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Glycotechnology for Decontamination of Biological Agents: A Model Study Using Ricin and Biotin-Tagged Synthetic Glycopolymers

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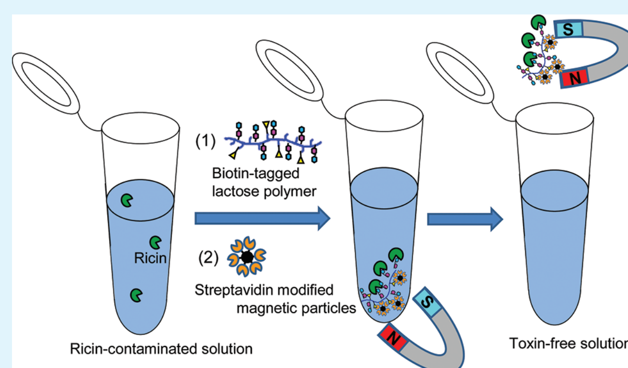
ABSTRACT: Two types of biotin-tagged glycopolymers carrying lactose or glucose in clusters along the polyacrylamide backbone were prepared and subjected to decontamination analyses with the plant toxin ricin. A buffer solution containing the toxin was treated with one glycopolymer followed by streptavidin-magnetic particles. Supernatant solutions were analyzed with surface plasmon resonance and capillary electrophoresis, and revealed that the lactose glycopolymer "captured" this toxin more effectively than the glucose polymer. Free toxin was not detectable in the supernatant after treatment with the glycopolymer and magnetic particles; >99% decontamination was achieved for this potentially fatal biological toxin.



KEYWORDS: *biological agent, biological toxin, ricin, glycopolymer, decontamination, lactose, magnetic particles, biotin*

INTRODUCTION

More than 1200 types of biohazard agents are recognized as "biological agents" that can be used in bioterrorism or biological warfare. They include prions, viruses, and some microorganisms such as bacteria and fungi as well as the toxins that they produce. Biological toxins include botulinum toxin, ricin, saxitoxin, and tetrodotoxin, which are ubiquitous in the natural environment and rapidly reproducible in bacteria, plants, or animals. Because of their potential danger in a wide variety of occupational settings, they must be kept under strict control. Hence, technologies for the detection, containment, and decontamination of toxins are important.[1,2] For detection of the deadly plant toxin ricin, which has been used for illegal purposes,[3,4] researchers have developed immunological,[1,2,5−7] genetic,[8−10] spectroscopic,[11−13] and enzymatic approaches.[14] Moreover, other new technologies are being developed for toxin detection.[15,16] Conversely, technologies for the containment and decontamination of toxins are underdeveloped.[17,18] Sodium hypochlorite, alkaline solutions and peroxides (e.g., H_2O_2) are used as universal decontaminants. However, these chemical oxidants are harmful and may damage electronic machines and the environment. As an alternative approach to decontamination, the use of enzymes or multifunctional polymers has been demonstrated.[17−20]

We recently developed a highly sensitive detection method for ricin which utilizes synthetic glyco-chips and nanoparticles.[21] The approach was based on a plant toxin binding very specifically to the host-cell oligosaccharide and thus entering into the host.[22−24] We subsequently found that synthetic glycopolymers bearing lactose clusters along the side chain could inhibit ricin−host cell binding and effectively adsorb the toxin.[25] Many biological toxins possess a common infection pathway starting from recognition of the host-cell oligosaccharide. Hence, synthetic glycotechnology is expected to contribute significantly in maintaining biological toxins under control by governmental organizations.[21,25−32]

We were previously engaged in efforts to develop a practical detection and adsorption methodology based on glycotechnology. In the present study, we introduced the concept of decontamination and tried to develop an advanced technology that enables us to remove biological toxins from contaminated scenes. In the present study, a plant toxin was treated with biotin-tagged glycopolymers at the first stage and streptavidin-modified magnetic particles at the second stage (Figure 1). The efficacy of the proposed decontamination approach was evaluated by means of surface plasmon resonance (SPR) and electrophoretic capillary (EC) analyses.

