Use of Lactose against the Deadly Biological Toxin Ricin

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ABSTRACT Developing a technology for detecting and decontaminating biological toxins is needed. Ricin from *Ricinus communis* is a highly poisonous toxin; it was formerly used for an assassination in London and in postal attacks in the United States. Ricin is readily available from castor beans and could be used as a biological agent. We propose using glycotechnology against the illegal use of ricin. Lactose (a natural ligand of this toxin) was incorporated into polyacrylamide-based glycopolymers at variable sugar densities (18−100%) and evaluated with surface plasmon resonance (SPR) spectroscopy and the real agent, ricin. Glycopolymers (18−65% lactose densities) effectively interfered with the toxin-lactoside adhesion event (>99% efficiency within 20 min). This supported the notion of using the mammary sugar lactose against a deadly biological toxin.

KEYWORDS: glycopolymer • biological toxin • decontamination • ricin • terrorism

INTRODUCTION

Ricin from castor beans (*Ricinus communis*) was used for an assassination in London in 1978 (1) and postal attacks in the United States in 2004 (2). Considering that its strong toxicity (LD_{50} is ca. 3−5 μg/kg in humans) is much stronger than that of hydrogen cyanide gas, and because of the heightened awareness of chemical and biological terrorism, developing a rapid detection and decontamination technology for this type of biological toxin would be supported by many governmental security agencies around the world.

Different approaches are available for the detection of biological toxins. These include immunochemical enzyme-linked immunosorbent assay (ELISA) methods (3−5), cell-free assays based on the measurement of ribonuclease activity (6), polymerase chain reaction (PCR) (7, 8), and mass spectrometric approaches (9, 10). These are protein-engineering technologies coupled with gene technologies.

We reported an alternative method based on glycotechnology (11). In this method, a natural infection process (12−14) is applied. This toxin consists of a B-chain having at least two β-lactoside-binding domains in addition to an A-chain having ribonuclease activity that can enter host cells by endocytosis. We prepared β-lactoside-chips from synthetic lacto-lipids, and used them for the rapid detection of ricin (11).

In addition to detection technologies, a decontamination technology that can remove or inactivate the biological toxins from contaminated scenes is important. For this purpose, sodium hypochlorite and alkaline solutions are applied as popular decontaminants, but these reagents are harmful and often incompatible with sensitive equipment, personnel, and wounded persons (15). From environmental and technical viewpoints, a more environmentally friendly and sustainable decontamination technology is strongly desired. In this contribution, we propose a glycotechnology in which β-lactoside is incorporated into glycopolymers (Figure 1) and evaluated as a competitor to the B-subunit-β-lactoside binding event.

EXPERIMENTAL SECTION

Materials and Instruments. *p*NP β-lactoside and *p*NP β-D-glucopyranoside were purchased from Sigma Chemical Company (St. Louis, MO). Ricin from *Ricinus communis* was obtained from Honen Corp (Japan), and was safely handled as prescribed by Japanese law at the National Research Institute of Police Science. Reactions for carbohydrate monomers, **1** and **2** were monitored by thin-layer chromatography (TLC) on Silica Gel 60 F_{254} (E. Merck, Darmstadt, Germany) and visualized by UV light and by spraying with 20% H_2SO_4 in ethanol (EtOH) followed by charring at 180 °C. The products were purified by flash column chromatography on Silica Gel 60 RP-18 (ODS-C18, Yamazen, Japan, 40−63 μm). SIA-kit AU chips were purchased from Biacore AB (Uppsala, Sweden). Optical rotations were measured with a JASCO DIP-1000 digital polarimeter (Tokyo, Japan) at ambient temperature, using a 10 cm micro cell. ^{1}H NMR (400 MHz) spectra were recorded on a Varian INOVA 400 spectrometer (Palo Alto, CA) for solutions in deuterium oxide (D_2O). Chemical shifts are given in ppm and referenced to *tert*-butyl alcohol (δ_H 1.23 in D_2O) as the internal reference. All data are assumed to be first order with apparent doublets and triplets reported as d and t, respectively. Resonances that appear broad are designated br. Electrospray ionization mass spectra (ESI-MS) were directly recorded using a SHIMADZU LC-MS 2010A

