Talanta 79 (2009) 1142–1148

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Development of a sensitive surface plasmon resonance immunosensor for detection of 2,4-dinitrotoluene with a novel oligo (ethylene glycol)-based sensor surface

Kazutaka Nagatomo [a], Toshikazu Kawaguchi [b], Norio Miura [b], Kiyoshi Toko [c], Kiyoshi Matsumoto [d,*]

[a] Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka-shi, Fukuoka 812-8581, Japan
[b] Art, Science and Technology Centre for Cooperative Research, Kyushu University, 6-1 Kasuga-kouen, Kasuga-shi, Fukuoka 816-8580, Japan
[c] Faculty of Information Science and Electrical Engineering, Kyushu University, 744 Motooka, Nishi-ku, Fukuoka-shi, Fukuoka 819-0395, Japan
[d] Faculty of Agriculture, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka-shi, Fukuoka 812-8581, Japan

ARTICLE INFO

Article history:
Available online 20 February 2009

Keywords:
2,4-Dinitrotoluene
Immunoassay
Surface plasmon resonance sensor
Hapten heterology
Oligo (ethylene glycol)
Self-assembled monolayer

ABSTRACT

A surface plasmon resonance (SPR) immunosensor for detection of 2,4-dinitrotoluene (2,4-DNT), which is a signature compound of 2,4,6-trinitrotoluene-related explosives, was developed by using a novel oligo (ethylene glycol) (OEG)-based sensor surface. A rabbit polyclonal antibody against 2,4-DNT (anti-DNPh-KLH-400 antibody) was prepared, and the avidity for 2,4-DNT and recognition capability were investigated by indirect competitive ELISA. The sensor surface was fabricated by immobilizing a 2,4-DNT analog onto an OEG-based self-assembled monolayer formed on a gold surface via an OEG linker. The fabricated surface was characterized by Fourier-transform infrared-refractive absorption spectrometry (FTIR-RAS). The immunosensing of 2,4-DNT is based on the indirect competitive principle, in which the immunoreaction between the anti-DNPh-KLH-400 antibody and 2,4-DNT on the sensor surface was inhibited in the presence of free 2,4-DNT in solution. The limit of detection for the immunosensor, calculated as three times the standard deviation of a blank value, was 20 pg mL^{-1}, and the linear dynamic range was found to be between 1 and 100 ng mL^{-1}. Additionally, the fabricated OEG-based surface effectively prevented non-specific adsorption of proteins, and the specific response to anti-DNPh-KLH-400 antibody was maintained for more than 30 measurement cycles.

1. Introduction

Recent concern with global terrorism and weapons of mass destruction strongly demands the development of novel analytical methods for identification and quantification of explosives. Among them, 2,4,6-trinitrotoluene (TNT) is one of the most important explosives and its detection has high priority in many fields including public security and health, environmental toxicology, landmine search, and anti-terrorism activity [1–3]. Effective detection of TNT-containing explosives can be performed by measurement of not only TNT itself, but also 2,4-dinitrotoluene (2,4-DNT) because 2,4-DNT is present in military-grade TNT as a decomposition compound and major impurity and has a higher vapor pressure and environmental stability than TNT [4,5]. These properties give 2,4-DNT a characteristic "chemical signature" and therefore, various sensors have been developed for the detection of 2,4-DNT based on different principles [6–8].

Explosive-related compounds have been measured by using GC–MS spectrometry, HPLC, and other instrumental analytical devices [9,10]. These instrumental analyses, however, require tedious pretreatments such as extraction. On the other hand, immunodetection techniques such as the enzyme-linked immunosorbent assay (ELISA) and fluorescent and chemiluminescent immunosensors, are more useful because they allow us to detect low- or sub-parts per billion (ppb) levels of explosives [11,12].

In recent years, the surface plasmon resonance (SPR)-based immunosensor has been recognized as a useful tool in environmental and biomedical analysis because it allows label-free monitoring of biomolecular interactions [13,14] and also can be improved for multichannel measurements and downsized for easy portability [15,16]. One of the key components of an immunosensor is the strong and specific immunoreaction, which when combined with different physical transducers, provides both sensitivity and selectivity. Another key is the fabrication of the sensor surface on gold, which greatly affects the sensitivity of the analytical methods. A number of immobilization methods have been used for surface fabrication, including physical adsorption [17], Langmuir–Blodgett desorption [18], and self-assembly [19], embedding in polymer or

* Corresponding author. Tel.: +81 92 642 3011; fax: +81 92 642 3011.
E-mail address: kmatsu@agr.kyushu-u.ac.jp (K. Matsumoto).