EXPERIMENTAL SECTION

Materials and Instruments. Ricin from *Ricinus communis* was obtained from Honen Corporation (Tokyo, Japan) and safely handled at the National Research Institute of Police Science with approval of

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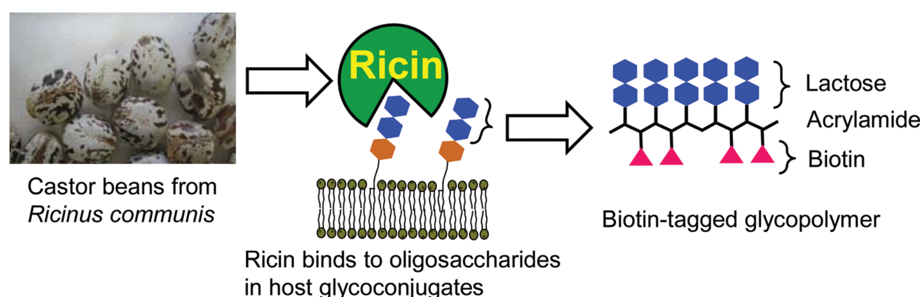


Figure 1. *Ricinus communis* toxin (ricin) binds to the host cell-surface oligosaccharides. This suggests the utility of synthetic glycopolymers as toxin decontaminants. We designed the biotin-tagged glycopolymers that carry lactose and biotin clusters along the side chain.

the Minister of Economy, Trade and Industry of Japan (http://www.meti.go.jp/polycy/chemical_management/cwc/200kokunai/202horitu_gaiyo.htm). D-(+)-Biotinyl-3,6,9-trioxaundecanediamine and streptavidin-coated magnetic particles with diameters of 1−4 μm (MagnaBind Streptavidin particles) were supplied from Thermo Scientific (Rockford, IL, USA). *p*-Acrylamidophenyl (*p*AP) β-lactoside and *p*AP β-D-glucopyranoside were prepared using an established pathway.[25] The reaction of a biotinylated monomer was monitored by thin-layer chromatography (TLC) on Silica Gel 60 F_{254} (Merck, Darmstadt, Germany) and visualized by ultraviolet (UV) light. Flash chromatography was carried out on Silica Gel 60 N (70−230 mesh; Kanto Chemical, Tokyo, Japan). Surface interaction analysis (SIA) Kit gold (Au) chips were purchased from Biacore AB (Uppsala, Sweden). ^{1}H NMR spectra were recorded on a Varian INOVA 400 system (Palo Alto, CA, USA) or a JEOL LA-600 (Tokyo, Japan) spectrometer for solutions in chloroform-*d* ($CDCl_3$) or deuterium oxide (D_2O). Chemical shifts are given in parts per million (ppm) and referenced to tetramethylsilane (TMS, δ_H 0.00) in $CDCl_3$ or *tert*-butyl alcohol (*tert*-BuOH, δ_H 1.23) in D_2O as the internal reference. All data were assumed to be first order with apparent doublets and triplets reported as d and t, respectively. Resonances that appeared broad were designated "br". Electrospray ionization mass spectra (ESI-MS) were directly recorded using a SHIMADZU LC-MS 2010A mass spectrometer (Kyoto, Japan). SPR experiments were undertaken on a BIAcore 2000 or BIAcore T-100 systems (BIAcore AB; GE Healthcare, Piscataway, NJ, USA), and all data were calculated using BIAevaluation commercial software (version 4.1). The sugar densities of glycopolymers were determined by ^{1}H NMR. CE was carried out using a Hewlett-Packard Model HP^{3D} capillary electrophoresis system (Waldbronn, Germany) with UV diode array detection, and monitored at 246 and 280 nm. Static light scattering (SLS) analyses were carried out using a Malvern Zetasizer Nano ZS system (Malvern Instruments, Malvern, UK). Weight-average molecular weights (M_w) were calculated using Nano HPPS v420 software by employing the differential index of refraction (*dn/dc*) reported to polyacrylamides.[33] 2,2′-Azobis(2-methylpropionamidine) dihydrochloride, acrylamide, acryloyl chloride, and other reagents were all commercially available.

