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A novel sugar-probe biosensor for the deadly plant proteinous toxin, ricin

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ABSTRACT

Because of the illegal use of highly toxic ricin from the castor-oil plant, *Ricinus communis*, in bioterrorism and suspected white powder cases, anti-terrorism measures for the toxin are urgently required. Here we demonstrate a facile and sensitive detection method using synthetic analogues of β-lactosyl- and β-D-galactosyl ceramides as the ligands based on the fact that ricin binds cell-surface oligosaccharides. Sugar-probes having lipoic acids as anchor functions were synthesized *via* either a chemical or chemoenzymatic way and were immobilized on the sensor chips by a self-assembled monolayer technique. Surface plasmon resonance (SPR) analysis using these carbohydrate probes allowed us to detect the toxin in a highly sensitive and facile manner (10 pg/mL, 5 min), being the best benchmark as a method for detecting the toxin. In addition, a visual monitoring method was developed, in which sugar-coated Au nanoparticles were utilized for discriminating ricin from other proteins in a facile manner, taking 10–30 min for judgment.

1. Introduction

From an actual case history, the strong toxicity of *Ricinus communis* (ricin: RCA_{60}) has become familiar since this plant toxin was used as an instrument for assassination in London (Crompton and Gall, 1980). Recently, the toxin was again illegally used, and mail contaminated with it was sent to the US Senate and the White House (The New York Times, 2004). The crisis of terrorism has very much increased in recent years (Niiler, 2002). Among various natural toxins involved in chemical and biological weapons (CBW), ricin is categorized as one of the extremely toxic proteins (Organization for the Prohibition of Chemical Weapons (OPCW): http://www.opcw.org/). The oral toxicity is estimated to be 500–1000 times stronger than potassium cyanide, which releases a deadly poison, cyanide gas. Also, for the sake of national security, the significance of "biohazard sensors" is being greatly increased (National Nanotechnology Initiative: http://www.nano.gov/). As a part of national policy on terrorism, we must address these urgent issues as part of the global mission against terrorism. Here we describe facile and reliable detection methods for the deadly toxin.

In our research project on developing biosensors targeting the Shiga toxins of *Escherichia coli O*-157 (Uzawa et al., 2002, 2005, 2007), we recently reported on a toxin sensor which applies a globobiosyl (Gb_2)-synthetic glycolipid as a carbohydrate probe with conventional sensing tools such as a quartz crystal microbalance (QCM) and surface plasmon resonance (SPR). This was based on the fact that the Shiga toxins recognize the host glycolipid at the initial stage of internalizing into the host cell (Kaper and O'Brien, 1998; Lindberg et al., 1987). This holds true also for the plant toxin, ricin. The castor-oil plant, *R. communis*, produces two similar proteins, ricin (RCA_{60}, ca. 60 kDa) and an agglutinin (RCA_{120}, ca. 120 kDa). Ricin is composed of A- and B-chains [A–B chains] which are connected to each other through a disulfide (S–S) bond (Montfort et al., 1987; Road et al., 1994). The A-chain is the actual toxin which has enzymatic *N*-glycanase activity in regard to ribosomal RNA, resulting in the inhibition of protein biosynthesis (Lynne and Smith, 2004). The B-chain has two carbohydrate-binding sites, each of which binds to the β-D-galactopyranoside (β-Gal) or β-D-*N*-acetylgalactosamine (β-GalNAc) residue in host glycoconjugates. The B-chain is considered to play an essential role in the internalization of the toxic A-chain. On the other hand, the agglutinin has a close homology in the peptide sequence to RCA_{60}. The agglutinin consists of a dimeric chain [(A–B)–S–S–(B–A)], in which the two (A–B) chains are covalently connected by a disulfide bond (Sweeney et al., 1997). The A-chain of the agglutinin cannot enter