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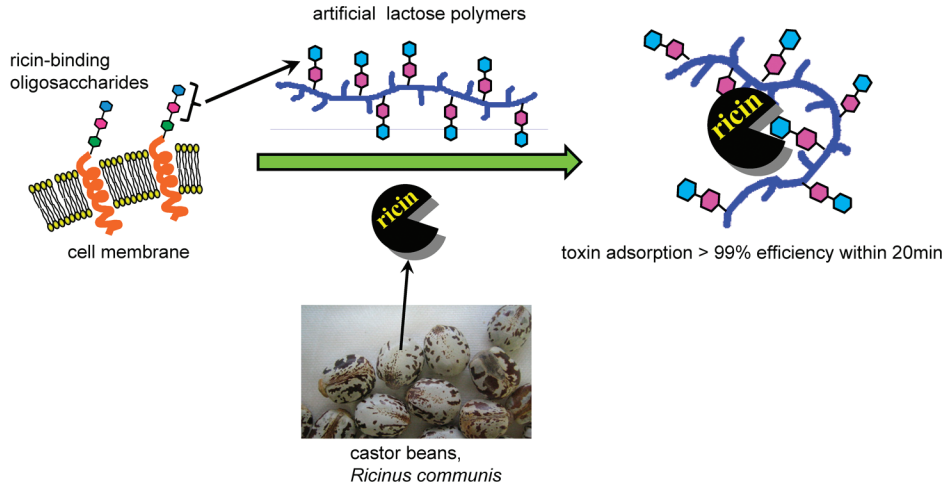


FIGURE 1. Application of the infectious process of ricin to toxin decontamination. Ricin from castor beans recognizes cell-surface oligosaccharides having β-D-Gal or β-D-GalNAc at the nonreducing terminal, and internalizes into the host cell. The recognition is species-specific, and therefore may be applicable to decontamination. Polyacrylamide-based glycopolymers were designed for the present study. Ricin adheres specifically to glycopolymers and the toxin-lactoside adhesion event is prevented, showing that the glycopolymers have adsorbed ricin.

mass spectrometer (Kyoto, Japan). Surface plasmon resonance (SPR) experiments were performed on a BIAcore 2000 or BIAcore T-100 (BIAcore AB, GE Healthcare, Piscataway, NJ), and all data were calculated with BIAevaluation commercial software (version 4.1). The sugar densities of the glycopolymers were determined by the phenol-H_2SO_4 method (16). Dynamic light scattering (DLS) analysis was carried out using a Malvern Zetasizer Nano ZS (Malvern Instruments, U.K.). 2,2′-Azobis(2-methylpropionamidine) dihydrochloride, acrylamide, acryloyl chloride, and other reagents were all commercially available.

Synthesis of *p*-Acrylamidophenyl β-Lactoside 1. To a solution of *p*-nitrophenyl (*p*NP) β-lactoside (200 mg, 0.43 mmol) in methanol (MeOH) (25 mL) and H_2O (5 mL) was added palladium carbon (Pd/C) (5 mg), and the reaction mixture was stirred vigorously under a hydrogen (H_2) atmosphere at room temperature for 6 h. The reaction mixture was filtered through a pad of Celite, and the filtrate was concentrated under diminished pressure to give *p*-aminophenyl (*p*AP) β-lactoside (166 mg, 89%) as an amorphous powder, which was used for the next step without further purification. To a solution of *p*AP β-lactoside (100 mg, 0.23 mmol) and potassium carbonate (K_2CO_3) (63.8 mg, 0.46 mmol) in water (0.5 mL) and tetrahydrofuran (THF) (1 mL) was added dropwise a solution of acryloyl chloride (24.4 μL, 0.30 mmol) in THF (1 mL) slowly at 0 °C, and the reaction mixture was stirred at room temperature for 3 h. The reaction mixture was concentrated under diminished pressure, and the residue was purified by chromatography (ODS) to give monomer **1** (77.6 mg, 69%). $[\alpha]_D$ −29.0° (*c* 0.13, H_2O). ^{1}H NMR (D_2O): δ 7.448 (d, aromatic H, *J* = 8.8 Hz, 2H), 7.148 (d, aromatic H, *J* = 8.8 Hz, 2H), 6.414 (dd, −C*H*=CH$_2$, *J* = 10.2 and 17.0 Hz, 1H), 6.313 (br d, −CH=C*H*$_2$, *J* = 17.0 Hz, 1H), 5.860 (br d, −CH=C*H*$_2$, *J* = 10.2 Hz, 1H), 5.134 (d, H-1, *J* = 7.6 Hz, 1H), 4.474 (d, H-1′, *J* = 7.6 Hz, 1H), 3.929 (br d, H-4′, *J* = 3.4 Hz, 1H), 3.665 (dd, H-3′, *J* = 3.4 and 9.8 Hz). ESI-MS (positive); 510.1 $[M + Na]^+$, 526.0 $[M + K - 1]^+$. ESI-MS (negative); 486.3 $[M - H]^-$.