Synthesis of 1-Acrylamido-11-D-(+)-biotinamido-3,6,9-trioxaundecane 2. To a solution of D-(+)-biotinyl-3,6,9-trioxaundecanediamine **1** (21.3 mg, 0.05 μmol) in THF (0.66 mL) was added slowly a solution of acryloyl chloride (6 μL) in THF (20 μL) at 0 °C. The reaction mixture was stirred at room temperature for 2.5 h. The reaction mixture was concentrated under diminished pressure, and the residue purified by chromatography (silica gel, $CHCl_3$/MeOH = 10/1) to give biotin monomer **2** (16 mg, 66%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.900 (br s, N*H*CO), 6.827 (br t, N*H*CO), 6.607 (br s, N*H*CO), 6.295 (dd, CH=C*H*$_2$, *J* 1.8 and 17.0 Hz), 6.182 (dd, C*H*=CH$_2$, *J* 10.0 and 17.0 Hz), 5.648 (br s, N*H*CO), 5.621 (dd, CH=C*H*$_2$, *J* 1.8 and 10.0 Hz), 4.57−4.47 (m, methine of biotin), 4.35−4.27 (m, methine of biotin), 3.74−3.40 (m, −OC*H*$_2$C*H*$_2$O−), 3.20−3.10 (m, methine of biotin), 2.900 (dd, -SC*H*$_2$CH- of biotin, *J* 4.8 and 12.8 Hz), 2.745 (d, -SC*H*$_2$CH- of biotin, *J* 12.8 Hz), 2.412 (br s, −CONHC*H*$_2$CH$_2$O- of ethylene glycol spacer), 2.28−2.19 (m, biotin), 1.84−1.60 (m, biotin), 1.53−1.38 (m, biotin). ESI-MS (positive); 473.1 $[M]^+$, 495.1 $[M+Na]^+$.

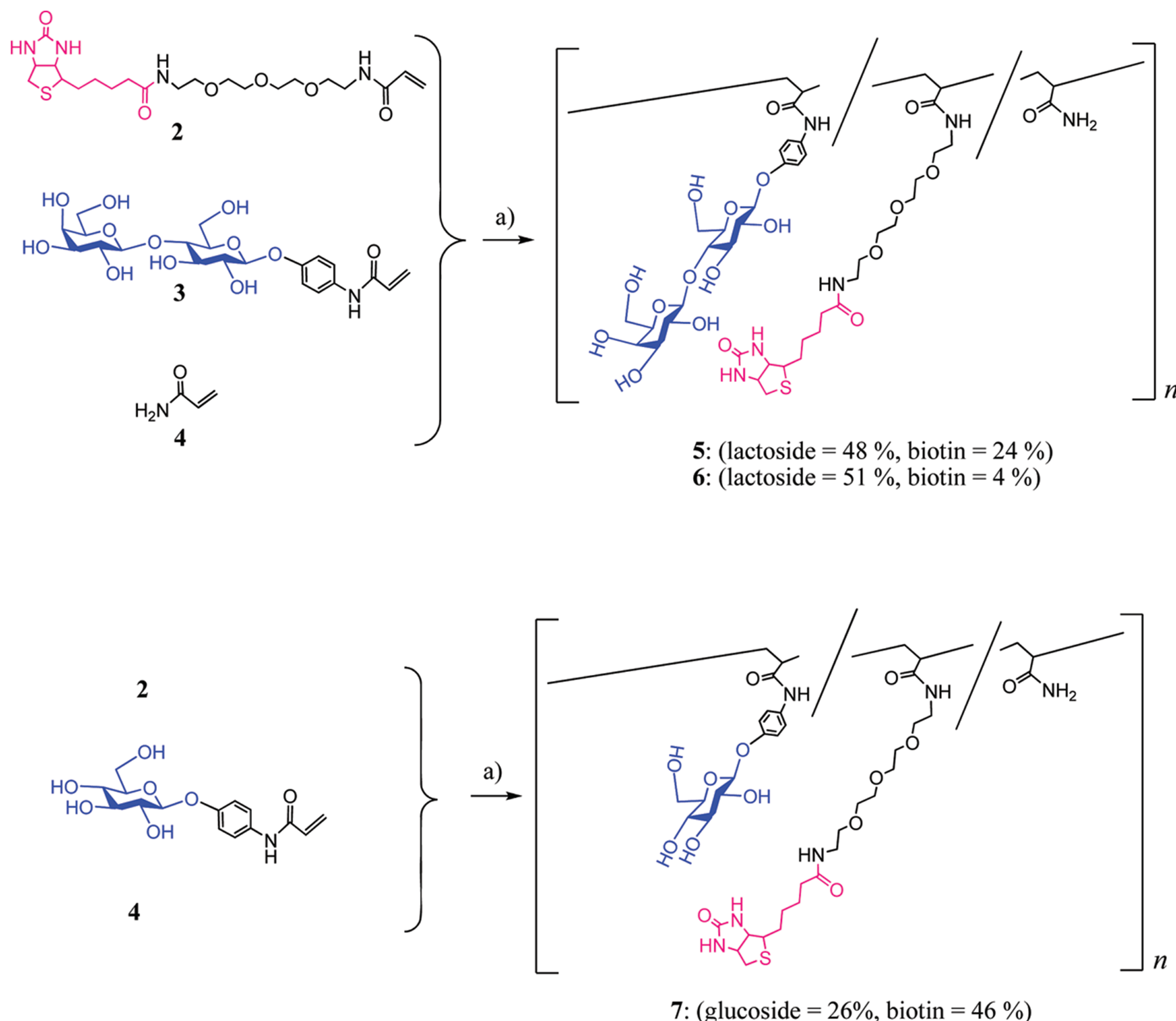
Synthesis of Glycocopolymer 5. To a solution of biotin monomer **2** (7.57 mg, 16 μmol), carbohydrate monomer **3** (10 mg, 18.4 μmol) and acrylamide **4** (0.28 mg, 3.9 μmol) in dimethyl sulfoxide (DMSO, 100 μL) was added 2,2-azobis(2-amidinopropane) dihydrochloride (0.1 mg, 0.38 μmol) dissolved in DMSO (5 μL). The reaction mixture was frozen and degassed repeatedly under reduced pressure. The glass tube containing the reaction mixture was sealed under reduced pressure, and incubated at 60 °C for 4.5 h under ultrasonication. The reaction mixture was dialyzed in water (*M*w 8,000 cut off) at 70 °C for 1 day, and lyophilized to give glycocopolymer **5** (7.18 mg, 40%) as a white powder: $M_w = 7.4 \times 10^5$ (SLS analyses); mol fraction of sugar unit = 48% and mol fraction of biotin unit =24% (determined by ^{1}H NMR). ^{1}H NMR (600 MHz, D_2O) δ 7.55−6.60 (br, aromatic H), 4.99 (br, Glc H-1), 4.47 (br, Gal H-1 + biotin), 4.23 (br, methine of biotin), 4.11−2.98 (br, sugar H + −OC*H*$_2$C*H*$_2$O− of ethylene glycol spacer + biotin), 2.85 (br, biotin), 2.66 (br, biotin), 2.53−1.95 (br, −C*H*CH$_2$− of acrylamide + −CONHC*H*$_2$CH$_2$O− of ethylene glycol spacer + NHCOC*H*$_2$CH$_2$CH$_2$CH$_2$− of biotin), 1.95−1.37 (br, −CHC*H*$_2$− of acrylamide + biotin), 1.37−1.05 (br, biotin).