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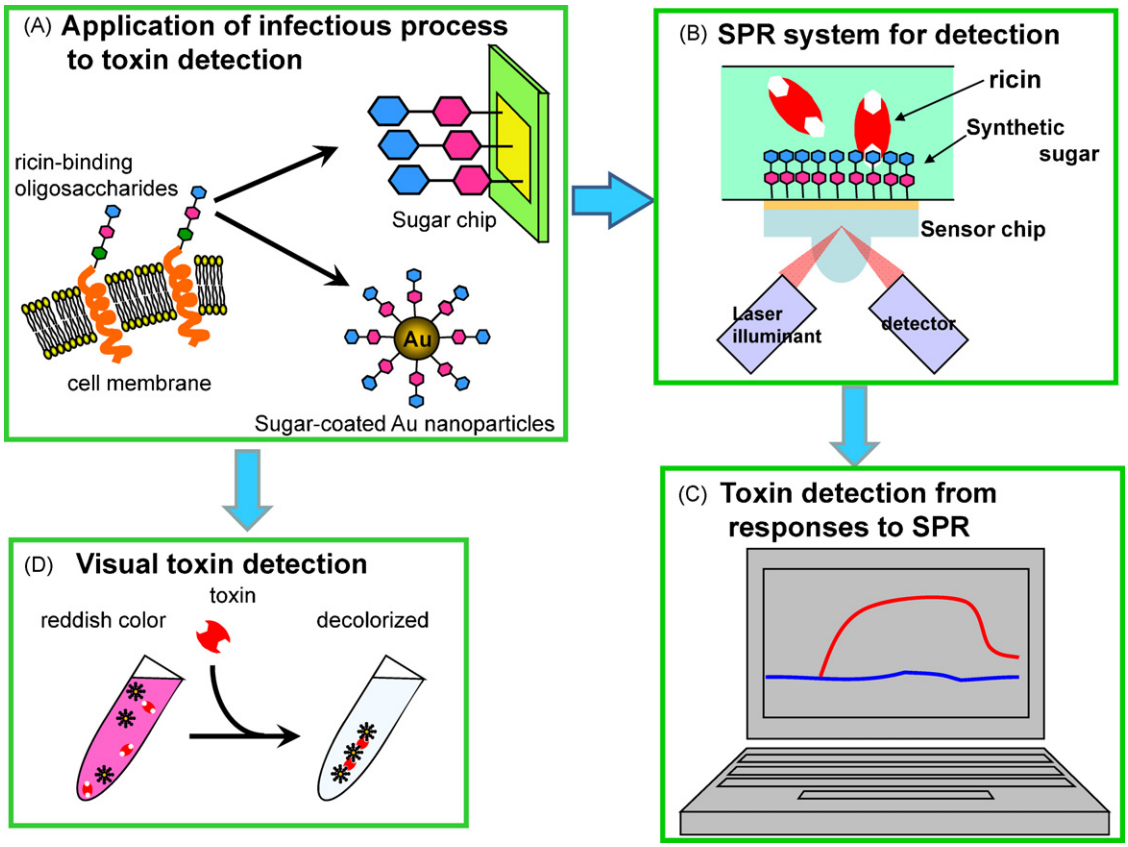


Fig. 1. Application of infection process to ricin detection. Ricin recognizes cell-surface oligosaccharides having β-Gal or β-GalNAc at the non-reducing terminal and internalizes into the host cell. The recognition is species-specific and thus may be applicable to detection. The different sugar-based nano-materials were designed for the present study, i.e., sugar chips for SPR detection and sugar-coated Au nanoparticles for visual detection (A). Ricin adheres specifically to the sugar chip and responds to an SPR system (B and C). The Au nanoparticles coated with sugar are dispersed in water as a colloidal solution, and the colloid color may change in the presence of the toxin (D).

into cells and, therefore, the toxicity is much lower than that of ricin.

In the present study, we apply a naturally occurring infection mechanism to ricin detection, which involves species-specific recognition of the β-Gal binding interaction (Fig. 1A). Namely, ricin adheres to an SPR chip coated with synthetic β-Gal derivatives which serve as the analogue of a natural ligand (Fig. 1B). The interaction can be determined by an SPR system from an increase in the optical property (Fig. 1C) (Phillips and Cheng, 2007). As a simpler on-site detection tool, convenient use of sugar-coated Au nanoparticles is also proposed (Fig. 1D). Common synthetic sugars are employed in the two methods to give a set of complementary approaches to the conventional immunological way (Pilch and Zilinskas, 2005).