0039-9140/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2009.02.018

membranes [20], each having advantages and limitations. Recently, oligo (ethylene glycol) (OEG)-based self-assembled monolayers (SAMs) have received considerable attention for stable immobilization of a variety of receptor molecules with good control over the size and orientation and without the problem of non-specific adsorption [21,22].

In the present work, we demonstrate the development of a high-performance SPR immunosensor for detection of 2,4-DNT, a possible chemical signature for explosives. We prepared polyclonal antibody with a high affinity for 2,4-DNT and the novel OEG-based high-performance sensor surface. They were applied for the SPR detection of 2,4-DNT based on the indirect competitive principle. This detection principle is suitable for highly sensitive detection of low-molecular weight analytes [23,24].

2. Experimental

2.1. Materials

2-Aminoethanol, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC), *p*-nitrophenyl phosphate disodium salt (*p*-NPP), *N*-hydroxysuccinimide (NHS), and gelatin were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). 2,4-DNT was obtained from TCI (Tokyo, Japan). Bovine serum albumin (BSA), rabbit immunoglobulin G (rabbit IgG), *N*-(2,4-dinitrophenyl) glycine (2,4-DNPh-Gly), 2,4-dinitrobenzoic acid (DNBA), 2,4-dinitrobenzenesulfonate sodium salt (2,4-DNBS), ovalbumin (OVA), and alkaline phosphatase (ALP)-labeled anti-rabbit IgG were obtained from Sigma (St. Louis, MO, USA). 1,3-Dinitrobenzene (1,3-DNB) was purchased from Kishida Chemical Co., Ltd. (Osaka, Japan). *N*-(2,4,6-trinitrophenyl) glycine (TNP-Gly) was obtained from Funakoshi Co. (Tokyo, Japan). 1,2-Diaminoethane and 2,6-dinitrotoluene (2,6-DNT) was purchased from Wako (Osaka, Japan). TNT, 2-amino-4,6-dinitrotoluene (2ADNT) and 4-amino-2,6-dinitrotoluene (4ADNT) were purchased from Supelco (Bellefonte, PA, USA). DNPh-KLH conjugate was purchased from Merck (Dalmstadt, Germany) as DNPh-KLH-400, which was guaranteed to have a hapten concentration higher than 400 mol/mol KLH. Freund's complete adjuvant was obtained from Difco (Detroit, MI, USA). A rabbit (6 weeks old, female) was purchased from Kyoyu (Fukushima, Japan). The Hi-Trap protein G column was purchased from GE Healthcare (Buckinghamshire, UK). As reagents for the gold surface modification, HS-C_{11}-EG_6-COOH [$HS(CH_2)_{11}(OCH_2CH_2)_6OCH_2COOH$] was obtained from SensoPath Technologies (Bozeman, MT, USA), and H_2N-EG_{12}-COOH [$H_2N(CH_2CH_2O)_{12}(CH_2)_2COOH$] was purchased from Quanta BioDesign (Powell, OH, 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).

2.2. Apparatus

ELISA measurements were performed using 96-well immunoplates (Nunc, No. 446612, Roskilde, Denmark) and a microplate reader (Wallac 1420, PerkinElmer Life Science Japan, Tokyo, Japan). SPR measurements were performed using the BIAcore J surface plasmon resonance biosensor (Uppsala, Sweden). The sensor chip used was an SIA Kit Au (GE Healthcare). Fourier-transform infrared-refractive absorption spectrometry (FTIR-RAS) was performed using a FT/IR-6300 spectrometer (JASCO, Tokyo, Japan) with RAS (PRO410H, JASCO).

2.3. Preparation of coating antigen–protein conjugates

DNPh–OVA: Ten milligrams of OVA was dissolved in 1 mL of 4% $NaHCO_3$ and 1 mL of 2,4-DNBS solution (16 mg mL^{-1}) was added to the solution and stirred over night at 40 °C. The reaction mixture was dialyzed against distilled water for 10 h at room temperature (RT) and lyophilized to produce DNPh–OVA conjugate.