Synthesis of Glycocopolymer 6. To a solution of biotin monomer **2** (1.89 mg, 0.29 μmol), carbohydrate monomer **3** (10 mg, 18.4 μmol) and acrylamide **4** (1.13 mg, 15.7 μmol) in DMSO (100 μL) was added 2,2-azobis(2-amidinopropane) dihydrochloride (0.1 mg, 0.38 μmol) dissolved in DMSO (5 μL). The reaction mixture was processed in the same way as described for **5** to give glycocopolymer **6** (9.8 mg, 75%) as a white powder: $M_w = 2.6 \times 10^6$ (SLS analyses); mol fraction of sugar unit = 51% and mol fraction of biotin unit = 4% (determined by ^{1}H NMR).^{1}H NMR (600 MHz, D_2O) δ 7.50−6.60 (br, aromatic H), 5.00 (br, Glc H-1), 4.46 (br, Gal H-1 + biotin), 4.18 (br, methine of biotin), 4.12−2.93 (br, sugar H + −OC*H*$_2$C*H*$_2$O− of ethylene glycol spacer + biotin), 2.81 (br, biotin), 2.63 (br, biotin), 2.57−1.98 (br, −C*H*CH$_2$− of acrylamide + CONHC*H*$_2$CH$_2$O− of ethylene glycol spacer + NHCOC*H*$_2$CH$_2$CH$_2$CH$_2$− of biotin), 1.98−1.30 (br, −CHC*H*$_2$− of acrylamide + biotin), 1.30−1.13 (br, biotin).