2. Materials and methods

2.1. General

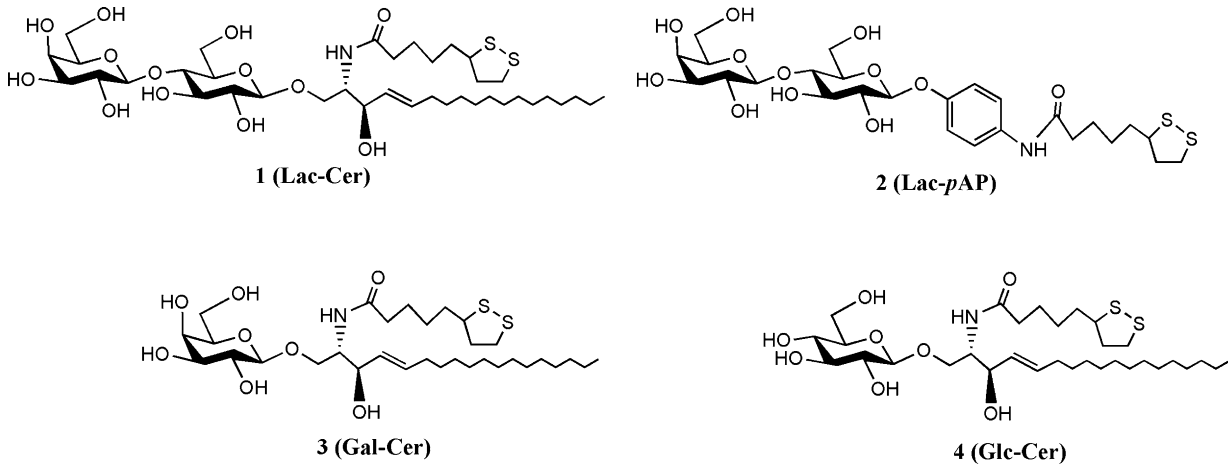
Jack bean (*Canavalia ensiformis*), soybean (*Glycine max*), peanut (*Arachis hypogaea*) lectins, bovine serum albumin and γ-globulin were supplied by Sigma (St. Louis, MO, USA). Osage orange lectin (*Maclura pomifera*) was purchased from Vector laboratories (Burlingame, Canada). *Maackia amurensis* and *Sambucus sieboldiana* lectins were obtained from Seikagaku Corp. (Tokyo, Japan). Sphingolipid ceramide *N*-deacylase (SCDase, E.C. 3.5.1.69) was obtained from TAKARA Bioscience (Shiga, Japan). The Au nanoparticles with diameters of 20 nm (colloidal gold particles; EM.GC15) were from British BioCell International Inc. (Cardiff, UK) or from Sigma Chemicals. The SIA-kit AU was purchased from BIAcore AB (Uppsala, Sweden). All SPR experiments were performed on a BIAcore 2000 or BIAcore T-100 (BIAcore AB, Uppsala, Sweden) and kinetic parameters were calculated with BIAevaluation commercial software (Version 4.1).

2.2. Synthesis of sugar-probes and handling of ricin

Sugar-probes were synthesized using SCDase (Kita et al., 2000), followed by the introduction of a lipoic acid anchor (see Supplementary Scheme S1). Ricin was safely handled as prescribed by Japanese law at National Research Institute of Police Science (NRIPS). For further details, see Supplementary information.

2.3. SPR detection of ricin

The assembled sugar chips were examined in this study. 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 through all the 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 concentration of 10 pg/mL was then injected into the SPR BIAcore T-100 system for 5 min at a flow rate of 10 μL/min. Also, 100 μL of ricin (1 μg/mL) were injected into the SPR BIAcore 2000 system for 10 min at a flow rate of 10 μL/min. All SPR data were analyzed with BIAevaluation software (ver. 4.1).