Synthesis of *p*-Acrylamidophenyl β-D-Glucopyranoside 2. To a solution of *p*NP β-D-glucopyranoside (200 mg, 0.66 mmol) in MeOH (12 mL) and H_2O (6 mL) was added Pd/C (5 mg), and the reaction mixture was stirred vigorously under a H_2 atmosphere at room temperature for 3 h. The reaction mixture was processed in the same way as described above to afford an amorphous powder, *p*AP β-D-glucopyranoside (180 mg, quant.). To a solution of *p*AP β-D-glucopyranoside (180 mg, 0.66 mmol) and K_2CO_3 (184.4 mg, 1.33 mmol) in water (0.5 mL) and THF (1 mL) was added dropwise a solution of acryloyl chloride (80 μL, 0.98 mmol) in THF (1 mL) slowly at 0 °C, and the reaction mixture was processed following the same procedure as described for **1** to give monomer **2** (154.5 mg, 72%). $[\alpha]_D$ −11.8° (*c* 0.13, H_2O). ^{1}H NMR (D_2O): δ 7.444 (d, aromatic, *J* = 8.8 Hz, 2H), 7.146 (d, aromatic, *J* = 8.8 Hz, 2H), 6.415 (dd, −C*H*=CH$_2$, *J* = 10.0 and 17.2 Hz, 1H), 6.313 (dd, −CH=C*H*$_2$, *J* = 1.4 and 17.2 Hz, 1H), 5.858 (dd, −CH=C*H*$_2$, *J* = 1.4 and 10.0 Hz, 1H), 5.113 (d, H-1, *J* = 7.6 Hz, 1H), 3.923 (dd, H-6a, *J* = 2.0 and 12.4 Hz, 1H), 3.745 (dd, H-6b, *J* = 5.6 and 12.4 Hz, 1H), 3.66 − 3.45 (m, H-2, H-3, H-4 and H-5, 4H). ESI-MS (positive); 363.9 $[M + K - 1]^+$. ESI-MS (negative); 324.0 $[M - H]^-$.

Synthesis of Glycopolymers 3. To a solution of monomer **1** (10 mg, 18.4 μmol) and acrylamide (3.9 mg, 55.2 μmol) in degassed water (80 μL) was added 2,2-azobis(2-amidinopropane) dihydrochloride (0.2 mg, 0.76 μmol) in degassed water. The reaction mixture was frozen and degassed repeatedly under reduced pressure. The glass tube containing the mixture was sealed under reduced pressure, and incubated at 60 °C for 4 h under ultrasonication. The reaction mixture was dialyzed in water (M_w 8000 cut off) for 2 days, and lyophilized to give copolymer **3** (9.0 mg, 65%) as a white powder: M_w 2.2 × 10^5 (DLS analysis); mol fraction of sugar unit = 0.18 (as determined by ^{1}H NMR). ^{1}H NMR (D_2O): δ 7.45−6.70 (br, aromatic H), 5.59 (br s, H-1), 4.47 (br s, H-1′), 4.02−3.45 (br, sugar H), 2.58−1.97 (br, −C*H*−CH$_2$−), 1.97 − 1.34 (br, −CH−C*H*$_2$−).

