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Preparation and characterization of a polyclonal antibody from rabbit for detection of trinitrotoluene by a surface plasmon resonance biosensor

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Abstract

A polyclonal antibody against trinitrophenyl (TNP) derivatives was raised in rabbit, and the antibody was applied to detection of trinitrotoluene (TNT) using a surface plasmon resonance (SPR) biosensor. TNP-keyhole limpet hemocyanine (TNP-KLH) conjugate was injected into a rabbit, and a polyclonal anti-TNP antibody was realized after purification of the sera using protein G. Aspects of the anti-TNP antibody against various nitroaromatic compounds, such as cross-reactivities and affinities, were characterized. The temperature dependence of the affinity between the anti-TNP antibody and TNT was also evaluated. The quantification of TNT was based on the principle of indirect competitive immunoassay, in which the immunoreaction between the TNP-β-alanine-ovalbumin (TNP-β-ala-OVA) and anti-TNP antibody was inhibited in the presence of free TNT in solution. TNP-β-ala-OVA was immobilized to the dextran matrix on the Au surface by amine coupling. The addition of a mixture of free TNT to the anti-TNP antibody was found to decrease the incidence angle shift due to the inhibitory effect of TNT. The immunoassay exhibited excellent sensitivity for the detection of TNT in the concentration range of 3×10^{-11} to 3×10^{-7} g/ml. To increase the sensitivity of the sensor, anti-rabbit IgG antibody was used. After flowing the mixture of free TNT and anti-TNP antibody, anti-rabbit IgG antibody was injected, and the incidence angle shift was measured. Amplification of the signal was observed and the detection limit was improved to 1×10^{-11} g/ml.
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Keywords: Trinitrotoluene; Immunoassay; Surface plasmon resonance sensor; Anti-trinitrophenyl-protein antibody; Landmine detection

1. Introduction

There are more than 100 million landmines buried throughout the world, and 3 million new landmines are buried every year. Antipersonnel landmines cause extensive injury to more than 20,000 people a year. However, only 100,000 landmines are removed each year [1].

It is very difficult to detect a buried landmine. Dogs are used as on-site detectors. Dogs, however, need extensive training, and they cannot concentrate on detection for more than two hours [2]. Thus, it is very important to develop a novel technology for detecting landmines effectively. In recent years, various kinds of detection technologies based on physical sensors, such as ground-penetrating radar (GPR) have been developed [3,4]; however, it is difficult to detect landmines using only a physical sensor.

TNT detection methods using fluorescence quenching, capillary electrochromatography and HPLC have been reported [5–7], and detection methods using an antigen–antibody reaction, such as enzyme-linked immunosorbent assay (ELISA) and fluorescent and chemiluminescent immunosensors, are more useful and are able to detect parts per billion (ppb) levels of TNT [8–10]. These methods using antigen–antibody reactions are sensitive, but are they not suitable for on-site detection.

On the other hand, a surface plasmon resonance (SPR) sensor is small and portable, hence suitable for on-site detection. One of the most sensitive and promising sensors for detecting landmines is an immunosensor using a portable SPR sensor [11,12]. It might be possible to achieve highly sensitive on-site detection of TNT and its related compounds using this method. One

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of the key components of an immunosensor is the high affinity antigen–antibody immunoreaction, which in combination with different physical transducers, provides both sensitivity and selectivity.

In recent years, we have focused on the development of highly sensitive SPR sensing methods for the detection of TNT using an antigen–antibody reaction [13–15]. In general, the detection of small molecules by direct SPR detection is difficult because they have insufficient mass to effect a measurable change in the refractive index. Therefore, we proposed SPR immunoassays for the detection of TNT based on the principle of indirect competitive immunoreaction [13–15]. In an indirect competitive immunoreaction, the affinity characteristics among the antigen (analyte), antibody and solid-phase antigen are very important to achieve highly sensitive detection. In a previous paper, we compared the efficiency of the immunoreactions between a commercial antibody (from goat) and a laboratory-prepared antibody (from rabbit) that was raised with differently designed immunogens [15].

In the present study, we investigated the factors of temperature, solid-phase antigens, response time and the regeneration method for the detection of TNT, and also evaluated the affinity constant between the antibody and antigen (analyte) and/or solid-phase antigen. Moreover, we tried to amplify the SPR response by using anti-rabbit IgG.