Scheme 1. Structures of synthetic sugar-probes **1**–**4**. Compounds **1**, **3**, and **4** have a ceramide moiety mimicking natural glycolipids, while compound **2** has an aromatic spacer at the reducing end.

*2.4. Detection of ricin using **1**-coated Au nanoparticles*

To 200 μL of the Au nanoparticle (ca. 20 nm) suspension prepared in a micro-plate well was added 100 μL of ricin at concentrations of 0, 1, 5, 10, 25, and 50 μg/mL in 10 mM HEPES buffer (pH 7.5) containing 0.15 M NaCl at room temperature, and the decolorizing processes were observed at regular intervals (0, 2, 4, 6, 8, 10, and 30 min) (see Supplementary Fig. S2). Similarly, seven plant lectins including RCA_{120}, jack beans, soybeans, peanut agglutinin, Osage orange trees, *Maackia*, and *Salvia*, as well as two reference proteins (bovine serum albumin and γ-globulin) were also examined (see Supplementary Fig. S3). Each protein concentration of 10 μg/mL was used in this study, and the other conditions were the same as described above.

3. Results and discussion

3.1. Design of synthetic sugars and sugar chips for ricin detection

As candidates for optimal β-Gal probes, we prepared β-lactosyl- (**1** and **2**) and β-galactosyl (**3**) derivatives. As a reference, β-glucopyranoside (**4**) was also prepared (Scheme 1). One type (compounds **1**, **3**, and **4**) carries a ceramide mimic at the aglycon. Another type (compound **2**) is a simpler phenyl glycoside derived from *p*-nitrophenyl (*p*NP) glycoside. They have a lipoic amide as the side group, and the disulfide function is usable for immobilization onto Au surfaces by way of a self-assembled monolayer (SAM) technique. The ceramide-type of glycoside was prepared in a chemoenzymatic manner *via* selective de-*N*-acylation (Kita et al., 2000) and regioselective amidation (lipoylation) of natural glycosyl ceramides. Their structures and purities were checked by means of ^{1}H NMR and electrospray ionization (ESI) mass spectroscopy. They were immobilized on Au surfaces *via* the established SAM technique, and the derived sugar chips were analyzed by X-ray photoelectron spectroscopy (XPS) and then analytically evaluated by means of an SPR system.

3.2. Binding affinity and highly sensitive detection of ricin; laboratory-installed SPR analysis

SPR analysis has shown that ricin binds to any sugar chips derived from β-lacto- and β-galactosides (**1**–**3**) although the apparent affinity constants (Ka) are largely different among the sugars. In general, this toxin favors β-lactoside **1** carrying a ceramide mimic rather than **2** having an aromatic group or **3** having a monosaccharide-based β-galactoside. The Ka values of **1** and **3** were estimated to be 1.5×10^9 and 2.3×10^6, respectively, exhibiting a large difference with each other. The affinity of **1** was ca. 88 times stronger than that of **2** (1.7×10^7), even though the sugar moiety was identical. The Ka value of **4** (8.2×10^4) was 18,000 times lower than that of **1**. Ricin obviously may recognize a ceramide moiety in addition to a β-lactoside moiety.

In order to check whether the toxin specifically binds to β-lactoside, an inhibitory experiment was carried out. In the presence of a polyacrylamide-based β-lactoside copolymer (lactose content = 25%), the SPR responses were suppressed in a dose-dependent and competitive way (see Supplementary Fig. S1). These data supported the fact that a sugar chip derived from β-lactoceramide mimic **1** can serve as one of the effective sugar-probes. Owing to the strong sugar affinity of ricin, we are able to detect it at the minimum amount of 10 pg/mL within 5 min (Fig. 2).

