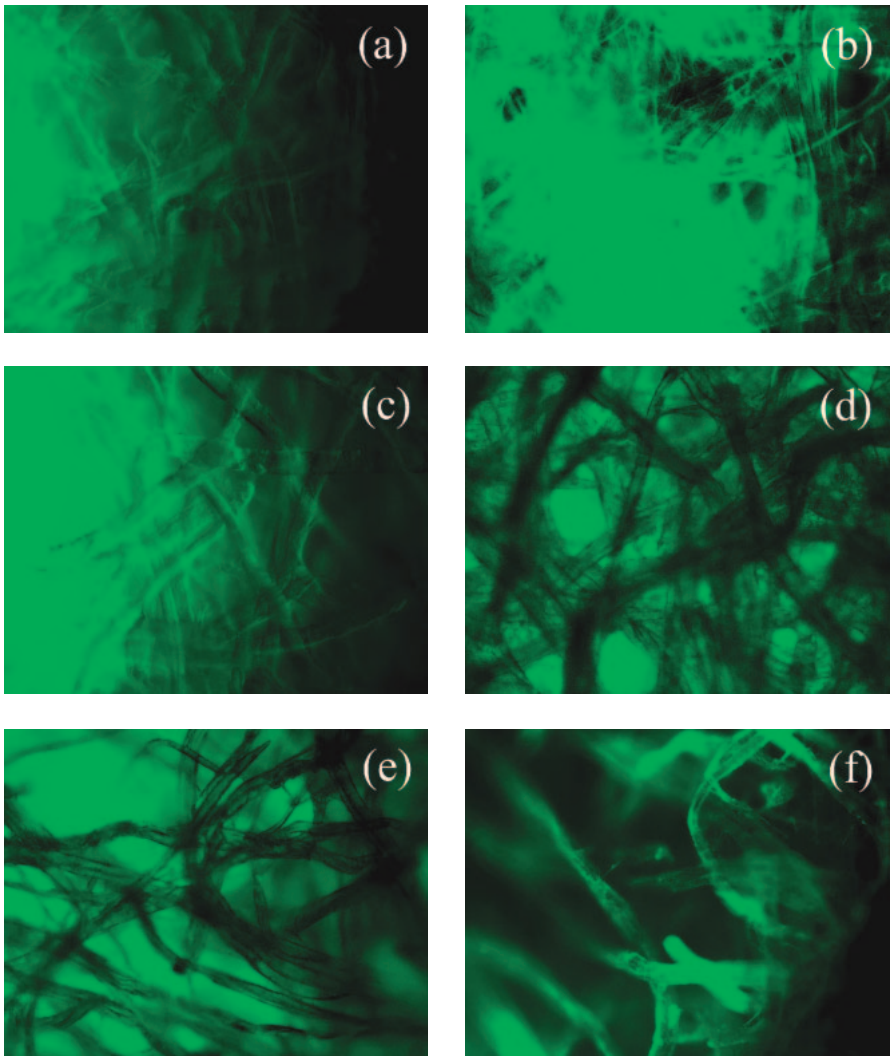


Fig. 9. Fluorescence observed from adsorbed FITC-labeled lectin on membranes. For ConA-FITC: (a) non-modified cellulose membrane, (b) membrane with immobilized glycoconjugate polymers having mannose, and (c) membrane with immobilized glycoconjugate polymers having lactose. For RCA_{120}-FITC: (d) non-modified cellulose membrane, (e) membrane with immobilized glycoconjugate polymers having mannose, and (f) membrane with immobilized glycoconjugate polymers having lactose.

using a Bio-Rad Protein Assay kit purchased from Bio-Rad.

Synthesis. 6-(2-Aminoethylthio)hexyl 4-*O*-(β-D-Galactopyranosyl)-β-D-glucopyranoside Hydrochloride (2): Compound **2** was prepared following the method described in the literature:[22] ^{1}H NMR (D_2O) δ 3.09 (dd, 2H, –CH_2–N–), 2.74 (dd, 2H, –S–CH_2–), 2.49 (dd, 2H, –CH_2–S–).