2. Experimental

2.1. Materials

Alkaline phosphatase (ALP) was obtained from Roche Diagnostics Japan Co. Ltd. (Tokyo, Japan). Bovine serum albumin (BSA), *p*-nitrophenyl phosphate disodium salt (*p*-NPP), 2,4,6-trinitrobenzene sulfonate sodium salt (TNBS), sodium sulfate, *N*-hydroxysuccinimide (NHS) and gelatin were obtained from Nacalai Tesque Inc. (Kyoto, Japan). Ovalbumin (OVA), keyhole limpet hemocyanin (KLH), ALP-labeled anti-rabbit IgG, anti-rabbit IgG, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC), methylamine, ethylamine and butylamine were obtained from Sigma (St. Louis, MO, USA). 2,4,6-Trinitrotoluene (TNT) was supplied by Chugoku Kayaku Co. Ltd. as a 10.7 ppm aqueous solution. 2,4,6-Trinitrophenol (TNP-OH) and 1,3-dinitrobenzene (DNB) were purchased from Kishida Chemical Co. Ltd. (Osaka, Japan). 2,4-Dinitrotoluene (DNT) and *bis*(2-methoxyethyl) ether were obtained from Wako Pure Chemicals Ind. Ltd. (Osaka, Japan). 2-Amino-4,6-dinitrotoluene (2-amino-DNT) and 4-amino-2,6-dinitrotoluene (4-amino-DNT) were purchased from Supelco (PA, USA). 2,4,6-Trinitrophenyl-glycine (TNP-gly) and 2,4,6-trinitrophenyl-β-alanine were obtained from Cosmo Bio Co. Ltd. (Tokyo, Japan). Freund's complete adjuvant was obtained from Difco (Detroit, MI, USA). Rabbits (6 weeks old, female) were purchased from Charles River (Yokohama, Japan). The Hi Trap Protein G column and PD-10 column were purchased from Amersham Bioscience (Piscataway, NJ, USA). All other reagents were of analytical-reagent grade. All buffer solutions were prepared using water purified with a Milli-Q filter (Millipore, Bedford, MA, USA) system.

2.2. Apparatus

SPR measurements were performed using the Biacore J-2000 surface plasmon resonance biosensor (Uppsala, Sweden). The sensor chip used was a CM5 (Biacore). ELISA measurements were performed using 96-well immunoplates (NUNC, No. 446612, Roskilde, Denmark) and a microplate reader (Spectra 1, Wako, Osaka, Japan). Spectrophotometric measurements were performed by using a Shimadzu Multi Spec 1500 (Kyoto, Japan).

2.3. Preparation of TNP-OVA, TNP-glycine-OVA, and TNP-β-alanine-OVA conjugates

TNBS (1 mg/ml H_2O, 1 ml) was reacted with 1 ml of 480 mM $NaHCO_3$ solution (pH 8.5) containing 10 mg OVA for 2 h at 40 °C. After the reaction, the preparation was dialyzed against five changes of H_2O at 4 °C for 3 days, and then lyophilized to produce TNP-OVA conjugate (9.4 mg yield). The molar ratio of the combined TNP moieties to OVA was estimated to be approximately 13.8 mol-TNP/mol-OVA using the molar adsorption coefficient of TNP-NH ($1.1 \times 10^4\ cm^{-1}\ M^{-1}$) [16].

TNP-glycine-OVA and TNP-β-alanine-OVA conjugates were prepared as follows. In 1 ml of *bis*(2-methoxyethyl) ether, 25 mg (88 μmol) of TNP-glycine or 26 mg (91 μmol) of TNP-β-alanine and 13 mg (111 μmol) of NHS were dissolved. Fifty milligrams (352 μmol) of sodium sulfate were added, and the mixture was cooled to 0 °C. To the mixture were added 34 mg (177 μmol) of EDC, and the reaction mixture was allowed to warm to room temperature and stirred overnight. Ten mg of OVA were dissolved in 1 ml of 25 mM borate buffer (pH 8.0). At intervals of 30 min, three portions of the NHS-ester solution (each 62 μl) were added slowly to the reaction mixture under intense stirring. After the reaction, the mixture was dialyzed against three changes of H_2O at 4 °C, and then lyophilized to produce TNP-glycine-OVA conjugate (TNP-gly-OVA) or TNP-β-alanine-OVA (TNP-β-ala-OVA).

2.4. Preparation of TNP-KLH conjugate

TNBS (1 mg/ml H_2O, 1 ml) was reacted with 1 ml of 480 mM $NaHCO_3$ solution (pH 8.5) containing 10 mg KLH for 2 h at 40 °C. After the reaction, the preparation was dialyzed against four changes of H_2O at 4 °C for 2 days, and then lyophilized to produce TNP-KLH conjugate.

2.5. Synthesis of TNP-derivatives

TNP-derivatives were synthesized according to our previous report [17].