3.3. Discriminative affinity between ricin and the agglutinin to sugar-probes

As already mentioned, ricin and the agglutinin have a similar protein sequence and β-Gal recognition of each other. In fact, the present SPR analysis showed that the agglutinin (RCA_{120}) made a similar response to the β-lactoside chip derived from **1**. The two homologues, however, showed a largely different response to the

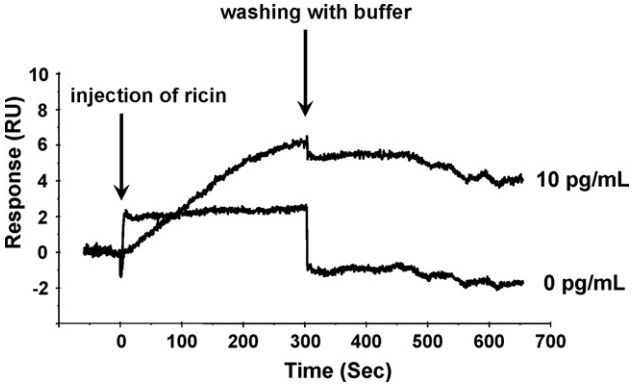


Fig. 2. SPR responses of ricin at different concentrations (0 and 10 pg/mL) to a sugar chip derived from **1**. One resonance unit (1 RU) was calculated as being equivalent to ca. 1 pg of the toxin protein binding on a 1 mm^2 area of a glyco-chip surface.

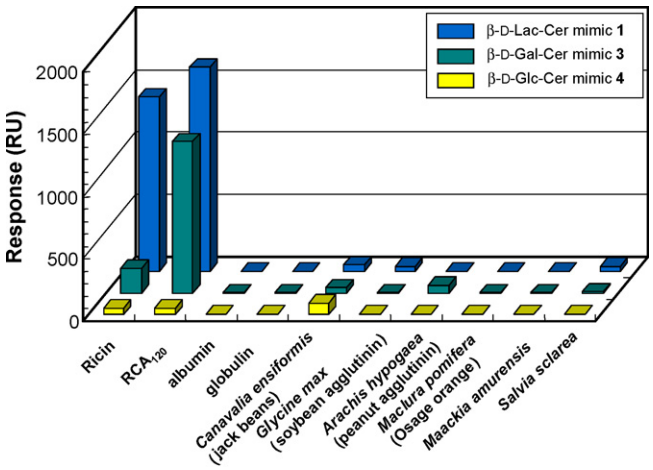


Fig. 3. Discriminative analyses of ricin and different proteins using SPR. Ricin is specific to only the lactosyl chip and the agglutinin has an affinity for the lactosyl- and galactosyl chips, while other proteins respond little to the sugar chips.

sugar chip derived from β-galactoside **3** (Fig. 3). The agglutinin (RCA_{120}) showed high affinity to both **1** and **3**, while ricin showed high affinity only to **1**. The two proteins can be discriminated clearly in this way using the two sugar chips and the SPR system.

Next, we examined the response of other carbohydrate-specific lectins from jack beans (*C. ensiformis*), soybeans (*G. max*), peanut agglutinin (*A. hypogaea*), Osage orange trees (*M. pomifera*), Amur maackia trees (*M. amurensis*), and clary sage (*Salvia sclarea*) as well as two reference proteins (bovine serum albumin and γ-globulin) (Fig. 3). All the proteins examined here showed much poorer binding affinity to the sugar chips, and this result allows us to detect ricin in a highly discriminative manner. Therefore, the present analysis is highly specific to ricin, and is a reliable tool to detect the biological toxin.

3.4. On-site ricin detection applying Au nanoparticles

To develop a simpler and handy analytical tool, we applied Au nanoparticles (de la Fuente et al., 2001; Otsuka et al., 2001; El-Boubbou et al., 2007; Sun et al., 2004). Each of the synthetic sugars (**1**, **3**, and **4**) was introduced onto commercially available Au nanoparticles with diameters of ca. 20 nm in the same way as the preparation of the SPR sugar chips. We have found that the obtained sugar-coated Au nanoparticles became red-colored colloidal suspensions in water, which are stable at ambient temperature for at least 2 years.