Synthesis of Glycocopolymer 7. To a solution of biotin monomer **2** (6.8 mg, 14 μmol), *p*-acrylamidophenyl β-D-glucopyranoside[25] (5.9 mg, 17.5 μmol) and acrylamide **4** (0.25 mg, 3.5 μmol) in DMSO (100 μL) was added 2,2-azobis(2-amidinopropane) dihydrochloride (0.1 mg, 0.38 μmol) dissolved in DMSO (5 μL). The reaction mixture was processed in the same way as described for **5** to give glycocopolymer **7** (8.0 mg, 61%) as a white powder: $M_w = 4.8 \times 10^5$ (SLS analyses); mol fraction of sugar unit =26% and mol fraction of biotin unit = 46% (determined by ^{1}H NMR).^{1}H NMR (400 MHz, D_2O) δ 7.58−6.86 (br, aromatic H), 5.04 (br, Glc H-1), 4.55 (br, biotin), 4.36 (br, methine of biotin), 3.90 (br), 3.80−3.01 (br, sugar H + −OC*H*$_2$C*H*$_2$O− of ethylene glycol spacer + biotin), 2.93 (br, biotin), 2.73 (br, biotin), 2.57−1.96 (br, −C*H*CH$_2$− of acrylamide + −CONHC*H*$_2$CH$_2$O− of ethylene glycol spacer + −NHCOC*H*$_2$CH$_2$CH$_2$CH$_2$− of biotin), 1.96−1.44 (br, −CHC*H*$_2$− of acrylamide + biotin), 1.44−1.0 (br, biotin).

Ricin Decontamination Study. We set the initial ricin concentration at 16.5 nM, at which the toxin could be readily detected by SPR and CE analyses. Ricin (1 μg/mL, 200 μL, 16.5 nM) was added to the buffer solution [10 mM HEPES buffer (pH 7.5)

Scheme 1. (a) 2,2′-Azobis(2-methylpropionamidine) Dihydrochloride in H_2O under Ultrasonication, 2:3:4:Initiator in ca. 4.1:4.7:1:0.1 in Feed Ratio (mol %) for Polymer 5, 2:3:4:Initiator in ca. 1:63:54:1.3 for Polymer 6, and 2:Glc Monomer: 4:Initiator in ca. 4:5:1:0.1 for Polymer 7; Yield = 40% for 5, 75% for 6, and 61% for 7



containing 150 mM NaCl] of glycopolymer **5** (100 μg/mL, 200 μL, 135 nM), and incubated at room temperature with gentle shaking for 20 min. Streptavidin-coated magnetic particles (2.5 mL, streptavidin 20 nmol) were treated with 10 mM HEPES buffer (pH 7.5) containing 150 mM NaCl, and the magnetic particles dispersed in the same buffer (100 μL). The dispersed magnetic particles (100 μL, streptavidin 20 nmol (200 μM)) were added to a mixture of ricin and **5**. The resulting mixture (total volume 500 μL, [ricin] = 6.6 nM, [**5**] = 54 nM, [sugar residue in polymer **5**] = 52 μM based on the sugar residue, [biotin residue in polymer **5**] = 26 μM based on the biotin residue, and [streptavidin on the magnetic particles] = 40 μM) was mixed well, and incubated at room temperature with gentle shaking for 40 min. A neodymium magnet was used to collect the magnetic particles in the mixture. The supernatant was diluted twice with the same buffer prior to SPR and CE analyses.

SPR Analyses. The SPR analysis was carried out in the manner established by our research team way to evaluate the efficiency of the decontamination.[21,25] Briefly, lactoside-modified chips were prepared in the following manner. Lactoside derivatives having lipoic acids at the termini were synthesized using our method and immobilized onto SPR sensor surfaces with a self-assembled monolayer (SAM) technique. Prepared chips were washed with methanol and Mili-Q water (Millipore, Billerica, MA, USA), dried, and set up to the SPR system. The running buffer used in the experiments was 10 mM HEPES (pH 7.5) containing 150 mM NaCl filtered with a 0.22-μm filter and degassed before use in all SPR experiments. The SPR system could be used to detect a shift in refractive index if the analytes (the samples being analyzed) were bound and dissociated from the surfaces of the sensor chips. The buffer was run in the SPR system until the baseline became stable. Fifty microliters of standard ricin (3.3 nM) or the supernatant after separation by a magnet was injected into the BIAcore 2000 or BIAcore T-100 system for 5 min at a flow rate of 10 μL/min. All SPR data were analyzed with BIAevaluation software (ver. 4.1).