2.6. Immunization

A rabbit was immunized with TNP-KLH conjugates according to the following procedure. Conjugates dissolved in PBS (0.3 mg/ml) were emulsified with an equal volume of Freund's complete adjuvant. On days 0, 14, 28, 42 and 56, 2 ml of the

prepared mixture was intracutaneously injected into the rabbit. On days 0, 35, 49 and 63, the rabbit was bled from an ear vein, and the antisera were collected by centrifugation of the blood samples. The antisera were tested by direct enzyme-linked immunosorbent assay (direct-ELISA). Ninety-six-well immunoplates were coated with 100 μl of TNP-KLH conjugate (10 μg/ml in 50 mM carbonate buffer, pH 9.6) overnight at room temperature. The plates were then washed three times with PBS containing 0.05% Tween 20 (PBST) and treated with 150 μl of 1% gelatin for 1 h at room temperature, and again washed three times with PBST and reacted with antisera at eight different dilutions (1/1000–1/128000 in PBS), which were added to the wells (50 μl/well) and incubated for 2 h at room temperature. The plates were washed again three times with PBST, and a solution of ALP-labeled anti-rabbit IgG (2000-fold dilution in PBS) was added (100 μl/well) and incubated for 1 h at room temperature. The plates were washed again, and the substrate solution (2 μg/ml of *p*-NPP in 50 mM carbonate buffer, pH 9.6, containing 1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$) was added and incubated for 30 min at room temperature. Then absorbance at 405 nm was measured.

2.7. Preparation and purification of polyclonal anti-TNP-KLH antibody

Whole blood of the rabbit was collected by cardiocentesis 7 days after the last injection. The purification was performed according to our previous paper [15].

2.8. Indirect competitive ELISAs for TNP-derivatives

ELISAs for TNP-derivatives were performed as follows. Ninety-six-well immunoplates were coated with 100 μl of TNP-OVA conjugate (10 μg/ml in 50 mM carbonate buffer, pH 9.8) overnight at room temperature. The following day, the plates were washed three times with PBST and treated with 150 μl of 1% gelatin for 1 h at room temperature. The plates were washed three times with PBST and reacted with 100 μl of the equivalent mixtures of anti-TNP-KLH antibody (10 μg/ml) and serially diluted TNP-derivative antigen for 1.5 h at room temperature. The plates were washed three times with PBST, and then reacted with 100 μl of ALP-labeled anti-rabbit IgG (2000-fold dilution in PBS) for 1 h at room temperature. After washing three times with PBST, 100 μl of the substrate solution (2 μg/ml of *p*-NPP in 50 mM carbonate buffer, pH 9.8, containing 1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$) was added to each well and incubated for 30 min at room temperature. The absorbance at 405 nm was measured using a microplate reader. Immobilization procedures of TNP-β-ala-OVA and TNP-gly-OVA for the solid phase were almost the same except for the concentration of antigen–protein conjugates (TNP-β-ala-OVA: 50 μg/ml, TNP-gly-OVA: 10 μg/ml).

2.9. Association properties of anti-TNP-KLH antibody to various kinds of nitroaromatic compounds

The association of anti-TNP-KLH antibody to nitroaromatic compounds was investigated by indirect competitive ELISAs. The IC_{50} was defined as the concentration of added TNP derivative that yields 50% inhibition compared with no inhibition (100%). Molar cross-reactivities were related to TNT (100); namely all molar cross-reactivities were determined in relation to the TNT standard inhibition curve. The molar cross-reactivity was calculated using the IC_{50} of each derivative according to the following equation [18]:

$$CR = \left(\frac{IC_{50}^*}{IC_{50}}\right) \times 100$$

where CR is molar cross-reactivity (%), IC_{50}^* is the IC_{50} of the TNT standard (M), and IC_{50} is the IC_{50} of derivatives (M).

2.10. Immobilization of antigen–protein conjugate on chip surface

The CM5 sensor chip consists of a gold-coated glass slide embedded in a plastic support platform. The gold film is covered with a covalently bound dextran (carboxymethylated dextran) layer to which biomolecules can be immobilized. The dextran matrix increases the binding capacity of the surface. In addition, the non-specific binding of proteins to a bare gold surface is minimized. The most generally applicable immobilization strategy is amine coupling, in which the ligand is coupled via a primary amino group. Amine coupling introduces NHS esters into the surface matrix by modification of the carboxymethyl groups with a mixture of NHS and EDC. These esters then react spontaneously with amines and other nucleophilic groups on the ligand to form covalent links [19]. In practice, we injected 190 μl of a mixture of equal amounts of 0.4 M EDC and 0.1 M NHS, and then 180 μl of TNP-β-ala-OVA (0.5 mg/ml in 10 mM acetate buffer, pH 4.5) was injected several times. Non-reactive active esters were deactivated by addition of 0.1 M ethanolamine hydrochloride (190 μl). The flow rate was maintained at 30 μl/min.

2.11. Indirect competitive SPR measurement for TNT

For highly sensitive detection, we used an indirect competition method, which is effective for detecting chemical substances with low molecular weights [13–15]. The refractive index (or resonance angle) of the CM5 sensor chip modified with the TNP-protein conjugate depends on the concentration of free antigen in the mixture of free antigen and antibody, and this small change can be detected using SPR. The schematic diagram of the indirect inhibition SPR measurement is shown in Fig. 1. In this method, TNP-protein conjugates were immobilized on the sensor chip and rinsed with PBS (State A in Fig. 1). Then, the anti-TNP-KLH antibody (appropriate concentration) solution was allowed to flow over the chip for 3 min, and the shift in the incidence angle ($\Delta\theta_0$) caused by the association of the anti-TNP antibodies to the immobilized TNP-protein conjugates was measured (State B). After 3 min, the flow solution was changed to the carrier buffer (PBS), which was allowed to flow for another 2 min, and then the sensor chip was regenerated by sequential injections of 60 μl of 0.5 M NaOH and 30 μl of 10 mM glycine-HCl, pH 1.5 (State C). After the chip was