Synthesis of Glycopolymers 4. To a solution of monomer **1** (10 mg, 18.4 μmol) and acrylamide (1.3 mg, 18.4 μmol) in degassed water (80 μL) was added 2,2-azobis(2-amidinopropane) dihydrochloride (0.1 mg, 0.38 μmol) in degassed water following the same procedure as described for copolymer **3** to give glycopolymer **4** (9.8 mg, 87%) as a white powder: M_w 2.5 × 10^5 (DLS analysis); mol fraction of sugar unit =0.65 (as determined by the phenol-H_2SO_4 method). ^{1}H NMR (D_2O): δ 7.45 − 6.55 (br, aromatic H), 6.2 − 5.3 (br, H-1), 4.44 (br s, H-1′), 4.1 − 3.4 (br, sugar H), 2.60 − 1.38 (br, −C*H*-CH$_2$− + −CH−C*H*$_2$−).

Synthesis of Glycopolymers 5. To a solution of monomer **1** (15 mg, 27.6 μmol) and acrylamide (0.65 mg, 9.18 μmol) in degassed water (100 μL) was added 2,2-azobis(2-amidinopropane) dihydrochloride (0.48 mg, 1.84 μmol) in degassed water following the same procedure as described for the synthesis of copolymer **3** to give glycopolymer **5** (7.5 mg, 72%) as a white powder. The product was insoluble in water, methanol, and other solvents. None of the characteristic data are available.

Synthesis of Glycopolymers 6. To a solution of monomer **1** (27 mg, 49.7 μmol) in degassed water (100 μL) was added 2,2-azobis(2-amidinopropane) dihydrochloride (0.13 mg, 0.49 μmol) in degassed water. The reaction mixture was processed in the same way as described for **3** to give homopolymer **6** (24.0 mg, 95%) as a white powder. The product was insoluble in water, methanol, and other solvents. None of the characteristic data are available.

Synthesis of Glycopolymers 7. To a solution of monomer **2** (20.5 mg, 63.3 μmol) and acrylamide (4.5 mg, 63.3 μmol) in degassed water (200 μL) was added 2,2-azobis(2-amidinopropane) dihydrochloride (0.34 mg, 1.3 μmol) in degassed water. The reaction mixture was processed in the same way as described for **3** to give reference polymer **7** (20.5 mg, 82%) as a white powder: M_w 1.2 $\times$ 10^5 (DLS analysis); mol fraction of sugar unit =0.50 (as determined by ^{1}H NMR spectrum). ^{1}H NMR (D_2O): δ 7.45 $-$ 6.65 (br, aromatic H), 5.06 (br, H-1), 3.95 $-$ 3.35 (br, sugar H), 2.60 $-$ 1.96 (br, $-CH-CH_2-$), 1.96 $-$ 1.24 (br, $-CH-CH_2-$).

Preparation of Lactose Chips for the Evaluation of Ricin Adsorption. The SIA Kit Au sensor substrates were cleaned with an ozone cleaner for 30 min to remove any organic adsorption, then immersed into an ethanol solution (2 mL) containing ca. 100 nmol of β-lactosyl ceramide mimic derivative having lipoic acid (11). After 24 h, the gold substrates were washed extensively with absolute ethanol and water three times and dried under a nitrogen atmosphere to give the lactose-modified sensor chips.

SPR Detection of Ricin. The above assembled sugar chips were used in this study. The running buffer used in the experiments was 10 mM HEPES (pH 7.5) containing 150 mM sodium chloride (NaCl), filtered with a 0.22 μm filter and degassed before use in all SPR experiments. The SPR system detects and measures changes in the refractive index when the analytes (the samples being analyzed) bind to and dissociate from the sensor chip surfaces. The buffer was run in the SPR system until the baseline was stable. Fifty microliters of ricin at a concentration of 250 ng/mL was then injected into the BIAcore T-100 system or the BIAcore 2000 system for 5 min at a flow rate of 10 μL/min. All SPR data were analyzed with BIAevaluation software (ver. 4.1).

