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Synthesis of an Artificial Glycoconjugate Polymer Carrying P^k-Antigenic Trisaccharide and its Potent Neutralization Activity against Shiga-like Toxin

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Abstract—Fluorescence-labeled glycoconjugate polymers carrying carbohydrate segments of a globotriaosyl ceramide (Gb_3) were synthesized and subjected to biological assays using *Escherichia coli* O-157 strains and Shiga-like toxins (Stx-I and Stx-II). For the fluorescence labeling, a new polymerizable fluorescent monomer with a TBMB carbonyl chromophore (Ex. 325 nm, Em. 410 nm) was designed. A glycosyl monomer of the trisaccharide segment of Gb_3 was prepared from *p*-nitrophenyl β-lactoside and copolymerized with acrylamide and the fluorescent monomer to prepare a fluorescence-labeled glycoconjugate copolymer carrying [α-D-galactopyranosyl-(1→4)-β-D-galactopyranosyl]-(1→4)-β-D-glucopyranoside. The polymer showed potent neutralization activity against Stx-I and also binding activity onto *E. coli* O-157 strains.

Introduction

Artificial glycoconjugate polymers carrying biologically active carbohydrates as pendant groups constitute a new class of biomimetic and biomedical materials.[1] They have provided access to many new methodologies in cell cultivation,[2] tumor diagnosis,[3] and detection and trapping of viruses and toxins.[4,5] Their wide range of utility can be ascribed primarily to the widely occurring carbohydrate-binding proteins on the surfaces of cells, bacteria, and viruses. Moreover, multivalency or cluster effects of the carbohydrates integrate the binding affinity of glycoconjugate polymers to carbohydrate-binding proteins and contribute much to extend their potential utility.[6–8] In our continuous efforts to synthesize and apply artificial glycoconjugate polymers, we reported several convenient ways to incorporate biologically active oligosaccharides into polystyrenes, polyacrylamides, and polyglutamates.[9,10]

In the present study, our interest was focused on a P^k-antigenic trisaccharide, [α-D-galactopyranosyl-(1→4)-β-D-galactopyranosyl]-(1→4)-β-D-glucopyranoside. This trisaccharide constitutes the carbohydrate chain of Gb_3 ceramide which is known to be a ligand of Shiga-like toxins (Stx-I and Stx-II, or verotoxins).[11–14] We describe in this paper the syntheses of a series of fluorescence-labeled glycoconjugate polymers carrying the trisaccharide and its components (α-D-galactopyranoside, and β-lactoside). A new polymerizable fluorescence compound was also designed and incorporated into each glycoconjugate polymer. Their biological activities were assayed using Shiga-like toxins (Stx-I and Stx-II) and *Escherichia coli* 0-157 strains.

Results and Discussion

Synthesis of a polymerizable glycosyl monomer of the Gb_3 trisaccharide segment

Syntheses of a Gb_3 ceramide and its analogues have already been accomplished by several groups.[15] The

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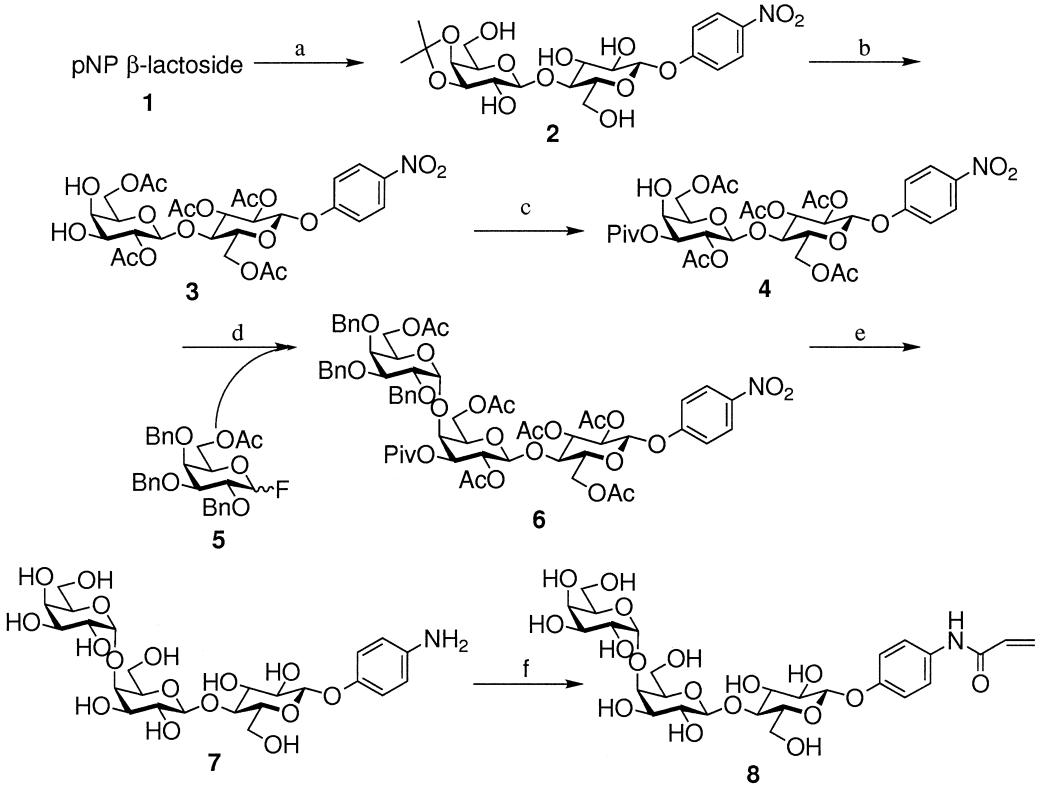
reported synthetic methods, designed for the syntheses of glycosyl ceramides, could not be applied conveniently for the present purpose to prepare glycoconjugate polymers, in which introduction of a polymerizable group at the anomeric center becomes a key step. In the present study we adopted a synthetic strategy starting from pNP-β-lactoside **1** (Scheme 1) towards *p*-nitrophenyl (pNP) glycoside of the Gb_3 trisaccharide, since the nitro group is convertible into an amino group leading to the desired glycoconjugate polymer.[16]

Chemical manipulations on **1** were, however, found to have to be approached cautiously since the pNP group was labile under both basic and acidic conditions employed for the usual benzylation (benzylbromide, NaH/DMF), isopropylidenation (refluxing in dimethoxy propane–acetone, *p*-toluenesulfonic acid), and deisopropylidenation (heating in 70% acetic acid) processes. Isopropylidenation of **1** was thus carried out using trimethylsilyl chloride (TMSCl) and acetone.[17] The reaction proceeded regioselectively to afford **2** in a nearly quantitative yield. After per-*O*-acetylation, de-*O*-isopropylidenation was performed using TMSCl and ethylene glycol[18] in MeOH (room temperature) to afford a key compound **3** as a crystalline solid (74% yield, mp = 148°C). The OH-3′ group of **3** was protected by a pivaloyl group, and the galactosylation for **4** was carried out using $SnCl_2$ and $AgClO_4$ as activators[19] and 6-*O*-acetylated galactose derivative **5** as the donor. After being purified on silica gel column chromatography, 4′-*O*-α-D-galactosyl lactoside **6** was obtained in 62% yield (α:β = > 95:5, no β-glycosidic linkage could be detected in the ^1H NMR analysis). In the above glycosylation, 6-*O*-acetylated donor **5** was used to aim at the α-selective glycosylation owing to the 6-*O*-acyl participation.[20,21] The donor was conveniently synthesized from methyl 2,3,4,6-tetra-*O*-benzyl-D-galactopyranoside via acid-catalyzed regioselective acetolysis at C-1 and C-6 (Scheme 2). De-protection of **6** using sodium methoxide in methanol (room temperature, 2 h) followed by hydrogenolysis with palladium hydroxide in methanol gave a trisaccharide **7** with a terminal amino group, which was then converted into the desired monomer **8** carrying the Gb_3 trisaccharide segment.