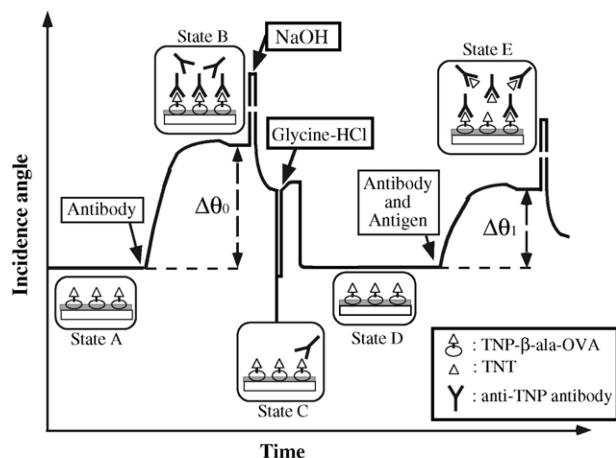


Fig. 1. Response transients of the TNP-β-ala-OVA immobilized sensor to the anti-TNP-KLH antibody without antigen ($\Delta\theta_0$) and with antigen ($\Delta\theta_1$).

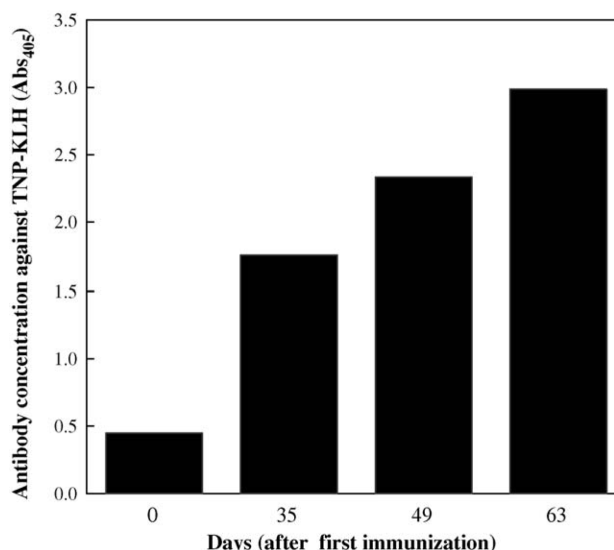


Fig. 2. Time course of concentration of antigen-specific antibody in the sera from a rabbit immunized with TNP-KLH conjugate. The data in the figure are shown using 128,000-fold diluted sera with PBS.

rinsed with the carrier buffer and the signal base line was recovered (State D), 90 μl of an equivalent mixture of anti-TNP-KLH antibody (20 μg/ml) and an appropriate amount of TNP was allowed to flow over the chip to realize a decreased incidence angle shift ($\Delta\theta_1$) with inhibition by TNT (State E). This cycle was performed for the mixture of anti-TNP antibody and serially diluted TNT. Quantitative determination of the antigen concentration can be made from the difference of $\Delta\theta_0$ and $\Delta\theta_1$. The flow rate was constantly maintained at 30 μl/min, and all the procedures were carried out at room temperature.

2.12. Enhancement of signal change using anti-rabbit IgG

In order to enhance the incidence angle shift, anti-rabbit IgG was used. The procedures were almost the same as above, except that after the injection of the mixture of anti-TNP-KLH antibody, analyte and rinse, 90 μl of anti-rabbit IgG (10 μg/ml) was injected. The subsequent procedures were the same.

3. Results and discussion

3.1. Immunization property

The results of the immunization of the rabbit with TNP-KLH conjugate are shown in Fig. 2. The antisera titers of the immunized rabbit increased, especially after booster immunization. On the basis of these results, at day 63 the rabbit was immunized intracutaneously for the last time. A whole blood sample from the rabbit was collected at day 70. The concentration of the anti-TNP-KLH antibody after Protein G treatment was estimate to be about 13 mg/ml of serum, standardized as human IgG.