6-(2-*N*-Acryloylaminoethylthio)hexyl 4-*O*-(β-D-Galactopyranosyl)-β-D-glucopyranoside (3): Compound **3** was prepared following the method described in the literature:[22] ^{1}H NMR (D_2O) δ 6.10 (m, 1H, –CH=C), 5.66 (brd, 1H, –C=CH), 4.35 (m, 2H, H-1′,1), 3.82 (m, 1H, H-4′), 3.68 (m, 1H, H-3′), 3.56 (m, 2H, H-3,5), 3.45 (m, 2H, H-2,4), 3.38 (m, 2H, –CH_2–N–), 3.20 (m, 3H, H-2, –O–CH–), 2.64 (m, 2H, –CH_2–S–), 2.49 (m, 2H, –S–CH_2–), 1.51 (m, 4H, –CH_2– × 2), 1.28 (m, 4H, –CH_2– × 2).

2,3,4,6-Tetra-*O*-acetyl-α-D-mannosyl Chloride (4): Compound **4** was prepared following the method described in the literature:[22,23] ^{1}H NMR ($CDCl_3$) δ 5.42 (dd, 1H, H-3), 5.31 (m, 1H, H-5), 5.25 (m, 2H, H-2,1α), 5.01 (d, 1H, $J_{1\beta,2}$ = 8.8 Hz, H-1β).

5-Hexenyl α-D-Mannoside (5): Compound **5** was prepared following the method described in the literature:[25] ^{1}H NMR (D_2O) δ 5.71 (m, 1H, –CH=C), 4.91 (dd, 2H, –C=CH_2), 4.66 (d, 1H, $J_{1\alpha,2}$ = 1.1 Hz, H-1), 3.73 (dd, 2H, H-2,6a), 3.64 (m, 3H, H-3,6a, –OCH–), 3.55 (t, 1H, H-4), 3.43 (m, 1H, H-5), 3.32 (m, 1H, –OCH–), 1.97 (m, 2H, –CH_2–C=C), 1.50 (m, 2H, –OC–CH_2–), 1.38 (m, 2H, –CH_2–).

6-(2-Aminoethylthio)hexyl α-D-Mannoside Hydrochloride (6): Compound **6** was prepared following the method described in the literature:[22] ^{1}H NMR (D_2O) δ 3.09 (dd, 2H, –CH_2–N–), 2.74 (dd, 2H, –S–CH_2–), 2.49 (dd, 2H, –CH_2–S–).

6-[2-(*N*-Acryloylamino)ethylthio]hexyl α-D-Mannoside (7): Compound **7** was prepared following the method described in the literature:[22] ^{1}H NMR (D_2O) δ 6.24 (m, 1H, –CH=C, –C=CH), 5.66 (brd, 1H, –C=CH), 4.77 (m, 2H, H-1), 3.83 (m, 2H, H-2,4), 3.72 (m, 1H, H-3,6a, –OCH–), 3.53 (m, 1H, H-5), 3.44 (m, 4H, H-5,6b, –OCH–, –CH_2–N–), 2.68 (m, 2H, –CH_2–S–), 2.57 (m, 2H, –S–CH_2–), 1.61 (m, 4H, –CH_2– × 2), 1.41 (m, 4H, –CH_2– × 2).

***N*-(6-Aminohexyl)acrylamide (8):** Hexamethylenediamine (500 mg, 4.30 mmol) was dissolved in methanol (20 mL) and the solution was cooled to 0 °C. Acryloyl chloride (384 μL, 4.73 mmol) was added to the solution dropwise and the solution was

stirred at 0 °C for 30 min. The mixture was concentrated. Purification by silica-gel column chromatography with 2:1 (v/v) chloroform–methanol was carried out on the residue to give **8** (481 mg, 55.8%): ^{1}H NMR (D_2O) δ 6.26 (brd, 1H, –CH=C), 6.17 (brd, 1H, –C=CH), 5.74 (brd, 1H, –C=CH), 3.26 (brs, 2H, $–CH_2–N–C–$), 2.98 (brs, 2H, $N–CH_2–$), 1.66 (brs, 2H, $–CH_2–C–N–$), 1.55 (brs, 2H, $–CH_2–$), 1.38 (brs, 4H, $–CH_2– \times 2$).