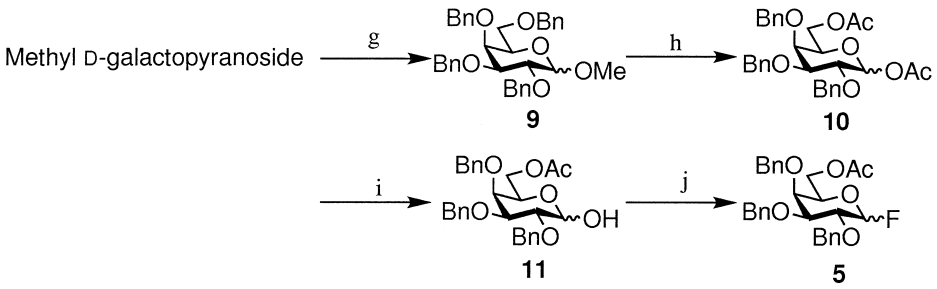
Design and synthesis of a new polymerizable fluorescent monomer

Fluorescence-labeled glycoconjugate polymers are expected to provide useful tools to investigate carbohydrate–protein interactions.[22] A convenient method to incorporate a fluorescence chromophore into glycoconjugate polymers has already been reported by Nishimura et al., who applied an *N*-allyl dansyl derivative as



Reagents and Conditions: a) TMSCl, acetone, flowing N_2, 4 h, 97%; b) (i) Ac_2O, pyridine, Et_3N, DMAP, 24 h (ii) ethylene glycol, TMSCl, CH_2Cl_2, MeOH, 5 h, 74% (2 steps); c) PivCl, Et_3N, DMAP, CH_2Cl_2, 1.5 h, 82%; d) $AgClO_4$, $SnCl_2$, MS4A, CH_2Cl_2, 10 h, 62%; e) (i) NaOMe, MeOH, 6 h (ii) $Pd(OH)_2/C$, H_2, HCl, MeOH, 6 h, 92% (2 steps); f) acryloyl chloride, Et_3N, MeOH, 0°C→ r.t., 1.5 h, 83%

Scheme 1. Synthesis of a polymerizable glycosyl monomer carrying Gb_3 trisaccharide segment.



Reagents and Conditions: g) NaH, BnBr, DMF, 0°C, 12 h, 95%; h) Ac_2O, H_2SO_4, 0°C, 2 h, 82%; i) 2-aminoethanol, DMSO, EtOAc, 48 h, 81%; j) HFPDEA, CH_2Cl_2, 0°C, 2 h, 95%

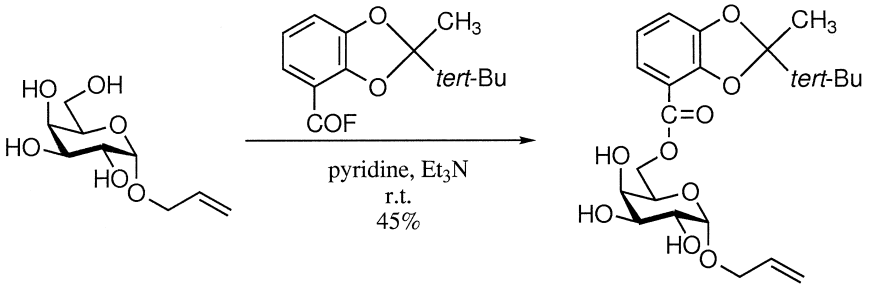
Scheme 2. Synthesis of a glycosyl donor **5**.

a polymerizable fluorescence monomer.[23] More recently, Kanie and Wong et al. applied BODIPY chromophore in the preparation of a fluorescence-labeled lyso G_{M3}/poly-L-glutamic acid conjugate.[24] Dansyl and BODIPY fluorescence is known to be sensitive to micro environmental changes including solvents and pH. The changeable fluorescence character is advantageous to monitor the binding events between carbohydrates and proteins in a simple manner. The unstable fluorescence intensity, however, often incurs problems in a quantitative assay such as that presented in this paper. Here, we utilize a new fluorescence chromophore of TBMB carboxylic acid (2-*tert*-butyl-2-methyl-1,3-benzodioxole-4-carboxylate) that shows strong and stable fluorescence intensity under various conditions.[25] The stable fluorescence is considered to reflect the characteristic structure of TBMB fluorophore. It has neither an amino nor a polyacene group, which is common for other fluorescence agents, but has bulky alkyl substituents (*tert*-Bu and Me) in the vicinity which may have a role in protecting the chromophore from an intermolecular interaction with solvents and other chromophores to cause micro environmental changes.

In order to prepare a TBMB carboxylate monomer which is copolymerizable with the glycosyl monomer **8** in aqueous solution, TBMB carboxylic acid was coupled with allyl α-D-galactopyranoside (Scheme 3). The chemical conversion was performed using racemic TBMB carbonyl fluoride[26] and allyl α-D-galactopyranoside in pyridine. The selective 6-*O*-acylation should be ascribed to the bulky substituent (*tert*-Bu) of TBMB carboxylic acid. The fluorescence monomer **12**, obtained as a colorless powder, was found to be soluble in water, chloroform, methanol, dimethyl sulfoxide, and dimethylformamide, which are popularly used for polymerization reactions. The solubility in both water and methanol is particularly useful in separating the unreacted monomer from the polymer during the reprecipitation in methanol and dialysis in water. The aqueous solution of the monomer shows fluorescence maximum at Ex. 325 nm and Em. 410 nm, the intensity of which is comparable to that of quinine sulfate (Ex. 350 nm, Em. 450 nm). The fluorescence was found to be stable in the pH range between 4.5 and 12.0 (the intensity change within 5%).

Fluorescence-labeled glycoconjugate polymers carrying Gb_3 carbohydrate segments

The fluorescence monomer **12** (less than 0.1 mol equiv to glycosyl monomers) was used as a 'seasoning' in the radical copolymerization between **8** (1 mol equiv) and acrylamide (2.9 mol equiv). After being precipitated in methanol, dialyzed (Mw 3500 cut-off) in water for 3 days and lyophilized, a fluorescence-labeled glycoconjugate polymer carrying the Gb_3 segment (glycopolymer **III** in Figure 1) was obtained as a colorless powder. Copolymerization with other glycosyl monomers gave the corresponding fluorescence glycopolymers **I**



Scheme 3. Preparation of a new polymerizable fluorescence monomer **12** bearing TBMB carboxylate chromophore.