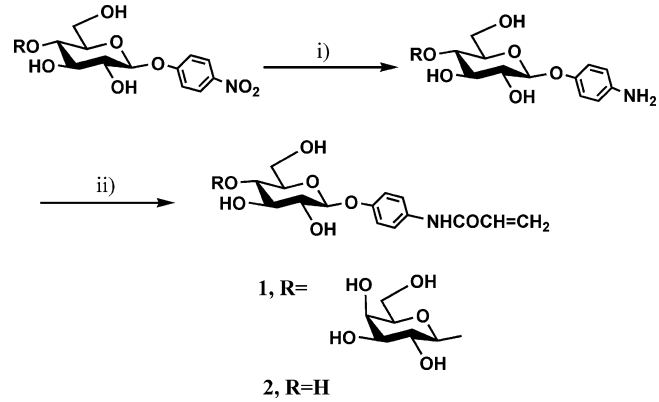
Inhibition Assay. Two hundred microliters of ricin solution (initial concentration, 500 ng/mL, 8.4 nM) in 10 mM HEPES (pH 7.5) containing 150 mM NaCl was added to 200 μL of copolymer **3** (sugar density, 18%) at various initial concentrations of 4.8 nM (1.4 μM based on the sugar residue), 25 nM (7 μM based on the sugar residue), 50 nM (14 μM based on the sugar residue), and 100 nM (28 μM based on the sugar residue). The mixture was incubated for 60 min at 25 °C. The final concentration for ricin was 4.2 nM; the final concentrations were 2.4 nM (0.7 μM based on the sugar residue), 12.5 nM (3.5 μM based on the sugar residue), 25 nM (7 μM based on the sugar residue), and 50 nM (14 μM based on the sugar residue) for polymer **3** (total amount, 400 μL).

The mixture (50 μL) was injected into the SPR system for 5 min at a flow rate of 10 μL/min. The remaining ricin was detected as a response of SPR, and ricin adsorption calculated using the following equation: [SPR response at the end of the dissociation in the presence of glycopolymers as the inhibitors]/[SPR response at the end of the dissociation in the absence of glycopolymers as the inhibitors] $\times$ 100 (%).

RESULTS AND DISCUSSION

We reported the potent activity of artificial glycopolymers carrying P^k-antigenic trisaccharide (Gb_3) for neutralizing Shiga toxins (17, 18), regioselectively sulfated glycopolymers as selectin blockers (19), and sialyl Le^x and other cell-surface oligosaccharide mimic polymers based on the concept of the "carbohydrate module approach" (20). Similar approaches have been reported by other research teams. These include the inhibitory effects of globotriose or lactose glycopolymer against Shiga toxin (21), and the inhibition of cholera toxin by polypeptide-based glycopolymers (22). In addition, synthetic studies have been directed to polyacrylamide-based glycopolymers, including sulfated saccharides (23) and trimannose (24). Acrylamide-based glycopolymers can be readily prepared, and are considered to be less allergenic than peptide-based polymers. We therefore used acrylamide-based glycopolymers for ricin adsorption.

Scheme 1. Synthesis of Carbohydrate Monomers 1 and 2[a]



[a] (i) Pd, H_2, (ii) CH_2=CHCOCl, K_2CO_3, THF, trace H_2O.

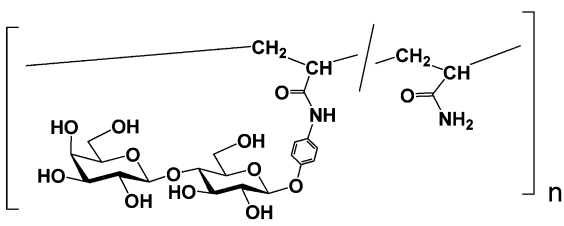
Table 1. Polymerization Conditions of 3−7[a]

polymer	feed ratio (sugar: acrylamide)	time (h)	yield (%)	M_n[b]	M_n/M_w	sugar density (sugar: acrylamide)
3	1:3	4	65	2.2 $\times$ 10^5	1.6	18:82[c]
4	1:1	4	87	2.5 $\times$ 10^5	1.6	65:35[d]
5	3:1	4	72	[e]		[e]
6	1:0	4	95	[e]		[e]
7	1:1	4	82	1.2 $\times$ 10^5	1.6	50:50[c]