Upon addition of ricin into the Au colloidal suspension of **1**, the reddish color changed to purple within 5 min and then almost disappeared in an additional 5 min while depositing the aggregates (Fig. 4). The color change was observed at a toxin concentration of 3.3 μg/mL in 10 min and 1.7 μg/mL in 30 min (see Supplementary Fig. S2). When an excess amount of reducing lactose (ca. 10^6 mol equiv. to ricin) was added to the final mixture, the reddish color was recovered reversibly. Therefore, the observed change is based on the 'toxin-to-sugar' interaction, similar to the case of the SPR response. In this case, the cross-linking property of ricin aggregates the nanoparticles (illustration in Fig. 4c). Owing to this inherent property, we are able to detect the toxin in an amount which is much less than the reported lethal dose level [LD_{50} value = ca. 200 μg/person] (Pilch and Zilinskas, 2005).

Ricin showed little response to Au suspensions derived from **3** and **4** (Fig. 5). The toxin obviously recognizes the disaccharide structure of β-lactoside. On the other hand, RCA_{120} responded to both

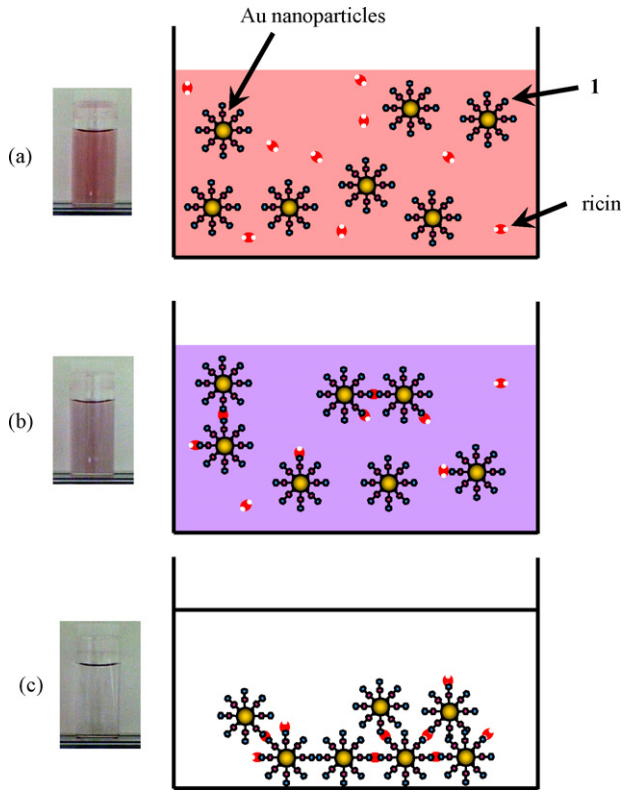


Fig. 4. Color change of the colloidal suspension in the presence of ricin after (a) 1 min, (b) 5 min, (c) 10 min and a schematic illustration of the hetero-aggregation of the nanoparticles due to cross linking. Three hundred microliters of ricin (10 μg/mL) was added to 600 μL of **1**-coated Au nanoparticle suspension in this study. In this illustration, the A-subunit in ricin is neglected for facile understanding of the cross-linking interaction which led to aggregation. The average sizes of the aggregates derived from Au nanoparticles and RCA_{120} were estimated by dynamic light scattering (DLS). The data supported the fact that the agglutinins and the sugar-coated Au nanoparticles cross-linked with each other. This should hold true also in the case of the ricin made of the same B-subunits carrying two carbohydrate-binding sites.