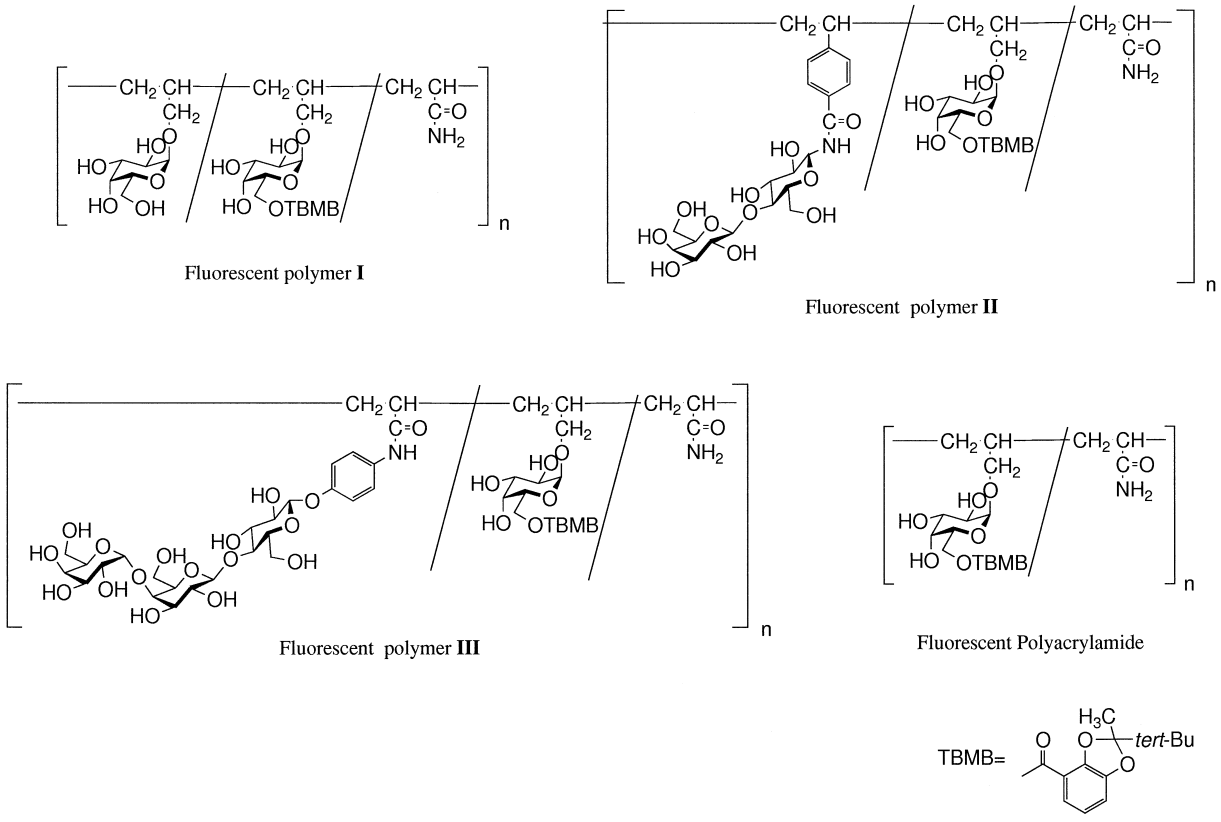


Figure 1. Chemical structures of fluorescence-labeled glycoconjugate polymer **I–III**. The molar ratio of each polymer component is cited in Table 1.

and **II**, respectively. The molar ratio of each component in these polymers was determined as summarized in Table 1 based on the ^{1}H NMR spectra (see Experimental). The polymers show respective fluorescence maximum at Ex. 325 nm and Em. 409 nm similar to that of the fluorescence monomer. The fluorescence intensity was found to show excellent linearity against the concentration in the range at least between 0.001 and 0.10 mg/mL for each fluorescence-labeled polymer.

Biological activities of glycoconjugate polymers I–III

Influence of fluorescence chromophore on lectin binding. In order to evaluate the possible influence of a TBMB carbonyl chromophore on a carbohydrate–protein interaction, association constants (K_a) of **I** and **II** with RCA_{120} lectin (β-D-galactose binding lectin) were compared with those of the corresponding non-labeled polymers (Table 2). The binding assays using FITC-labeled RCA-lectin allowed us to determine the affinity constants of **I** and **II** to be 1.6×10^4 and 2.1×10^5 M^{-1} and those of the corresponding non-fluorescent polymers as 1.7×10^4 and 1.6×10^5 M^{-1}, respectively (Table 2). Though some increase in the K_a value was observed for fluorescence polymer **II** compared with the non-fluorescence **II**, the fluorescence chromophore was found not to impair the binding with this lectin. The change in TBMB carboxylate fluorescence upon the lectin binding was too small in the present case to determine the K_a values (Fig. 2).

The data in Table 2 also indicate that all glycoconjugate polymers **I–III** have higher binding affinity with RCA_{120} than monomeric allyl α-D-galactopyranoside. This can be rationalized by the carbohydrate cluster effect as described before. Polymer **II** has apparently higher binding affinity than polymer **I**, reflecting the higher binding affinity of this lectin to β-D-galactosides. The

Table 1. Radical copolymerization of flourescence monomer **12** with glycoside monomer and acrylamide[a]

Polymer no.	Glycoside monomer mg (mmol)	Monomer **12** mg (mmol)	Acrylamide mg (mmol)	APS[b] mg	TMEDA[c] μL	Yield mg (%)	Mol fraction[d] of glycosyl unit	Relative fluorescence intensity (50 μg/mL) Ex. 325 nm, Em. 410 nm
I	40.2 (0.18)	8.0 (0.018)	64.7 (0.91)	12.7	55.3	77 (68)	0.10	29
II	100 (0.22)	9.6 (0.022)	78.0 (1.10)	15.1	59.8	161 (86)	0.14	25
III	50.0 (0.08)	3.4 (0.008)	16.5 (0.23)	3.5	14.0	35 (50)	0.20	13

[a] All reactions were carried out in degassed water at 30°C for 1.5–2.0 h.
[b] APS = ammonium peroxodisulfate, 5 mol%.
[c] TMEDA = *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, 30 mol%.
[d] Determined by ^{1}H NMR spectra.

Table 2. Association constants of fluorescence-labeled and non-labeled glycoconjugate polymers with RCA_{120} lectin

Polymers	K_a [M^{-1}][a]
Allyl α-D-galactopyranoside	5.7×10^3
Non FL-polymer **I**	1.7×10^4
FL-polymer **I**	1.6×10^4
Non FL-lac polymer **II**	1.6×10^5
FL-lac polymer **II**	2.1×10^5
FL-polymer **III**	1.5×10^5

[a] Values are expressed as per molar sugar unit. Derived from Stern–Volmer plots for five diluted solutions of polymers against FITC-labeled lectin solution (0.01 mg/mL phosphate buffer) (see Experimental for details).

fluorescence polymer **III**, however, shows a similar binding affinity to that of polymer **II**, though it has a non-reducing terminal α-D-galactopyranoside linkage. This suggests that the lectin binding can occur also at the internal lactose residue or may be simply due to the difference in the aglycon structure between **II** and **III**.