CE Experiment. A fused silica capillary (75 μm i.d. × 72 cm; Agilent Technologies, Wilmington, DE, USA) and a running buffer (50 mM borate-sodium buffer, pH 9.5) were used. Data were collected in all experiments in positive polarity mode at an applied voltage of 30 kV with the capillary cooled to 15 °C. All the data were analyzed using HP 3D-CE ChemStation. Sample solutions and running buffers were filtered through a 0.22 μm filter cartridge (Millipore), and were hydrodynamically injected (50 mbar for 6 s for sample and 50 mbar for 4 s for buffer).

Safety Considerations. Ricin is highly toxic if inhaled or digested. This compound should be handled with special care. After examination, it must be decomposed using sodium hypochlorite or an autoclave.

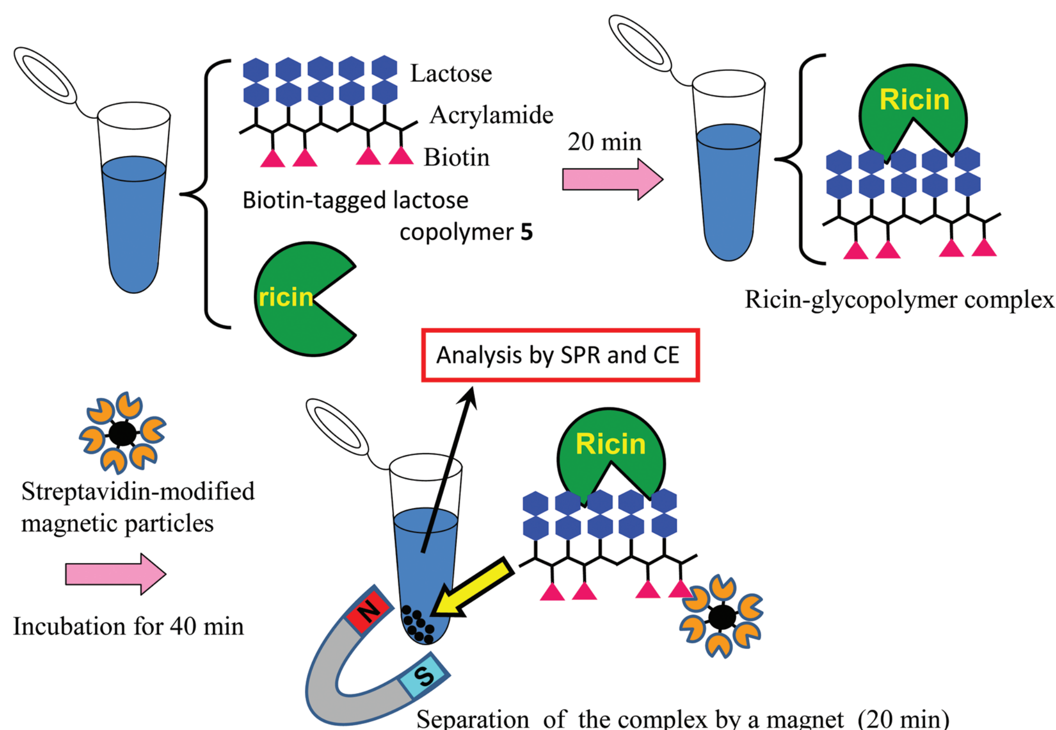


Figure 2. Decontamination of the deadly plant toxin ricin. Ricin specifically binds to cell-surface oligosaccharides containing β-D-galactopyranoside (β-D-Gal) and β-D-*N*-acetylgalactosaminide (β-D-GalNAc). This process can be applied to toxin decontamination. The toxin binds to the glycopolymer **5** containing β-lactoside. The resulting complex can be removed using the streptavidin-modified magnetic particles. The supernatant solution is then analyzed by surface plasmon resonance and capillary electrophoresis techniques.

RESULTS AND DISCUSSION

Ricin is composed of single A- and B-chains (1:1). The A-chain has *N*-glycosidase activity. The B-chain has two carbohydrate-binding domains; these can recognize the β-D-galactopyranoside (β-Gal) and *N*-acetyl β-D-galactosaminide (β-GalNAc) residues in host oligosaccharides. Hence, the B-chain binds specifically to host cell-surface oligosaccharides by recognizing β-D-Gal or β-D-GalNAc residues at the nonreducing terminal, and enables the A-chain to enter into host cells by endocytosis.[22−24] We therefore designed a glycopolymer bearing β-lactoside and biotin for toxin recognition and for transfer of the toxin−polymer complex onto streptavidin-modified magnetic particles.

