Elimination Filter for Verotoxins —Highly Adsorbing by Glycoconjugate Polymer—

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Glycoconjugate polymer having globotriose, which is a ligand for Verotoxins (VTs: VT1 and VT2), was immobilized to the filter paper. By flowing VTs solution through the filter paper, the elimination ability of the filter paper for VT1 and VT2 was evaluated. The globotriose-immobilizing filter effectively diluted the starting concentration of VT1 (1 μg/mL) and VT2 (1 μg/mL) to about one hundred thousandth and about one thousandth parts of the starting concentration, respectively.

Infection with *Escherichia coli* O157 causes hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), and neurological damages in humans.[1] Verotoxins produced by *E. coli* O157 are composed of one toxic subunit (A subunit) and five sugar recognizing subunits (B subunits). The A subunit (32 kDa) acts as an *N*-glycosidase that cleaves an adenine residue in 28S ribosomal RNA of a host cell, making the cell unable to synthesize proteins. The B subunits recognize glycolipids on the cell surface and then VTs are incorporated into the cell. VTs circulating in the blood bind to globotriaosylceramide (Gb3) on the cell surface of renal endothelial cells or red blood cells and then impair renal function.[2]

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.[3]

In the previous reports,[4] two kinds of glycoconjugate polymers, having either lactose or mannose, were synthesized and immobilized to filter papers. D-Lactose possesses potency as a ligand for *Ricinus communis* agglutinin (RCA120) and D-mannose possesses potency as a ligand for *Canavalia ensiformis*, concanavalin A (ConA). The protein adsorption was evaluated using these modified membranes and lectins. The proteins were effectively adsorbed to these polymers due to the "glycoside cluster effect."

Here, we report a filter with immobilized glycoconjugate polymer, which can neutralizes pathogenic agent and the adsorption ability of the filter was evaluated. Glycoconjugate polymer containing globotriose, as a ligand for VTs,[5–7] and an amino group, as an immobilizing point to a filter membrane, was synthesized and bound covalently to a filter paper. The elimination ability of the filter for VT1 and VT2 was evaluated.

Monomers (Figure 1) were synthesized according to the following procedure described in literature.[8] Gb3 monomer **1** was the ligand for VTs and Lac monomer **2** was used as control for sugar recognition of VTs. The amine monomer **3** makes a reactive point on the carboxymethylated filter paper.

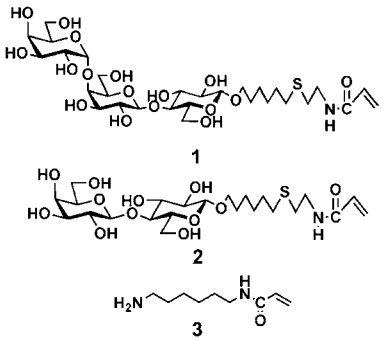

Figure 1. Monomers.

Table 1. Results of polymerization

Polymer	Monomer ratio		Total yield	Polymer composition[a]	Sugar content /mol %	M_w[b] /kDa
	Glycosyl monomer **1** or **2**	Amine monomer **3**				
G	1.00 (**1**)	0.05	89	1:0.13	97	248
L	1.00 (**2**)	0.05	93.1	1:0.08	97.6	302

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

Glycosyl monomers **1** and **2** were copolymerized with amine monomer **3** at room temperature using *N*,*N*,*N*′,*N*′-tetramethylethylenediamine and ammonium peroxodisulfate as initiators. The copolymerization data are summarized in Table 1. Polymer **G** or **L**, which carries globotriose or lactose and amino groups, was immobilized to the filter paper and was utilized as multivalent affinity ligands.

The filter papers, made from cellulose, were soaked in 1 M NaOH and saturated NaCl solution to activate the hydroxy groups of cellulose. Then, bromoacetic acid was added to the solution and carboxymethylated. The content of the carboxymethyl group introduced to the filter paper was determined by titration to be 41.1 μmol/paper (41.9 nmol/mm^2).

The glycoconjugate polymers were immobilized to the carboxymethylated filter papers by the covalent bonding of amide linkage. Polymer **G** or **L** was immobilized to the filter paper by condensation reaction between the carboxyl group of the filter paper and the amino group of glycoconjugate polymer (Figure 2). The filter paper had 0.70 mg of Polymer **G** per paper (Gb3: 0.99 μmol/paper), or 0.60 mg of Polymer **L** per paper (lactose: 1.0 μmol/paper).

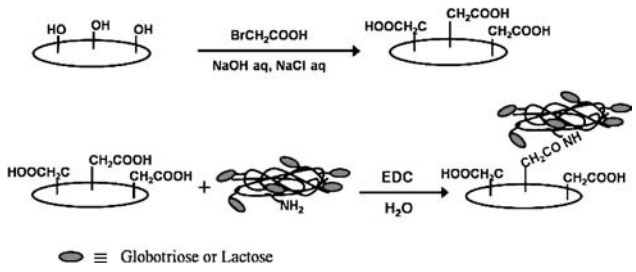

Figure 2. Activation of filter papers and immobilization of glycoconjugate polymers.