Neutralization activity against Shiga-like toxins for human ACHN cells. A globotriaosyl ceramide Gb_3 is known to be a ligand of Shiga-like toxins (Stx-I and Stx-II).[10–14] The selective renal toxicity of these toxins is assumed to reflect the high abundance of Gb_3 ceramide in kidney tissues.[27] In the present section, the biological activity of polymers **I**–**III** was assayed using human kidney cells (ACHN cells) against Stx-I and Stx-II. ACHN cells cannot survive in the presence of Shiga-like toxins. The number of cells alive in the presence of each toxin and a glycoconjugate polymer was estimated (see Experimental for details) as a measure of neutralization activity of the polymer against Shiga-like toxins. Since these polymers have no ceramide group, we expected that the biological assay would afford clear insight into the carbohydrate–toxin interaction. The assay has revealed that the polymer **III** has powerful neutralization activity ($CD_{50}=0.001$–0.005 μg/mL, in the order of 1×10^{-9} M per trisaccharide unit) against Stx-I (Fig. 3). Polymers **I** and **II** showed no activity at all against Stx-I nor against Stx-II. Moreover, the polymer **III** was found to have no activity against Stx-II, though Gb_3 ceramide is thought to be a weak ligand also of Stx-II. This result means that the trisaccharide segment of Gb_3 is the real ligand of Stx-I but not of Stx-II.

Toone et al. reported that the binding constant of monomeric Gb_3 triaoside with Stx-I was in the order of $K_a=1\times10^3$ M^{-1} using 1-*O*-methyl globotriaoside.[27] The low affinity constant, however, could not explain the species-selective adhesion of this toxin onto kidney cells (estimated to be in the order of $K_a=1\times10^9$ M^{-1}). The

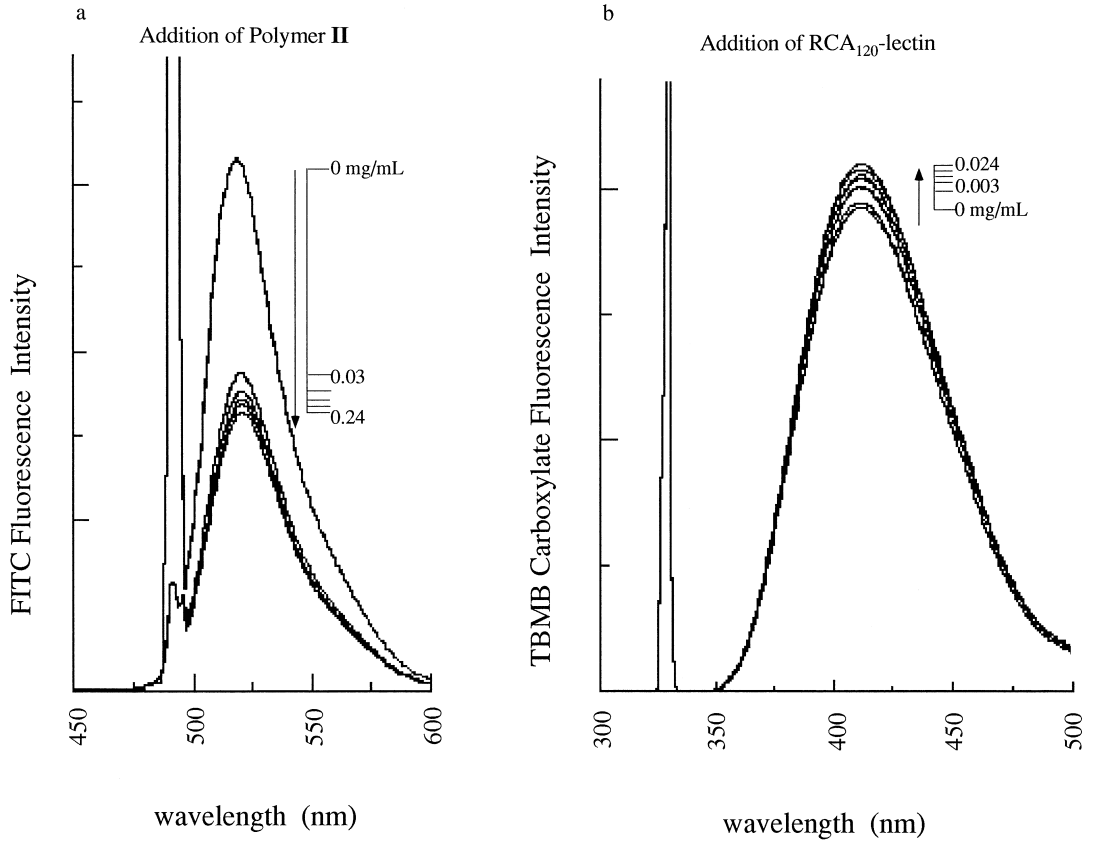


Figure 2. Fluorescence change of FITC (a) and TBMB carboxylate (b) chromophores upon the polymer **II**-RCA_{120} lectin binding. In (a), an aliquot of a fluorescence polymer-**II** solution was added to the buffer solution of FITC-labeled lectin, while in (b), an aliquot of non-fluorescent lectin solution was added to the solution of TBMB-labeled polymer-**II**.

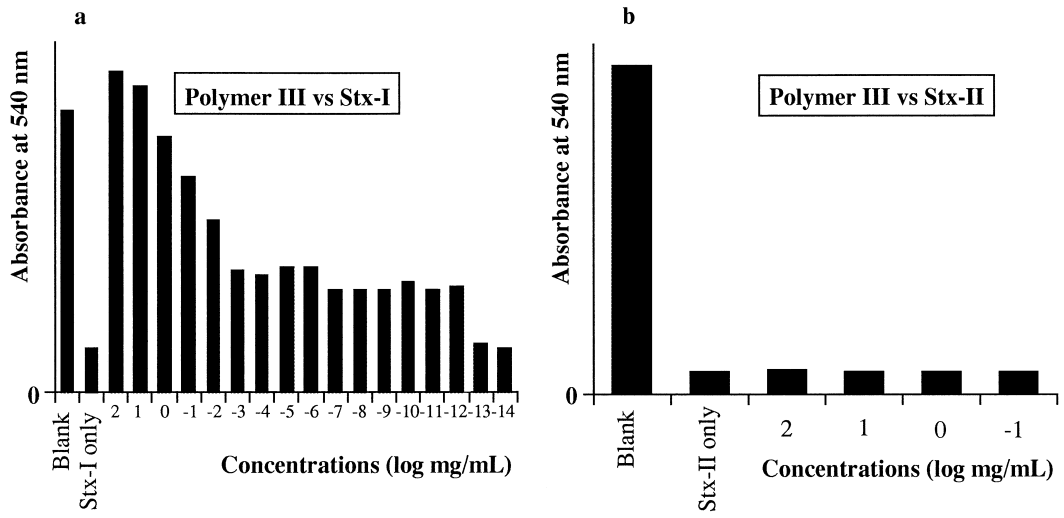


Figure 3. Neutralization activity of polymer-**III** against Shiga-like toxin-I (a) and Shiga-like toxin-II (b). Absorbance at 540 nm represents the number of ACHN cells alive in the presence of Stx-I or Stx-II and polymer-**III**. Blank indicates the condition free from both toxin and polymer, and Stx-I only indicates the absence of the polymer-**III**.