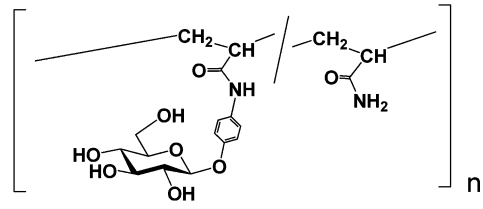
[a] Molar ratio of the initiator to the monomer = 0.01% mol in each case. [b] Determined by DLS. [c] Determined by ^{1}H NMR. [d] Determined by the phenol-H_2SO_4 method (16). [e] Not determined because of their poor solubility in water.

Carbohydrate monomers (**1** and **2**) were prepared from *p*NP (*p*-nitrophenyl) β-D-glucopyranoside and β-lactoside, respectively, as previously reported (Scheme 1) (17, 18, 25). They were converted into polyacrylamide-based glycopolymers (**3**−**7**) under the polymerization conditions summarized in Table 1. Glycopolymers **3**−**6** carried β-lactoside at different sugar densities (18−100 mol %), and glycopolymer **7** carried β-D-glucopyranoside (50 mol %) (Figure 2). Number-averaged molecular weights (M_n) were estimated by DLS as summarized in Table 1.

To evaluate these synthetic glycopolymers, competitive binding assays were carried out using our previously described β-lactoside-chips to an SPR analysis (11). Figure 3 shows a typical SPR response to ricin (4.2 nM) using copolymer **3** (sugar density, 18%) at different competitor concentrations (0.7−14 μM based on the sugar residue). Ricin in the absence of **3** gave the highest SPR signal ([ricin] = 4.2



copolymer **3**: Lac/acrylamide = 18:82
copolymer **4**: Lac/acrylamide = 65:35
copolymer **5**: Lac/acrylamide = 75:25 in a feed ratio
homopolymer **6**: Lac/acrylamide = 100:0 in a feed ratio



copolymer **7**: Glc/acrylamide = 50:50

FIGURE 2. Structures of glycopolymers (3−7) having different sugar densities.

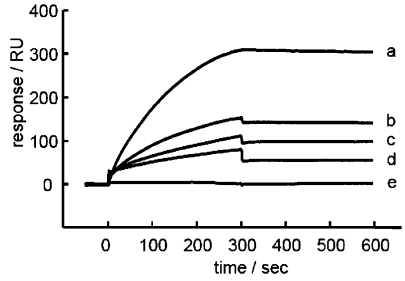


FIGURE 3. SPR responses to ricin on the surfaces of lactose chips (11) in the presence of different concentrations of glycopolymer 3: (a) ricin (4.2 nM); (b) ricin (4.2 nM) + 3 (0.7 μM based on the sugar residue); (c) ricin (4.2 nM) + 3 (3.5 μM based on the sugar residue); (d) ricin (4.2 nM) + 3 (7 μM based on the sugar residue); (e) ricin (4.2 nM) + 3 (14 μM based on the sugar residue). Inhibition time = 60 min.

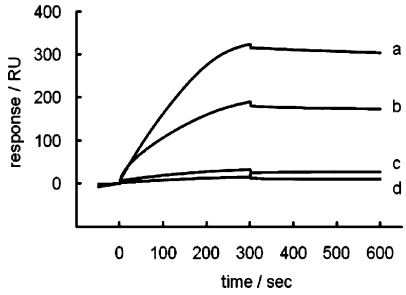


FIGURE 4. SPR responses to ricin on the surfaces of lactose chips (11) in the presence of different concentrations of glycopolymer 4: (a) ricin (4.2 nM); (b) ricin (4.2 nM) + 4 (0.23 μM based on the sugar residue); (c) ricin (4.2 nM) + 4 (0.69 μM based on the sugar residue); (d) ricin (4.2 nM) + 4 (1.1 μM based on the sugar residue). Inhibition time = 60 min.

nM, [**3**] = 0 μM, Figure 3a), which was in good accordance with the data from our previous study (11). In the presence of glycopolymers, SPR responses were dose-dependently suppressed (Figure 3b−e). At a concentration of 14 μM based on the sugar residue of inhibitor **3**, binding to the SPR chip was completely inhibited ([ricin] = 4.2 nM, [**3**] = 14 μM based on the sugar residue, Figure 3e). That is, free toxin was no longer in the mixture, showing that glycopolymer **3** had adsorbed ricin.