3.2. Association properties of anti-TNP antibody to various kinds of nitroaromatic compounds

First, the association properties of the raised antibody to nitroaromatic compounds were evaluated by the data of indirect competitive ELISAs. Midpoints (IC_{50}) and molar cross-reactivities are listed in Table 1. As shown in Table 1, dinitroaromatic compounds and *N*-(2,4,6-TNP)-alkylamines (TNP-ba and TNP-ha) showed cross-reactivities of less than 1% or no association, when the cross-reactivity of TNT was set as 100%. Because the anti-TNP-KLH antibody was raised in a rabbit with TNP-KLH conjugate as the immunogen, the formal loss of one nitrogroup effectively results in a decrease in the affinity, and the insertion of an alkyl chain of any length as a spacer would decrease the affinity. However, TNP-OH showed almost no association, and TNP-gly showed a relatively low association with the antibody, having a molar cross-reactivity of 58%. On the other hand, TNP-aha and TNA showed a much higher association with the anti-TNP antibody than with TNT: the molar cross-reactivities were about 14,000 and 1500%, respectively. The cross-reactivity of the antibody with TNP-aha was highest, in accordance with our previous report [17] which used an antibody raised from mouse with TNP-gly-BSA conjugates, whereas there was very little association with TNA in that case. The overall molar cross-reactivities are almost the same as our previous data, which were evaluated from the SPR responses [15]. Affinity–structure relationships between antigen–protein conjugates and raised antibodies are being investigated in our laboratory, but are not yet clarified.

3.3. Temperature dependence of the affinity between anti-TNP-KLH antibody and TNT

Temperature dependencies on the affinity between anti-TNP-KLH antibody and TNT were investigated by using ELISA. Two different conditions were adopted to measure the temperature dependencies; namely, one was 0.3 μg/ml of TNP-OVA immobilized on the surface of the wells and an antibody concentration of 0.03 μg/ml (condition A), and the other was 0.03 μg/ml of TNP-OVA and 0.1 μg/ml antibody (condition B). Competitive reactions among free TNT, bound TNP-OVA and anti-TNP-KLH antibody were performed for 1 h. Higher sensitivities were

Table 1
Association properties of anti-TNP-KLH antibody to nitroaromatic compounds

Nitroaromatic compounds	MW	IC_{50} (M)	Molar cross-reactivity (%)
Trinitrotoluene (TNT)	227	2.6×10^{-8}	100
N-(2,4,6-Trinitrophenyl)-6-aminohexanoic acid (TNP-aha)	342	$<2.9 \times 10^{-9}$	>13911
2,4,6-Trinitroaniline (TNA)	228	1.8×10^{-9}	1470
Trinitrophenol (TNP-OH)	229	3.1×10^{-5}	0.08
N-(2,4,6-Trinitrophenyl)-glycine (TNP-gly)	286	4.5×10^{-8}	58.1
1,3-Dinitrobenzene (1,3-DNB)	168	2.7×10^{-6}	0.97
2,4-Dinitrotoluene (2,4-DNT)	182	5.5×10^{-6}	0.48
2-Amino-4,6-dinitrotoluene (2-ADNT)	197	5.6×10^{-6}	0.47
4-Amino-2,6-dinitrotoluene (4-ADNT)	197	2.3×10^{-5}	0.11
2,6-Dinitrotoluene (2,6-DNT)	182	$>5.5 \times 10^{-5}$	<0.05
N-(2,4,6-Trinitrophenyl)-butylamine (TNP-ba)	284	N.I.[a]	–
N-(2,4,6-Trinitrophenyl)-hexylamine (TNP-ha)	312	N.I.[a]	–

[a] N.I.: not inhibited. Concentration-dependent inhibition was not observed in the range of the analyte concentrations studied (1.0×10^{-10} to 1.0×10^{-5} g/ml).

achieved at low temperature with both conditions. Only the results of condition B are shown in Fig. 3. About 15% inhibition was observed at concentrations as low as 10^{-11} g/ml (10 ppt) at 4 °C. The reasons for these phenomena are proposed to be depression of the non-specific binding of the antibody and the indicator antibody (ALP-labeled antibody) due to the lower temperature. The sensitivity was gradually lowered with increasing temperature, but the antibody was usable up to 50 °C without degradation (data not shown).

3.4. Selection of immobilized antigen–protein

The preferable antigen–protein for the solid phase was investigated by using indirect ELISAs. TNP-OVA, TNP-β-ala-OVA and TNP-gly-OVA conjugates were used. The molar ratios of the combined TNP moieties on OVA were 13.8, 12.1 and 5.1, respectively. The association constants, K_A, between each antigen–protein conjugate for solid phase and anti-TNP-KLH antibody were evaluated by using Stevens' method [20]. The K_A-values were 3.0×10^7 M^{-1} (TNP-OVA) and 5.3×10^6 M^{-1} (TNP-β-ala-OVA), respectively. Association of the anti-TNP-KLH antibody to TNP-gly-OVA (solid phase) was not observed. We selected TNP-β-ala-OVA as a solid phase antigen–protein conjugate considering the moderate affinity of TNP-β-ala-OVA to TNT.