strong neutralization activity of polymer **III** suggests that the polymer **III** inhibits the toxin–cell adhesion effectively by clustering trisaccharides around the polymer. Stx-I is known to construct a subunit structure composed of an A-subunit centered in five copies of a B-subunit. Provided that each of the five B-subunits has the same binding ability with the Gb_3 trisaccharide, it may be concluded that the binding of Stx-I is totally enhanced by carbohydrate clusters on cell surfaces and effectively inhibited by this polymer.

Adhesion activity onto *E. coli* O-157 strains. Recognition of cell membrane glycolipids or glycoproteins is an essential event also for microorganisms to initiate colonization and infections onto host organs.[28] Several types of carbohydrate receptors (adhesins) have already been identified for *E. coli* strains, which include a mannose-type adhesin and a P-type adhesin recognizing Gb_3 and Gb_4, globosyl ceramides.[14] To our best knowledge, however, the type of adhesin has not yet been identified for *E. coli* O-157 strains. Fluorescence-labeled glycoconjugate polymers are expected to provide useful tools for determining the species-specific interactions of carbohydrates with certain cells, bacteria, and viruses. In this section, fluorescence polymers **I**, **II** and **III** are subjected to a binding assay using *E. coli* O-157 strains as a preliminary test to speculate on the existence of adhesins on these bacterial cells.

The assay was carried out using simple and facile protocols as follows. Each of three *E. coli* O-157 strains (NM, H-45, H-7) was incubated in a medium containing a fluorescent glycoconjugate polymer for 90 min, collected by centrifugation, washed and solubilized. The incubation period was set at 90 min in order to investigate the polymer binding at the initial stage, when the adhesion may not have reached saturation. The fluorescence intensity was measured and applied to linear plots (0.001–1.0 mg/mL) standardized for the polymer concentration to calculate the amounts of fluorescence polymer (mg/mL) adsorbed onto the *E. coli* strain ($OD_{660} = 1.5$). In this assay, non-toxic *E. coli* O-157 strains having no plasmids of Shiga-like toxin produced were used to rule out possible polymer binding mediated by the toxins. A non-glycosylated polyacrylamide was also subjected to the same assay to estimate an expected non-specific binding ability of the copolymerized acrylamide moiety due to multivalency hydrogen bonding expressed in the polymer.

The assay data are summarized in Figure 4 which shows the polymer amounts (mg/mL of *E. coli* suspension, $OD_{660} = 1.5$) adsorbed onto each *E. coli* strain. Under the examined conditions, non-glycosylated polyacrylarnide was also adsorbed onto every strain, and

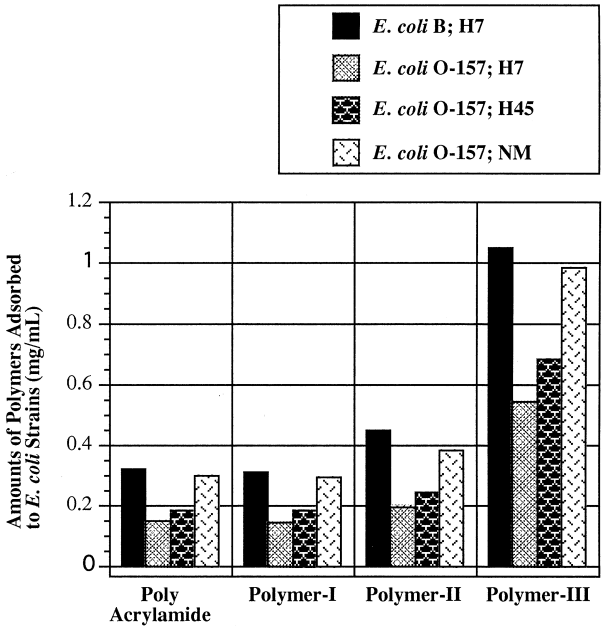


Figure 4. Relative adhesion activity of each polymer onto *E. coli* strains determined from the fluorescence intensity of strains.

this may be due to the non-specific binding onto ubiquitous surface lipoproteins or lipopolysaccharides of *E. coli* via multivalence hydrogen bonding as expected above. Polymer **I** carrying α-D-galactopyranoside was found to show similar, or even less binding than polyacrylamide. This indicates that the clustering monosaccharides in **I** have no binding affinity to any strains or even interfere with the non-specific binding of the polyacrylamide moiety. On the other hand, β-lactosides in polymer **II** and Gb_3 trisaccharide in polymer **III** showed apparent positive effects on the binding to every strain, particularly the latter to B;H7 and O-157;NM strains. These results imply a receptor-mediated binding mechanism arising from the existence or inducement of adhesion proteins of the P-type. The weak but substantial binding property of β-lactosides in polymer **II** may be explained in terms of the 'isoreceptor ligand'[28] for P-type adhesion which recognizes weakly not only the Gb_3 trisaccharide and Gb_4 tetrasaccharide but also the internal lactose moiety.

Conclusion

We have shown a general synthetic approach to a fluorescence-labeled glycoconjugate polymer carrying the Gb_3 trisaccharide segment. The fluorescence monomer can be used as a 'seasoning' in the radical copolymerization to prepare fluorescence-labeled glycoconjugate polymers in aqueous solution. The strong and stable fluorescence intensity allowed us to investigate the adhesion activity of these polymers onto *E. coli* O-157 strains as a preliminary test to speculate on the existence of adhesins. The test has revealed that the polymer carrying P^k-antigenic trisaccharides has high binding affinity with every *E. coli* O-157 strain studied here. Moreover, the same polymer showed potent neutralization activity against Shiga-like toxin-I for human ACHN cells but no activity against Shiga-like toxin-II. This means that the Gb_3 trisaccharide is the real carbohydrate ligand of Stx-I. For Stx-II, the lipophilic ceramide structure of Gb_3 may play a major role in the toxin–Gb_3 binding, or there may exist a certain oligosaccharide structure in globo- or ganglio-series as the real carbohydrate ligand of Stx-II.