Biotin-tagged lactose copolymers (**5** and **6**) and glucose copolymer **7** were prepared as previously reported by our research team (Scheme 1).[25] The lactose copolymers (**5** and **6**) have different biotin densities from each other. Oligoethylene-glycols were selected as spacers because they were expected to eliminate nonspecific binding.[34] A biotinyl monomer **2** having a 3,6,9-trioxaundecanediamine spacer was derived from biotinyl-3,6,9-trioxaundecanediamine **1** in 66% yield. Lactose monomer **3** was synthesized according to previously described methods by our research team.[25] Radical copolymerization was carried out in the biotin monomer **2**, lactose monomer **3**, and acrylamide **4** in ca. 4.1:4.7:1 feed ratio in the presence of 2,2′-azobis(2-amidinopropane) dihydrochloride (0.1 mol %), producing copolymer **5** in 40% yield. Another feed ratio (biotin:sugar:acrylamide in ca. 1:63:54) gave the lactose copolymer **6**. The M_w of copolymers **5** and **6** was estimated by SLS to be 7.4×10^5 and 2.6×10^6, respectively. The molar ratio of the polymer components (biotin, lactoside, acrylamide) was determined by ^{1}H NMR analyses to be 24:48:28 for **5** and 4:51:45 for **6**. The glucose copolymer (**7**, M_w = 4.8×10^5, biotin:sugar:acrylamide = 46:26:28) was also prepared in the same way as described for **5** and **6**.

A hypothetical decontamination process is summarized in Figure 2. The first process is treatment of ricin solution with glycopolymer to complete the lactose−toxin interaction. The second process is the biotin−streptavidin interaction to transfer the toxin−polymer complex onto the magnetic particles. The optimized processes are summarized as follows. A large excess of glycopolymer **5** is added to a ricin solution ([**5**] = 52 μM based on the sugar residue, [ricin] = 6.6 nM), and the reaction mixture incubated for 20 min. The resulting complex of ricin and glycopolymer **5** is mixed with streptavidin-modified magnetic particles for 40 min. The final complexes of the toxin, glycopolymer and magnetic particles are collected by a magnet for 20 min. The supernatant was analyzed by SPR and CE to ascertain the efficiency of the decontamination processes.

SPR analyses showed that the authentic ricin solution gave a typical SPR response (Figure 3a), whereas no SPR response appeared after treatment with excess lactose copolymer **5** (Figure 3c; ca. 7800 mol equiv. to the toxin solution; [**5**] = 52 μM based on the sugar residue, [ricin] = 6.6 nM) followed by treatment with the magnetic particles and magnet. This result showed that the concentration of the toxin was <10 ng/mL (the limit of detection in the SPR analysis). Thus, the concentration of the toxin in the mixture was negligible, and was certainly not a biohazard. That is, glycopolymer **5** almost completely "captured" the toxin. In this case, an excess amount of streptavidin on the magnetic particles was used for complete recovery of the complex (40 μM based on the streptavidin residue on the magnetic particles vs 26 μM based on the biotin residue in glycopolymer **5**). In contrast, the reference glycopolymer **7** carrying β-D-Glc showed a strong response

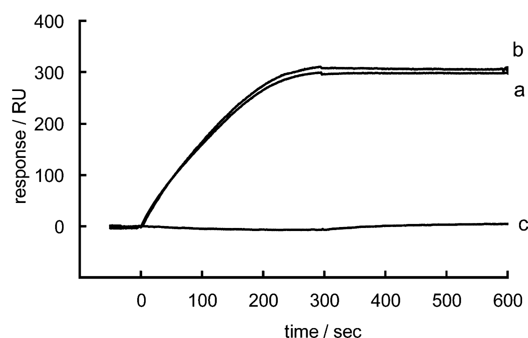


Figure 3. SPR responses of (a) standard (reference) ricin, (b) supernatant after the treatment with glucose copolymer 7, and (c) supernatant after treatment with lactose copolymer **5**.

to ricin in the SPR experiment (Figure 3b), the same as the standard (reference) ricin, even though glucose copolymer 7 was applied in large excess (ca. 1.2×10^9 mole equivalents to the toxin based on the sugar residue, [7] = 8200 mM based on the sugar residue, [ricin] = 6.6 nM). This result indicated that the glucose polymer could not effectively remove the toxin.