3.5. Detection of TNT by SPR

The TNP-β-ala-OVA immobilized chip was exposed to the flow of anti-TNP-KLH antibody at various concentrations (5–40 μg/ml). The incidence angle shifts were plotted against the concentration of the anti-TNP-KLH antibody, and the plots are shown in Fig. 4. The incidence angle shift increased linearly with increasing concentration of the anti-TNP-KLH antibody up to 20 μg/ml, and then the increase leveled off above this concentration. The anti-TNP-KLH antibody solution (20 μg/ml) was mixed with an equal volume of TNT of various concentrations

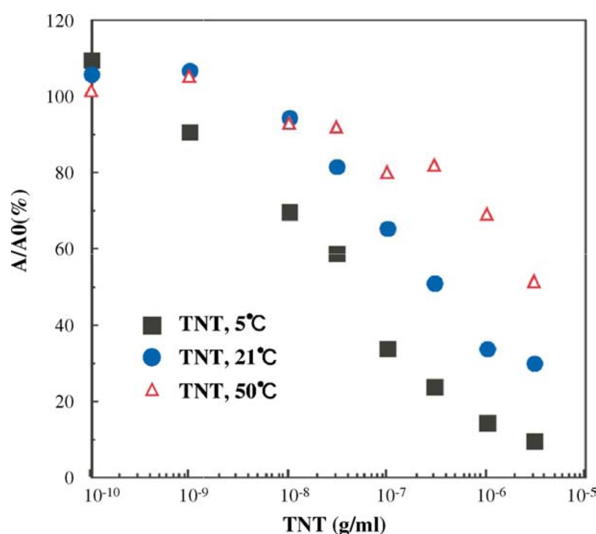


Fig. 3. Dependence on temperature of the affinity between anti-TNP-KLH antibody and TNT measured by indirect ELISAs. Concentrations of TNP-OVA (solid phase) and anti-TNP-KLH antibody were 0.03 μg/ml and 0.1 μg/ml, respectively.

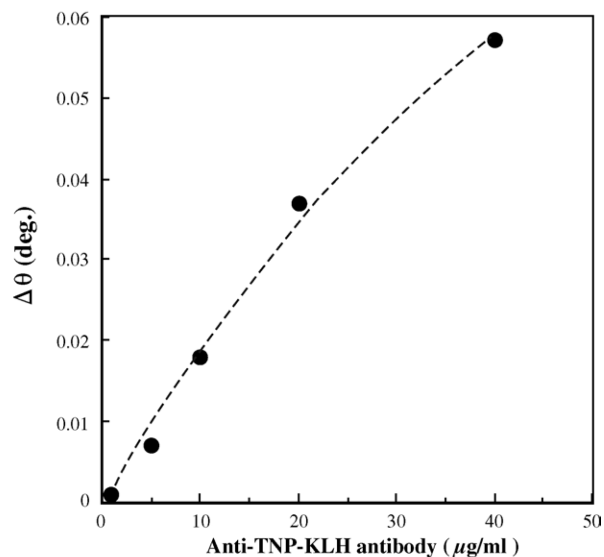


Fig. 4. Dependence of anti-TNP-KLH antibody concentration on the incidence angle shift ($\Delta\theta$) of TNP-β-ala-OVA immobilized sensor. Solid circles show experimental data, and the dashed line shows the theoretical curve using evaluated K_1-value ($K_1 = 1.7 \times 10^6$ M^{-1}).

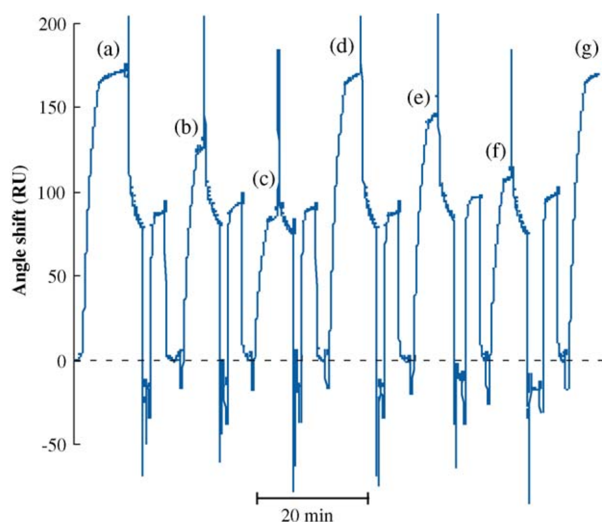


Fig. 5. SPR sensor-gram of a TNP-β-ala-OVA conjugate immobilized on the chip surface to the flow of 10 μg/ml anti-TNP-KLH antibody in the absence and in the presence of TNT solution in a random order of concentration. (a) Anti-TNP-KLH antibody (Ab) alone; (b) Ab + 10^{-10} g/ml TNT; (c) Ab + 10^{-7} g/ml TNT; (d) Ab alone; (e) Ab + 10^{-11} g/ml TNT; (f) Ab + 10^{-9} g/ml TNT; and (g) Ab alone. The carrier solution was PBS, and the flow rate was 30 μl/min.