Experimental

Materials and Methods. ^{1}H NMR (200 and 500 MHz) spectra were recorded at 40°C in $CDC1_3$ using an internal tetramethylsilane (TMS) standard unless otherwise stated. Fluorescence spectra were measured with a JASCO FP-550A spectrofluorometer at room temperature. IR was measured in the form of KBr disc for solid samples or film on KBr for liquid samples. TLC was performed on silica gel 60-F_{254} (Merck) detectable under UV lamp or 5% H_2SO_4 in EtOH. Silica gel column chromatography was performed on silica gel 60 (Merck 0.063–0.200 mm) using toluene–ethyl acetate as eluting solvents. *N*-*p*-Vinylbenzoyl β-lactosyl amine,[2] racemic TBMB carboxylic acid[26] and allyl α-D-galactopyranoside[29] were prepared by reported methods. FITC-RCA_{120} lectin from *Ricinus communis* and its non-labeled form were purchased from Sigma Chemical Company. All other chemicals for the syntheses of fluorescence glycoconjugate polymers were purchased from Wako Pure Chemical Industries Co., Ltd., and Tokyo Kasei Kogyo in Tokyo. All *E. coli* O-157 strains studied here are non-pathogenic ones producing no Shiga-like toxins. The cultures of each *E. coli* O-157 strain (H7, H45, and NM) were kindly offered by Professor M. Ohta of Medical School of Nagoya University. Syntheses of fluorescence monomer **12** and fluorescent glycoconjugate polymers **I**–**III** were performed as follows.

***p*-Nitrophenyl 3′,4′-*O*-isopropylidene-β-lactoside 2.** Trimethylchlorosilane (16 mL) was added to a suspension of *p*-nitrophenyl β-lactoside (4 g, 8.6 mmol) in acetone (50 mL). The mixture was stirred for 4 h under N_2 stream. The mixture was azeotroped repeatedly with toluene:EtOH (20:1, mL) to afford **2** (4.2 g, 97%) as a white crystal; $[\alpha]_D$ −48.1° (*c* 1.0; MeOH); mp > 290°C (decomposed); IR (cm^{-1}): 1516 (NO_2), 1086, 1065 (ketal). ^{1}H NMR (δ ppm, 500 MHz, $CDCl_3$): 8.15 and 7.13 (d×2, 4H of pNP group), 5.18 (d, 1H, $J_{1,2}$ = 7.8 Hz, GlcH-1), 4.40 (d, 1H, $J_{1,2}$ = 8.3 Hz, GalH-1), 4.24 (broad-s, 1H, GalH-4), 4.10 (broad-dd, 1H, GalH-3), 1.42 and 1.26 (s×2, 6H CH_3 of isopropylidene). FABMS: 656 $[M + m\text{-nitrobenzylalcohol} + 1]^+$.

***p*-Nitrophenyl 2,3,6,2′,6′-penta-*O*-acetyl-β-lactoside 3.** Triethylamine (5.0 mL) and 4-*N*,*N*-dimethylaminopyridine (10 mg) were added to a solution of **2** in Ac_2O: pyridine (25:50, mL) and stirred for 24 h. The mixture was azeotroped with toluene:EtOH (10:1) and diluted with $CHCl_3$. The organic layer was washed with 1 N aq HCl, satd aq $NaHCO_3$, and water, dried over $MgSO_4$, and concentrated. After silica gel column purification, excess ethylene glycol (1 mL) was added to a solution of penta-*O*-acetylated product (200 mg, 0.31 mmol) in CH_2Cl_2:MeOH (5:5, mL) followed by addition of trimethylchlorosilane (50 μL) and the solution was stirred for 5 h. The mixture was concentrated and diluted with $CHCl_3$. The organic layer was washed with water, dried over $MgSO_4$, and concentrated. Purification of the residue by silica gel column chromatography (toluene:EtOAc = 3:1) afforded **3** (141 mg, 74%) as a colorless crystal; $[\alpha]_D$ −19.8° (c 1.0; $CHCl_3$); mp 147–150°C; IR (cm^{-1}): 3481 (OH), 1747, 1234 (Ac), 1522 (NO_2). ^{1}H NMR (δ ppm, 500 MHz, $CDCl_3$): 8.20 and 7.06 (d×2, 4H, H of pNP group), 5.20 (d, 1H, $J_{1,2}$ = 7.4 Hz, GlcH-1), 4.37 (d, 1H, $J_{1,2}$ = 7.5 Hz, GalH-1), 3.87 (broad-d, 1H, GalH-4), 3.62 (broad-d, 1H, GaH-3), 2.15, 2.12, 2.09×2, 2.06 (s×5, 15H, CH_3 of acetyl group) FABMS: 674 $[M + 1]^+$.

***p*-Nitrophenyl 2,3,6,2′,6′-penta-*O*-acetyl-3′-*O*-pivaloyl-β-lactoside 4.** Pivaloyl chloride (0.43 mL, 3.5 mmol) was added to a solution of compound **3** (1.00 g, 1.66 mmol), triethylamine (0.68 mL, 2.49 mmol), and 4-*N*,*N*-dimethylaminopyridine (10 mg) in CH_2Cl_2 (100 mL) at 0°C and stirred for 1.5 h. The mixture was washed with water, dried over $MgSO_4$, and concentrated. Silica gel column chromatography (toluene:EtOAc = 5:1) afforded

4 (1.00 g, 82%) as a colorless crystal; $[\alpha]_D$ −19.8° (*c* 1.0; CH_2Cl_2); mp 175–179°C; IR (cm^{-1}): 3515 (OH), 1745, 1254 (Ac), 1707, 1232 (Piv), 1520 (NO_2). ^1H NMR (δ ppm, 500 MHz, $CDCl_3$): 8.20 and 7.07 (d×2, 4H, H of pNP group), 5.19 (d, 1H, $J_{1,2}=7.8$ Hz, GlcH-1), 4.48 (d, 1H, $J_{1,2}=8.3$ Hz, GalH-1), 4.84 (dd, 1H, $J_{2,3}=10.0$ Hz, $J_{3,4}=3.0$ Hz, GalH-3), 4.02 (broad-d, 1H, GalH-4), 2.12, 2.09×2, 2.06, 2.04 (s×5, 15H, CH_3, of acetyl group), 1.19 (s, 9H, CH_3 of pivaroyl group). FABMS: 757 $[M]^+$.

6-*O*-Acetyl-2,3,4-tri-*O*-benzyl-D-galactopyranosyl fluoride 5. 6-*O*-Acetyl-2,3,4-tri-*O*-benzyl-D-galactopyranose **11** was prepared starting from methyl D-galactopyranoside according to conventional methods for benzylation (benzylbromide, NaH in DMF), acid-catalyzed acetolysis (0.1% H_2SO_4, in acetic anhydride for 2 h), and deacetylation using 2-aminoethanol in DMSO–ethyl acetate mixture (room temperature, 48 h). The crude syrupy compound **11** was used for the next 1-fluorination as follows.