***N*-[6-(*N*-Acryloylaminohexyl)]-5-dimethylamino-1-naphthalenesulfonamide (9):** Hexamethylenediamine (4.31 g, 37.1 mmol) was dissolved in 1,4-dioxane (100 mL) and the solution was cooled to 0 °C. After adding dansyl chloride (1.00 g, 3.71 mmol), the solution was stirred overnight at 0 °C. The mixture was concentrated and the residue was extracted with chloroform. The chloroform solution was dried over anhydrous magnesium sulfate, filtered, and evaporated in vacuo. The syrupy residue was then dissolved in THF (60 mL) and sodium carbonate (789 mg, 7.44 mmol) was added to the mixture at 0 °C. Acryloyl chloride (909 µL, 7.44 mmol) was added dropwise to the solution, and the mixture was stirred at room temperature for 1 h. The mixture was concentrated and extracted with chloroform. The chloroform layer was washed successively with aqueous sodium hydrogencarbonate and brine, dried over anhydrous magnesium sulfate, filtered, and evaporated in vacuo. Purification by silica-gel column chromatography with 50:1 (v/v) of chloroform–methanol was carried out on the residue to give **9** (1.12 g, 74.7%): ^{1}H NMR ($CDCl_3$) δ 8.54 (d, 1H, H-7), 8.30 (d, 1H, H-9), 8.23 (d, 1H, H-2), 7.54 (m, 2H, H-3,8), 7.19 (d, 1H, H-4), 6.27 (d, 1H, –CH=C), 6.10 (m, 1H, –C=CH), 5.78 (brs, 1H, –NH–CO–), 5.60 (d, 1H, –C=CH), 3.23 (m, 2H, $–CH_2–N–S–$), 2.88 (m, 8H, $–CH_2–N–C–$, $–CH_3 \times 2$), 1.37 (m, 4H, $–CH_2–C–N–$, $–CH_2–C–S–$), 1.18 (m, 4H, $–CH_2– \times 2$).

Copolymerization. Solution of the glycosyl monomer **3** or **7**, amine monomer **8**, fluorescent monomer **9**, or acrylamide in deionized water or DMSO was degassed using a diaphragm pump, and TEMED (0.1 equiv for glycosyl monomer) and APS (0.04 equiv for glycosyl monomer) were added to the solution. The reaction mixture was continuously stirred overnight at room temperature. The resulting product was purified by reprecipitation with a mixed solution of methanol and water and lyophilized to give a water-soluble copolymer as a powder. This procedure was performed for each of the monomer ratios in Table 1.

Modification of Membrane. Carboxymethylation of the cellulose membrane was achieved by the addition of bromoacetic acid. Cellulose membranes (20 pieces) were soaked in a mixture of a 1, 3, and 6 M NaOH solution (30 mL) and saturated NaCl solution (30 mL), and then stirred for 1 h at room temperature. To the reaction mixture was added bromoacetic acid (0.10, 0.50, 1.0, or 2.0 g) and the mixture was then stirred overnight at room temperature. Next, the membranes were washed thoroughly and dried in air.

Determination of the Carboxyl Content in the Cellulose Membrane. The carboxyl content in the membrane was determined by titration. Initially, 4 pieces of the modified cellulose membranes were soaked for 30 min in a 1 M HCl solution. The membranes were washed thoroughly and cut in small pieces and placed in a 25 mL flask, and then water was added. Several drops of 0.04% phenolphthalein indicator were added to the flask. The solution containing the pieces of membrane was then titrated with a 2.0 mM NaOH solution. The concentration of the NaOH solution used was calibrated by titration with a commercial 0.1 M acetic acid solution.

Immobilization. Immobilization of glycoconjugate polymers to a carboxymethylated cellulose membrane was achieved by the condensation reaction. Carboxymethylated cellulose membranes (4 pieces) were soaked in a solution containing 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (11.4 mg) in water (20 mL) and the mixture was stirred for 2 h at room temperature. The glycoconjugate polymers (10 mg) were then dissolved in water (10 mL) and added to the mixture. The mixture was stirred overnight at room temperature. The membranes were filtered, washed thoroughly, and dried in air. Alternatively, the filtrate was concentrated and dialyzed. After dialysis, the solution was lyophilized and the amount of the polymers that had not been immobilized to the membranes was determined. The dried membranes were observed under an ultraviolet lamp (254 nm) to confirm whether the polymers had been immobilized to the cellulose membranes. The same procedure was performed for each polymer.

Protein Adsorption Assay. Protein quantification was performed using a Bio-Rad Protein Assay based on the Bradford method. The absorbance of protein solutions containing reagents was measured at 590 nm. Comparison to a standard curve provided a relative estimate of protein concentration.

The cellulose membranes with immobilized glycoconjugate polymers were set in the filter holder, and 200 µg mL^{-1} ConA or RCA_{120} PBS(−) solution (2 mL) was allowed to flow through the holder. The holder was then washed with PBS(−) solution (3 mL) and subsequently eluted with 0.2 M D-mannose, or D-lactose, in PBS(−) solution (5 mL). The protein content of each of the solutions was assayed using Bio-Rad Protein Assay reagents.

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