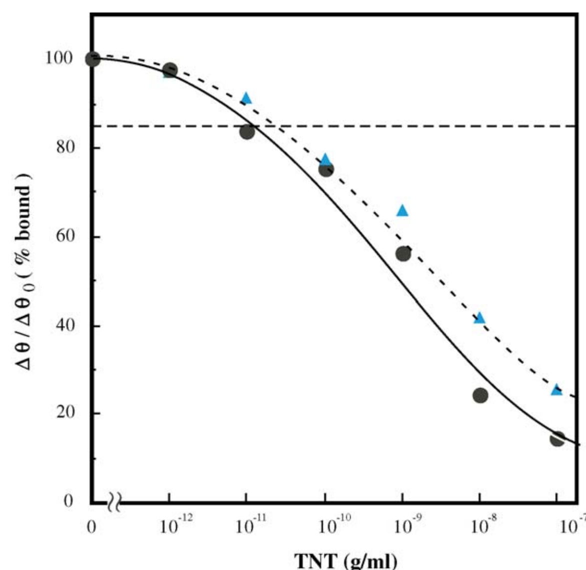
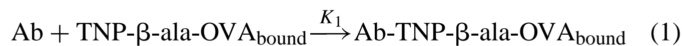


Fig. 6. Comparison of the results of TNT measurements by indirect competitive SPR immunosensor in the presence of anti-rabbit IgG antibody with those in the absence of anti-rabbit IgG antibody. The solid line shows data with anti-rabbit IgG and dashed line shows those without anti-rabbit IgG antibody.

and was incubated for 30 min at room temperature prior to flowing it over the TNP-β-ala-OVA-immobilized sensor chip. The final concentration of anti-TNP-KLH antibody was 10 μg/ml. After incubation, the anti-TNP-KLH antibody solutions containing TNT were allowed to flow over the sensor surface in a random order. Fig. 5 represents the sensor-gram observed for the immunoreaction of TNP-β-ala-OVA conjugate with anti-TNP-KLH antibody in the absence and presence of TNT. In the figure, curve (a) corresponds to the immunoreaction in the absence of TNT. Curves (b), (c), (e) and (f) correspond to the immunoreaction in the presence of 10^{-10}, 10^{-7}, 10^{-11} and 10^{-9} g/ml TNT, respectively. From the figure, it can be observed that the incidence angle shift for the immunoreaction was decreased in the presence of TNT. Moreover, the decrease in incidence angle shift is larger at a higher concentration of TNT. This indicates the inhibition of the immunoreaction in the presence of TNT. Curves (d) and (g) correspond to the immunoreaction in the absence of TNT, which showed almost similar immunoresponse to that of the curve (a), indicating good reproducibility of the immunoresponse. The above results clearly indicate that the decrease in the incidence angle shift in the presence of TNT is due to inhibition. The inhibition curves expressed as $100 \times (\Delta\theta/\Delta\theta_0)$, where $\Delta\theta$ and $\Delta\theta_0$ are the incidence angle shifts observed with TNT standard and blank samples, respectively, are shown in Fig. 6 (dashed line). The R.S.Ds. ($n = 3$) of each point were less than 4%. The lowest limit is defined as the concentration where the percent inhibition is increased by 15%, which is approximately three S.Ds., from the mean incidence angle shift observed in the absence of TNT. The lowest detection limit for TNT was approximately 3×10^{-11} g/ml (30 ppt). Experiments on the reproducibility of the immunoresponse indicate that the immunosystem exhibited good binding and regeneration characteristics, but that the response signal for repeated immunoreaction had decreased by about 10% at the end of 20 cycles. Though the antibody could be regenerated a sufficient number of times, disposable sensor chips are desirable to detect TNT from landmines, which are buried in soil.

3.6. Evaluation of affinity constants

Determination of an affinity constant, K_A, is frequently useful in the study of antigen–antibody interactions. We attempted to evaluate the affinity constant of the anti-TNP-KLH antibody to immobilized TNP-β-ala-OVA using SPR data. The analyses were performed according to the method reported by Sakai et al. [21,22]. They evaluated affinity constants relevant to an immunoassay system by assuming a Langmuir-type adsorption model for the immunoreaction. The present system involves two competitive immunoreactions as follows:



$$\mathrm{Ab} + \text{TNP-}\beta\text{-ala-OVA}_{\mathrm{bound}} \xrightarrow{K_1} \text{Ab-TNP-}\beta\text{-ala-OVA}_{\mathrm{bound}} \quad (1)$$

$$\mathrm{Ab} + \mathrm{TNT} \xrightarrow{K_2} \text{Ab-TNT} \quad (2)$$

Here, Ab indicates anti-TNP-KLH antibody, and K_1 and K_2 are affinity constants. Three assumptions are required to correlate the incidence angle shift with each immunoreaction. First, the antigen–antibody reaction proceeds by monovalency. Second, a Langmuir-type adsorption model can be adopted. Third, the incidence angle shift is proportional to the coverage of adsorption sites (immobilized TNP-β-ala-OVA) by the anti-TNP-KLH antibody. Based on these assumptions, we obtained the following equation for the first immunoreaction:

$$\frac{[\mathrm{Ab}]}{\Delta\theta_0} = \frac{[\mathrm{Ab}]}{\Delta\theta_{0,\max}} + \frac{1}{\Delta\theta_{0,\max} K_1} \quad (3)$$