Hexafluoropropene–diethylamine complex (Tokyo Kasei, 0.29 mL, 3.5 mmol) was added to a solution of 6-*O*-acetyl-2,3,4-tri-*O*-benzyl-D-galactopyranose (300 mg, 0.53 mmol) in toluene (15 mL) at 0°C and stirred for 2 h. The mixture was washed with satd aq $NaHCO_3$ and water, dried over $MgSO_4$, and concentrated. Silica gel column chromatography (toluene:EtOAc = 20:1) afforded **5** (289 mg, 95%; α/β = 70/30 determined ^1H NMR) as a syrup; ^1H NMR (δ ppm, 500 MHz, $CDCl_3$): 7.25–7.44 (m, 15H, aromatic H), 5.73 and 5.47 (d×2, 1H, $J_{1,2}=3.3$ Hz, $J_{1,F}=53$ Hz, H-1α), 5.32 and 5.06 (d×2, 1H, $J_{1,2}=6.7$ Hz, $J_{1,F}=53$ Hz, H-1β).

***p*-Nitrophenyl *O*-[6-*O*-acetyl-2,3,4-tri-*O*-benzyl-α-D-galactopyranosyl-(1→4)-2,6-di-*O*-acetyl-3-*O*-pivaloyl-β-D-galactopyranosyl)]-(1→4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside 6.** Flame-dried 4 Å molecular sieves were added to a stirred mixture of silver perchlorate (402 mg, 0.65 mmol) and $SnCl_2$ (368 mg, 0.65 mmol) in dry CH_2Cl_2 (10 mL). The mixture was cooled to 0°C and a solution of **4** (113 mg, 0.16 mmol) in dry CH_2Cl_2 (2.5 mL) was added. After 10 min, a solution of **5** (278 mg, 0.49 mmol) in dry CH_2Cl_2 (2.5 mL) was added, and the mixture was stirred for 10 h. The mixture was filtered through a pad of Celite. The filtrate was washed with satd aq $NaHCO_3$ and water, dried over $MgSO_4$, and concentrated. Silica gel column chromatography (toluene:EtOAc = 5:1) afforded **6** (124 mg, 62%) as a syrup; IR (cm^{-1}): 1747, 1228 (Ac and Piv), 1519 (NO_2). ^1H NMR (δ ppm, 500 MHz, $CDCl_3$): 8.20 and 7.03 (d×2, 4H H of pNP), 7.15–7.44 (m, 15H, aromatic H of benzyl group), 4.60–4.74 (d×6, 6H, CH_2 of benzyl group), 5.16 (d, 1H, $J_{1,2}=7.3$ Hz, GlcH-1), 4.70 (d, 1H, $J_{1'2'}=1.6$ Hz, GalH-1′), 4.47 (d, 1H, $J_{1,2}=8.3$ Hz, GalH-1), 4.82 (dd, 1H, $J_{2,3}=10.1$ Hz, $J_{3,4}=3.0$ Hz, GalH-3), 3.84 (broad-s, 1H, GalH-4), 2.06×2, 2.03, 2.00×2, 1.97 (s×6, 18H, CH_3 of acetyl group), 1.14 (s, 9H, CH_3 of pivaloyl group). FABMS: 1148 $[M+1]^+$.

***p*-Aminophenyl *O*-[α-D-galactopyranosyl-(1→4)-β-D-galactopyranosyl]-(1→4)-β-D-glucopyranoside 7.** To a stirred solution of **6** (60 mg, 50 μmol) in MeOH (3 mL) was added NaOMe (10 mg), and the mixture was stirred for 6 h. The mixture was neutralized with Amberlyst 15E-ion exchange resin, filtered, and concentrated. After silica gel column purification, the de-acylated product was dissolved in 10 N HCl (10 μL, 100 μmol) and MeOH (5 mL) containing 10% $Pd(OH)_2$ on carbon and hydrogenated at room temperature for 6 h. The filtrate through a pad of Celite was evaporated to afford **7** (29 mg, 92%) as a syrup; IR (cm^{-1}) 3419 (OH), 1228, 1076 (NH_2). ^1H NMR (δ ppm, 500 MHz, D_2O): 6.83 and 6.64 (d×2, 4H, H of pAP group), 4.82 (d, 1H, $J_{1,2}=8.1$ Hz, GlcH-1), 4.77 (d, $J_{1',2'}=3.2$ Hz, GalH-1′), 4.35 (d, 1H, $J_{1,2}=7.7$ Hz, GalH-1). FABMS: 749 [M + *m*-nitrobenzylalcohol + 1]$^+$.

***p*-*N*-Acryloylamidophenyl *O*-[α-D-galactopyranosyl-(1→4)-β-D-galactopyranosyl]-(1→4)-β-D-glucopyranoside 8.** A solution of **7** (25 mg, 40 μmol) and triethylamine (22 μL, 120 μmol) in MeOH (3 mL) was cooled at 0°C. After 10 min, acryloyl chloride (4 μL, 50 μmol) in CH_2Cl_2 (0.5 mL) was added, and the mixture was stirred at room temperature for 1.5 h. The mixture was concentrated and purified by TSK gel HW-40S column chromatography (water) to afford **8** (21 mg, 83%) as a white amorphous powder; IR (cm^{-1}) 3298 (OH), 1660 (amide), 1033, 827 (olefin). ^1H NMR (δ ppm, 500 MHz, D_2O): 7.54 and 7.23 (d×2, 4R aromatic H), 6.48 (dd, 1H, H-olefin), 6.40 (dd, 1H, H-*trans*), 5.95 (dd, 1H, H-*cis*), 5.19 (d, 1H, $J_{1,2}=7.8$ Hz, GlcH-1), 5.15 (d, $J_{1',2'}=3.6$ Hz, GalH-1′), 4.62 (d, 1H, GalH-1). FABMS: 803 [M + *m*-nitrobenzylalcohol + 1]$^+$.

Allyl 6-*O*-TBMB carbonyl-α-D-galactopyranoside 12. Racemic TBMB carboxylic acid (96.9 mg, 0.41 mmol) was dissolved in CH_2Cl_2 (3 mL) and treated with hexafluoropropene–diethylamine complex (100.6 mg, 0.45 mmol) at room temperature for 30 min. After being evaporated, the residue was purified on silica gel column (toluene:ethyl acetate = 1:1) to afford syrupy TBMB carbonyl fluoride[16] (96.5 mg, 99%). To the mixture of allyl α-D-galactopyranoside (100 mg, 0.45 mmol) and 4-*N*,*N*-dimethylaminopyridine (5 mg) in dry pyridine (10 mL) was added TBMB carbonyl fluoride (96.5 mg, 0.41 mmol) in pyridine (1 mL). The mixture was kept at room temperature for 6 h and concentrated in vacuo. The residue was chromatographed on silica gel (toluene: ethyl acetate = 3:1 to 1:10). The fractions containing **12** were collected and concentrated. The residue was dissolved in water (15 mL) and then lyophilized to afford a white glassy powder (62.4 mg, 45%); IR (cm^{-1}) 3365 (OH), 1618, 1351, 1330 (PhC(O)OR), 1081, 1058 (ketal). ^1H NMR (δ ppm, 200 MHz, $CDCl_3$): 7.34 (broad, 1H $J=8.8$ Hz, Ph-H), 6.80–6.88 (m, 2H, Ph-H), 5.76–6.00 (m, 1H, C–CH=C), 5.08–5.35 (m, 2H, C−C=CH_2), 5.00 (d, 1H, $J_{1,2}=3.5$ Hz, GalH-1), 4.4–4.7 (m, 2H, GalH-$6_{R,S}$), 1.62 (s, 3H, CH_3 of ketal), 1.08 (s, 9H, *tert*-Bu of ketal). FABMS: 439 $[M+1]^+$. UV (H_2O, 0.1 mM): 325 nm (ε = 5000, TBMB carboxylate ^1L_b transition), 242 nm (shoulder), 222 nm (ε = 17000, ^1L_a transition). Fluorescence (H_2O, 0.001 mM): Ex. (max) 325 nm, Em. (max) 410 nm.