Copolymer **4** (sugar density, 65%) inhibited toxin binding dose-dependently (Figure 4b−d), even though complete inhibition by **4** required a lower concentration than in the case of **3** ([ricin] = 4.2 nM, [**4**] = 1.1 μM based on the sugar residue and [**3**] = 14 μM based on the sugar residue). This showed that glycopolymers with higher sugar densities adsorbed the toxin more effectively.

Table 2. Inhibitory Activity per Sugar Residue

glycopolymer	IC_{100} (μM)
3	14
4	1.1
lactose[a]	100 000

[a] Free lactose was used.

Table 2 shows the IC_{100} values per sugar residue of each glycopolymer in comparison with lactose (a reducing disaccharide). In this case, IC_{100} means the concentration per sugar residue of the inhibitor required for complete inhibition of ricin-lactose adhesion on SPR chips. Polymer **4** showed a higher activity than polymer **3**; this result was in accordance with the results stated above. In contrast, the reducing monomeric lactose required a much higher concentration (100,000 μM) for complete inhibition. These results indicated that the multivalent or cluster effect (26) worked well in this case.

The activity of copolymer **5** and homopolymer **6** could not be evaluated because of their poor solubility in aqueous solution. Reference Glc polymer **7**, as well as pullulan [an α(1→4)Glc-based polymer; data not shown], showed no inhibition in the SPR measurement. This revealed that ricin did not exhibit affinity for the Glc residues in these reference polymers, and was specifically bound to the β-lactoside residue in **3** and **4**. We also confirmed the absence of nonspecific binding on SPR sensor chips by **3**, **4**, and **7**.

The SPR curves in Figure 5 show that the inhibition was complete by 60 min ([ricin] = 4.2 nM, [**4**] = 1.1 μM based on the sugar residue, Figure 5d). The use of **4** at higher concentrations resulted in the completion of inhibition in a shorter time (20 min, [ricin] = 4.2 nM, [**4**] = 1.6 μM based on the sugar residue).

CONCLUSIONS

The present study was the "starting point" of ricin decontamination R&D. For example, two pathways were proposed for ricin endocytosis. One is through lactose binding on the cell surface (12−14) and the other is through mannose receptors with mannose oligosaccharides on ricin (27). We synthesized a series of lactose-based glycopolymers

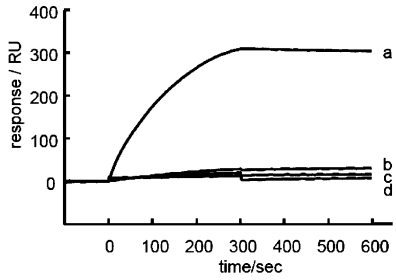


FIGURE 5. SPR responses to ricin on the surfaces of lactose chips (11) in the presence of glycopolymer 4 at different times: (a) 0, (b) 20, (c) 40, and (d) 60 min. Ricin = 4.2 nM, glycopolymer 4 = 1.1 μM based on the sugar residue.

having different sugar densities to show that synthetic Lac polymers effectively inhibit the toxin-lactoside binding event. Glycopolymers having higher lactose density showed higher activity, indicating their utility in decontamination technologies against this toxin. Studies on the mannose receptor will be addressed in a forthcoming study, probably in an identical manner as described in this contribution.

On the basis of glycotechnology, many sugar-based reagents and materials have been designed, developed, and applied. We have shown in this and a previous study (11) that glycotechnology can make significant contributions to the detection and decontamination of biological toxins. These studies also suggest that synthetic glycopolymers may be applicable for clinical purposes.

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