Here, [Ab] is the molar concentration of the anti-TNP-KLH antibody, $\Delta\theta_0$ is the equilibrium incidence angle shift for a given concentration of antibody and $\Delta\theta_{0,\max}$ is the maximum

incidence angle shift at full coverage. Assuming the latter immunoreaction to also be in equilibrium, Eq. (4) can be derived:

$$\frac{1}{\Delta\theta} = \frac{1}{\Delta\theta_0} + \frac{K_2[\text{TNP}]}{\Delta\theta_{0,\max} K_1[\text{Ab}]_0} \quad (4)$$

Here, $\Delta\theta$ and $\Delta\theta_0$ are the angle shifts in the presence and absence of TNT, respectively. $[\text{Ab}]_0$ is the initial molar concentration of anti-TNP-KLH antibody.

From Eq. (3), $[\text{Ab}]/\Delta\theta_0$ should be linear to [Ab], the slope and intercept giving the values of $\Delta\theta_{0,\max}$ and K_1, respectively. The data points in Fig. 4 are replotted in this way, and a linear correlation (correlation coefficient, $r = 0.915$) was obtained, giving $\Delta\theta_{0,\max} = 0.188^\circ$ and $K_1 = 1.7 \times 10^6\ \text{M}^{-1}$. From Eq. (4), the inverse $\Delta\theta$ should be linear to [TNT], K_2 being obtained from the slope and the known values of $\Delta\theta_{0,\max}$, K_1 and $[\text{Ab}]_0$. The data points of the SPR method using 10 μg/ml of antibody were replotted, and the linear correlation obtained gave $K_2 = 6.5 \times 10^9\ \text{M}^{-1}$. The value of K_1 ($1.7 \times 10^6\ \text{M}^{-1}$) corresponds to the K_A-value ($5.3 \times 10^6\ \text{M}^{-1}$) evaluated from indirect ELISA, and both values showed relatively good coincidence. The evaluation of the K_2-value by indirect ELISA is basically unavailable.

3.7. Enhancement of signal change using anti-rabbit IgG

Since the SPR signal reflects to the mass on the chip surface, we tried to enhance the incidence angle shift by using anti-rabbit IgG. The incidence angle shift was increased by about 40% when anti-rabbit IgG was added, as shown in Fig. 7, which is a sensor-gram of the enhancement with anti-rabbit IgG in the absence of TNT. Fig. 6 (solid line) shows the inhibition curves express as $100 \times (\Delta\theta/\Delta\theta_0)$, indicating a shift to the lower concentration

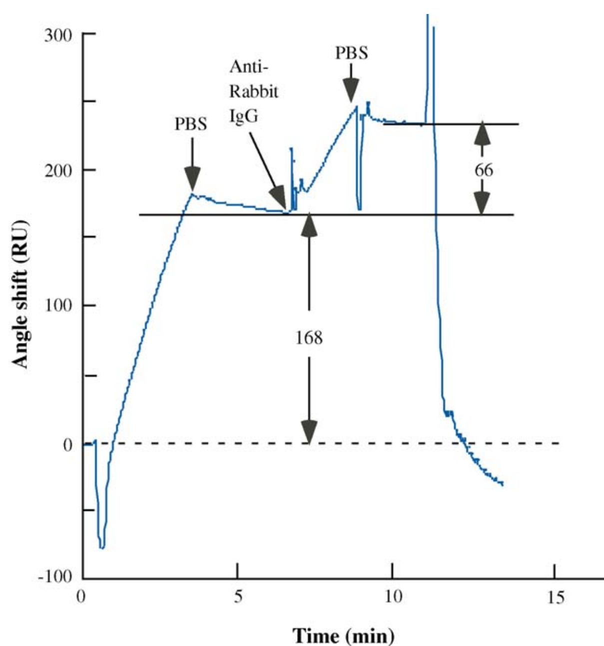


Fig. 7. SPR sensor-gram showing enhancement of the resonance signal due to the addition of anti-rabbit IgG. The concentrations of anti-TNP-KLH antibody and anti-rabbit IgG were both 10 μg/ml.

side. The lower detection limit for TNT shifted to 1×10^{-11} g/ml (10 ppt). The reason for this shift to a lower concentration may be caused by the addition of anti-rabbit IgG (MW ca. 150000), which expands the dynamic range of the SPR signal.

It is estimated that the TNT vapor concentration from soil residues is about 0.1 ppt [23], and the vapors must be concentrated 100 times to detect TNT contained in the vapors above landmines. Vapor-concentrating devices are now under construction.

The anti-TNP-KLH antibody used here is a polyclonal antibody and may contain several kinds of IgG. This was the reason for the very slow inhibition curve in SPR measurement. The preparation of a monoclonal antibody is now in progress. For the purpose of searching for explosives, however, polyclonal antibodies are useful in inhibition occurring in low concentrations. We are now studying approaches using both monoclonal and polyclonal antibodies.

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