Next, CE was applied to confirm the presence of free ricin in the mixture after treatment with lactose copolymer **5** and magnetic particles. Lactose polymer **5** and ricin could be detected at 246 nm and at 280 nm, respectively (Figure 4A, B). In contrast, a CE peak could not be detected for the ricin solution, indicating that complete removal of the toxin was achieved (Figures 4C, D). The glycopolymers were also not detected (limit of detection, <3.3 nM), i.e., the residual polymers in the supernatants were in negligible amounts. From the SPR and CE analyses, we postulated that ricin was captured by synthetic biotin-tagged glycopolymers bearing lactose in clusters and had multivalent interactions with this biological toxin. We thought that the resulting ricin−glycopolymer complex was transferred onto the magnetic nanoparticles by strong biotin−streptavidin interactions. After sedimentation using a magnetic field, the toxin was completely removed from the supernatant when the magnetic particles were applied in excess to the glycopolymers.

Lactose copolymer **6** had fewer biotin residues (4%) in the polymer, so ricin removal was incomplete. This finding showed that biotin content was also a determining factor for the efficiency of ricin decontamination.

CONCLUSIONS

Synthetic glycopolymers carrying lactose and biotin along the acrylamide side chain were prepared and applied for ricin decontamination. The copolymers effectively adsorbed this toxin, and the resulting toxin−polymer complex could be transferred onto streptavidin-modified magnetic particles. The efficiency of decontamination was >99% by means of SPR and CE analyses. The lactose copolymer with a high biotin density could be used to decontaminate the toxin. In comparison with conventional decontamination methodology using chemical oxidants, the described method utilized the more environmentally friendly sugars and biotin. This fact supports our idea of using simple sugars for maintaining these biohazards under governmental control.

Decontamination methodology based on toxin−sugar interactions is in its infancy. Conversely, developments in glycotechnology are rapid, and may help in the development of a validated method for tackling biological agents and toxins.

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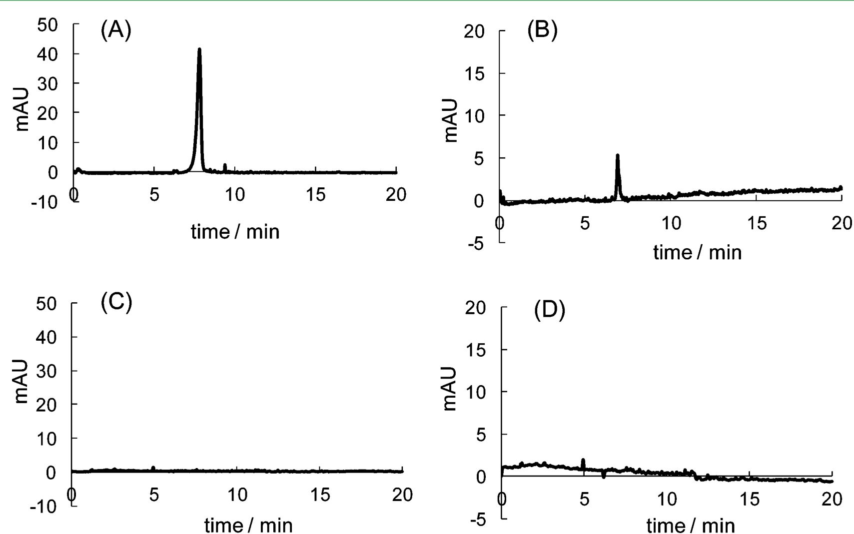

Figure 4. Capillary electrophoretic analyses of lactose copolymer **5**, ricin, and the supernatant sample (UV detection at 246 nm for the polymer and at 280 nm for ricin). A fused silica capillary (75 mm i.d. × 72 cm) and a running buffer (50 mM borate sodium buffer, pH 9.5) were used at 30 kV and 15 °C. (A) Standard solution of lactose copolymer **5** monitored at 246 nm; (B) standard solution of ricin monitored at 280 nm; (C) supernatant detected at 246 nm after treatment with **5** and streptavidin-modified magnetic particles; (D) supernatant detected at 280 nm after treatment with **5** and magnetic particles.

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