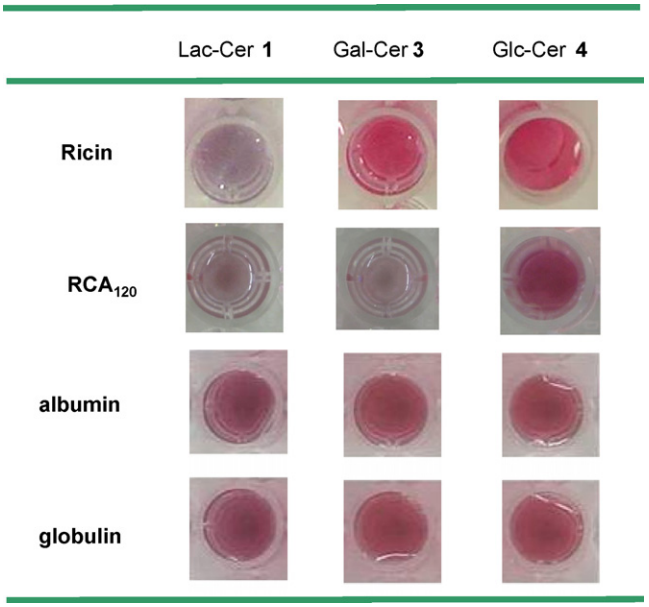


Fig. 5. Facile and discriminative detection of ricin with sugar-coated Au nanoparticles derived from **1**, **3**, and **4**. Hundred microliters of ricin (10 μg/mL) was added to 200 μL of the sugar-coated Au suspension in micro-plate plastic wells.

1 and **3**. To the other proteins, including six plant lectins and two referential proteins (albumin and globulin), none of the three sugars (**1**, **3**, and **4**) responded under standardized assay conditions (see Supplementary Fig. S3). These results completely matched the preceding SPR analyses.

The monitoring of biological toxins such as ricin is definitely required for protection against terrorism in public places, security checks at territorial borders, airports, big event venues, and executive facilities. At scenes of terrorism, detection should be performed on the spot, and then on-site test portions (or samples) should be immediately transported to police institutes for further detailed analysis and emergency lifesaving measures. Therefore, simple, reliable and highly sensitive detection methods are absolutely necessary for such deadly strong toxins.

It is highly important to be equipped with different approaches against the illegal use of the biological toxin. For example, immunochemical ELISA methods (Pilch and Zilinskas, 2005; Taitt et al., 2002; Leith et al., 1998) are available. The detection limit is in a range of 1–10 ng/mL or less (Pilch and Zilinskas, 2005). A handheld SPR biosensor was also developed using an anti-ricin antibody, which detected 200 ng/mL of ricin (Feltis et al., 2008). A mass spectrometric approach based on limited protease-catalyzed hydrolysis is also proposed (Seto and Kanamori-Kataoka, 2005). An analytical approach using naturally occurring oligosaccharides has also appeared. For example, lyposomes including GM1 and GD1b, natural ceramides, are prepared and applied (Gustafson, 2003; Puu, 2001). Our approach is based on non-natural and synthetic sugars applied to either sugar chips for high-sensitive SPR analysis or nano-sized particles for visual detection. The Ka values reach the order of $10^9\ M^{-1}$, showing a level ca. 100–1000 times higher than the case of liposome-based probes. Our present SPR method could detect 10 pg/mL of ricin, and the detection time was 5 min, being the best benchmark as a method for detecting the toxin. In the visual detection, we can detect ricin at amounts of less than 3.3 μg/mL in 10 min or 1.7 μg/mL in 30 min with no need of special equipments.

4. Conclusion

We have developed highly sensitive and facile detection methods for the biological toxin, ricin, by applying sugar-probes. The detection principle is based on the naturally occurring infection mechanism and the strong affinity that the toxin has for sugar. Using the two synthetic sugars, **1** and **3**, ricin can be discriminated from the agglutinin, RCA_{120}, and many other carbohydrate-binding proteins with this simple and handy tool. With the SPR device, we are able to analyze the toxin in a more precise and sensitive way (10 pg/mL, 5 min). With the sugar-coated Au nanoparticles, the toxin is detected in a preliminarily manner at amounts of less than 3.3 μg/mL in 10 min or 1.7 μg/mL in 30 min. The present Au nanoparticle approach is useful for detecting ricin as an on-site and "Yes" or "No" type biosensor in a range from 3.3 to 1.7 μg/mL in a short time, which may avert a lot of bioterrorist threats due to ricin. The proposed methodologies will contribute to a large extent to our safety.

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Appendix A. Supplementary data

Supplementary data about materials, synthesis of sugar-probes **1**–**4**, preparation of sugar chips, XPS analysis, binding specificity, determination of Ka, inhibitory study, preparation of sugar-coated Au nanoparticles, and DLS analysis can be found, in the online version, at doi:10.1016/j.bios.2008.07.049.

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