Fluorescence-labeled glycoconjugate polymers I–III. A solution of allyl α-D-galactopyranoside (40.2 mg, 0.18

mmol), acrylamide (64.7 mg, 0.91 mmol) and fluorescent monomer **12** (8 mg, 0.018 mmol) in water (2 mL) was degassed under reduced pressure. To the mixture was added *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (55.3 mg) in one portion and then ammonium peroxodisulfate (12.7 mg). The mixture was again degassed, and saturated with nitrogen gas, and the polymerization tube was sealed under reduced pressure, and incubated at 30°C for 3 h. The solution was poured into methanol (20 mL) in a centrifugation tube, and the mixture was centrifuged (5°C, 5000 rpm). The precipitate dissolved in water was again poured into methanol. The precipitate was dissolved in water (20 mL), dialyzed for 2 days in water (Mw 3500 cut off), and lyophilized to afford a white powder of polymer **I** (77 mg, 68%); IR (cm^{-1}) 3392 (OH), 2933, 1668 (amide), 1617, 1350, 1327 (PhC(O)OR), 1078, 1058 (ketal). ^1H NMR (δ ppm, 500 MHz, D_2O, 50°C): 5.0 (H-1 of sugar), 3.6–4.1 (H-2–H-6 of sugar), 3.4 (-CH_2-O-), 2.3–2.4 (-CH_2-CH-, main chain), 1.7–1.9 (-CH_2-CH-, main chain). UV (H_2O, 1 mg/mL): 322 nm (TBMB carboxylate, ^1L_b), 245 nm, 220 nm (TBMB carboxylate, ^1L_a). Fluorescence (H_2O, 0.1 mg/mL): Em. (max) 325 nm, Ex. (max) 409 nm. The molar fractions of monomer components are summarized in Table 1.

In the same manner as described above, polymers **II** and **III** were prepared from *N*-*p*-vinylbenzoyl β-lactosyl amine and *p*-acryloylamidophenyl 4-*O*-[3-*O*-α-D-galactopyranosyl-β-D-galactopyranosyl]-β-D-glucopyranoside, respectively. The molar ratio of every reagent and monomer was summarized in Table 1. Polymer **II**: IR (cm^{-1}) 3370 (OH), 2931, 1670 (amide), 1617, 1349, (PhC(O)OR), 1078, 1058 (ketal). ^1H NMR (δ ppm, 500 MHz, D_2O, 50°C): 7.3–7.5 (Phenyl), 5.3 (GlcH-1), 4.6 (GalH-1), 3.7–4.0 (H-2–H-6 of sugars), 2.3–2.4 (-CH_2-CH-, main chain), 1.7–1.9 (-CH_2-CH-, main chain). Fluorescence (H_2O, 0.1 mg/mL): Em. (max) 325 nm, Ex. (max) 409 nm. Polymer **III**: IR (cm^{-1}) 3394 (OH), 2931, 1668 (amide), 1618, 1351, 1328 (PhC(O)OR), 1078, 1059 (ketal). ^1H NMR (δ ppm, 500 MHz, D_2O, 50°C): 7.0–7.5 (Ph-H), 5.2 (GlcH-1β), 5.05 (GalH-1α), 4.4 (GalH-1β), 3.6–4.2 (H-2–H-6 of sugars), 2.3–2.6 (-CH_2-CH-, main chain), 1.7–1.9 (-CH_2-CH-, main chain). Fluorescence (H_2O, 0.1 mg/mL): Em. (max) 325 nm, Ex. (max) 409 nm.

Association constants (K_a) of each glycoconjugate polymer with RCA_{120} lectin. Based on the fluorescence changes in FITC-labeled lectin (Ex. 490 nm, Em. 518 nm), K_a value was determined for fluorescence and non-fluorescence glycoconjugate polymers according to the reported method[10–13] and expressed as the value per molar sugar unit. The molarity of the sugar unit was calculated for each polymer as listed in Table 1. Stern–Volmer plot ($F_o \cdot [S]/\Delta F$ versus $[S]$) was employed to determine the maximum fluorescence change ΔF_{max} and K_a as follows:

$$F_o \cdot [S]/\Delta F = F_o/\Delta F_{max} \cdot K_a + F_o \cdot [S]/\Delta F_{max}$$

where F_o, $[S]$, and ΔF are the initial fluorescence intensity, the molarity of the sugar unit of the polymer (mol/L), and change in the fluorescence intensity at the concentration, respectively. The fluorescence measurement was carried out for FITC-RCA lectin solution in 10 mM phosphate buffer (1.04×10^{-2} mg/mL, pH 7.2) at room temperature (air-conditioned at 20°C). After 0.3 μL aliquot of each polymer solution (10 mg/mL) was added to the lectin solution, the mixture was stabilized for 10 min and then the fluorescence spectra were measured. Attempts to determine the K_a based on the fluorescence change of TBMB carboxylate failed due to the weak fluorescence change upon the lectin addition (Fig. 3b).

Neutralization activity of glycoconjugate polymers I–III against Stx-I and Stx-II. Non-fluorescence polymers **I**–**III** and polyacrylamide were employed for the assay. The mixture of each Stx-I (250 pg/mL phosphate buffer solution (PBS)) and the diluted polymer solution (10^{-2}–10^{-14} μg/mL) was preincubated in a well at 37°C for 1 h. To the well were added ACHN cells (65 μL of 8×10^5 cells/mL in Eagle's medium containing 10% FCS), and the mixture was incubated at 37°C for 3 days. The mixture was treated with 0.014% neutral red solution (100 μL) at 37°C for 1 h, washed with PBS (2×200 μL), and then mixed with 0.5 N HCl–35% EtOH (100 μL). The absorbance at 540 nm was measured for each polymer at different concentrations.

Adhesion activity of I–III onto *E. coli* O-157. Each of the *E. coli* O-157 strains ($OD_{660} = 1.5$) in a medium (3 mL) containing each of the polymer solutions (0.1 mg/mL 0.1 N phosphate buffer pH 7.2, 3 mL) was incubated at 37°C for 90 min. The mixture was centrifuged (3000 rpm, 15 min), and the precipitate was washed twice with the same buffer. To the residue was added 5.5 N NaOH (100 μL) and kept for 30 min for solubilization. The phosphate buffer (2.4 mL) was added, and the fluorescence intensity at Ex. 320 nm and Em. 410 nm was measured. The amount of fluorescence polymers adsorbed onto each *E. coli* strain was determined using standard plots for the fluorescence intensity versus the concentration of polymers (0.00–0.1 mg/mL).

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