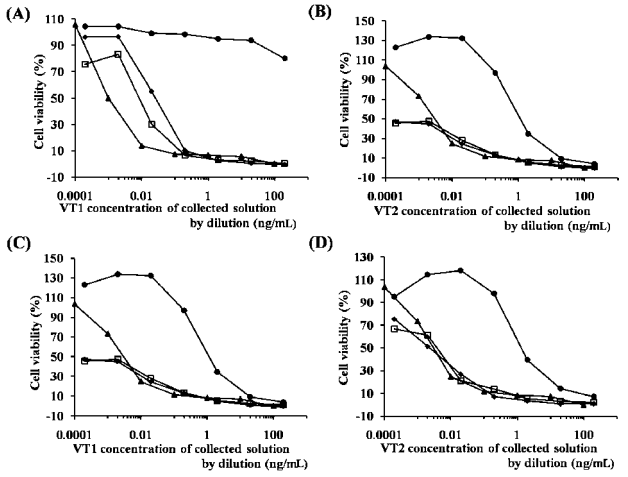

Figure 3. Estimation of Verotoxin adsorption ability by filter with immobilized polymers. The flowed toxins solutions are (A) 1 μg/mL VT1-PBS(−), (B) 1 μg/mL VT2-PBS(−), (C) 1 μg/mL VT1-1% BSA-PBS(−), (D) 1 μg/mL VT2-1% BSA-PBS(−). (●) is the filter immobilized with **G** and (◆) is filter immobilized with **L**. (□) is the carboxymethylated filter. (▲) is the standard concentration solution of VTs.

Verotoxins adsorption ability of the filters with immobilized polymers was evaluated. The filter papers (five pieces) were set in a filter holder. PBS(−) (phosphate buffered saline) solution was allowed to flow through the holder containing the filter papers. Carboxymethylated filter papers were used as standard and similarly set in filter holder. 1 μg/mL VT1-PBS(−) solution (1 mL, flow rate: 6 mL/min) was flowed through the holder, and the holder was washed with PBS(−) solution (4 mL, flow rate: 24 mL/min). The flowed solution (5 mL) was evaluated for cytotoxicity on Vero cells (Figure 3A).[9] As another method, the filter paper was soaked in 1% BSA-PBS(−) solution for blocking of non-specific adsorption. After 30 min, the filter papers were set in the filter holder and applied to the toxin adsorption test (Figure 3C).

This method was also applied to VT2 (Figures 3B and 3D). As shown in Figures 3A and 3B the filter paper with immobilized Polymer **G** adsorbed VT1 and VT2 efficiently. On the other hand, carboxymethylated filter papers did not have significant nonspecific adsorption without using 1% BSA solution and did not adsorb VTs. The filter paper with immobilized Polymer **L** also did not adsorb VTs. Therefore, VTs showed no adsorption to the skeleton of the glycoconjugate polymer and selectively recognized globotriose. By flowing once VTs solution through the filter paper with immobilized Polymer **G**, concentration of VT1 was diluted to about one hundred thousandth parts of starting concentration, while concentration of VT2 was diluted to about one thousandth parts of starting concentration. Interestingly, 1% BSA solution inhibited adsorption of VT1 for the filter papers and did not inhibit adsorption of VT2. This result indicated that the binding style of VT1 was different from that of VT2. For more effective adsorption of VTs, it is suggested that a larger number of filter papers are set in the filter holder, or VTs solution flow through the filter paper many times.

This study indicated that the filter paper with immobilized glycoconjugate polymer containing globotriose is usable for elimination filter of Verotoxins. The filter papers with glycoconjugate polymer eliminated VTs efficiently; 1 μg/mL solutions of VT1 and VT2 were diluted to about 10 pg/mL and 1 ng/mL, respectively. This simple application will be applied to the elimination of other pathogenic agents as a medical tool.

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References and Notes

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9 Verotoxins adsorption evaluation. The evaluation method was carried out as follows: (1) The cellulose membranes with immobilized glycoconjugate polymers were set in the filter holder, and 1 μg/mL of VT1 or 1 μg/mL of VT2 in PBS(−) (1 mL) was allowed to flow through the holder; (2) The holder was then washed with PBS(−) solution (4 mL); (3) the solution was diluted with medium; (4) subconfluent Vero cells were treated with the diluted samples; and (5) the remaining amount of VT in the flowed solution was evaluated by comparing with the number of living cells treated with standard VT solution by using a WST-8 Cell Counting Kit (Wako Pure Industries). The concentrations of remaining VTs were calculated from the standard concentration of VTs.

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