DNBA–OVA: Twenty-five milligrams of DNBA and 13 mg of NHS were dissolved in 1 mL of *N*,*N*-dimethylformamide. Thirty milligrams of sodium sulfate was added, and the mixture was cooled to 0 °C. Thirty-four milligrams of EDC was added to the reaction mixture and stirred over night to produce an NHS–DNBA solution. Twenty milligrams of OVA was dissolved in 2 mL of 25 mM borate buffer (pH 8.0, 1 mL). At intervals of 30 min, three aliquots of the NHS–DNBA solution (each 20 μL) were added slowly to the OVA solution during intense stirring. After reacting for 3 h, the mixture was dialyzed against distilled water for 10 h at RT and lyophilized to produce DNBA–OVA conjugate.

2.4. Immunization

A rabbit was immunized with DNPh-KLH conjugate according to the following procedure. DNPh-KLH-400 conjugate dissolved in PBS (0.6 mg mL^{-1}) was emulsified with an equal volume of Freund's complete adjuvant. On days 0, 14, 28, 42, and 56, 1 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 antiserum was collected by centrifugation of the blood sample. The antiserum was tested by direct-ELISA. Ninety-six-well immunoplates were coated with 100 μL of DNPh-KLH-400 conjugate (10 μg mL^{-1} in PBS) over night at RT. The plates were then washed three times with PBS containing 0.05% Tween 20 (PBST), treated with 150 μL of 1% gelatin for 1 h at RT, again washed three times with PBST, and reacted with antiserum at eight different dilutions (1:1000 to 1:128,000 in PBS), which were added to the wells (50 μL to each well), and incubated for 1 h at RT. The plates were washed again three times with PBST, and a solution of alkaline phosphatase-labeled anti-rabbit IgG (2000-fold dilution in PBS) was added (100 μL to each well) and incubated for 1 h at RT. The plates were washed again, and the substrate solution (2 mg mL^{-1} 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 RT. Then absorbance was measured at 405 nm.

2.5. Preparation and purification of polyclonal anti-DNPh-KLH-400 antibody

Whole blood of the rabbit was collected by cardiocentesis 7 days after the last injection. The purification was performed using a protein G immobilized affinity column according to our previous paper [25] with a minor modification.

2.6. Indirect competitive ELISA (ic-ELISA) for 2,4-DNT

Ic-ELISAs for 2,4-DNT were performed as follows. Ninety-six-well immunoplates were coated with 100 μL of DNPh–OVA or DNBA–OVA conjugate (0.025 μg mL^{-1} in PBS) over night at RT. The following day, the plates were washed three times with PBST and treated with 150 μL of 1% gelatin for 1 h at RT. The plates were washed three times with PBST, reacted with 100 μL of the equivalent mixtures of anti-DNPh-KLH-400 antibody (0.2 μg mL^{-1} in PBS), and serially diluted 2,4-DNT for 1.5 h at RT. 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 45 min at RT. After washing three times with PBST, 100 μL of the substrate solution (2 mg mL^{-1} 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 RT. The absorbance at 405 nm was measured using a microplate reader. The assays were performed in triplicate, and the experimental wells were compared with wells in which the primary antibody had been omitted.

2.7. Avidity of anti-DNPh-KLH-400 antibody to various kinds of nitroaromatic compounds

The avidity of anti-DNPh-KLH antibody to nitroaromatic compounds was investigated by ic-ELISAs using the DNPh–OVA conjugate as a coating antigen–protein conjugate. The ELISA procedures were the same as described in Section 2.6. The IC_{50} was defined as the concentration of added DNPh derivative that yields 50% inhibition compared with no inhibition (100%). Molar cross-reactivities were related to 2,4-DNT (100); namely, all molar cross-reactivities were determined in relation to the 2,4-DNT standard inhibition curve. The molar cross-reactivity of each derivative was calculated according to Weiler's equation [26]:

$$CR = \frac{IC^*_{50}}{IC_{50}} \times 100$$

where CR is molar cross-reactivity (%), IC^*_{50} is the IC_{50} of the 2,4-DNT standard (M), and IC_{50} is the IC_{50} of derivatives or related compounds (M).

2.8. Sensor chip fabrication

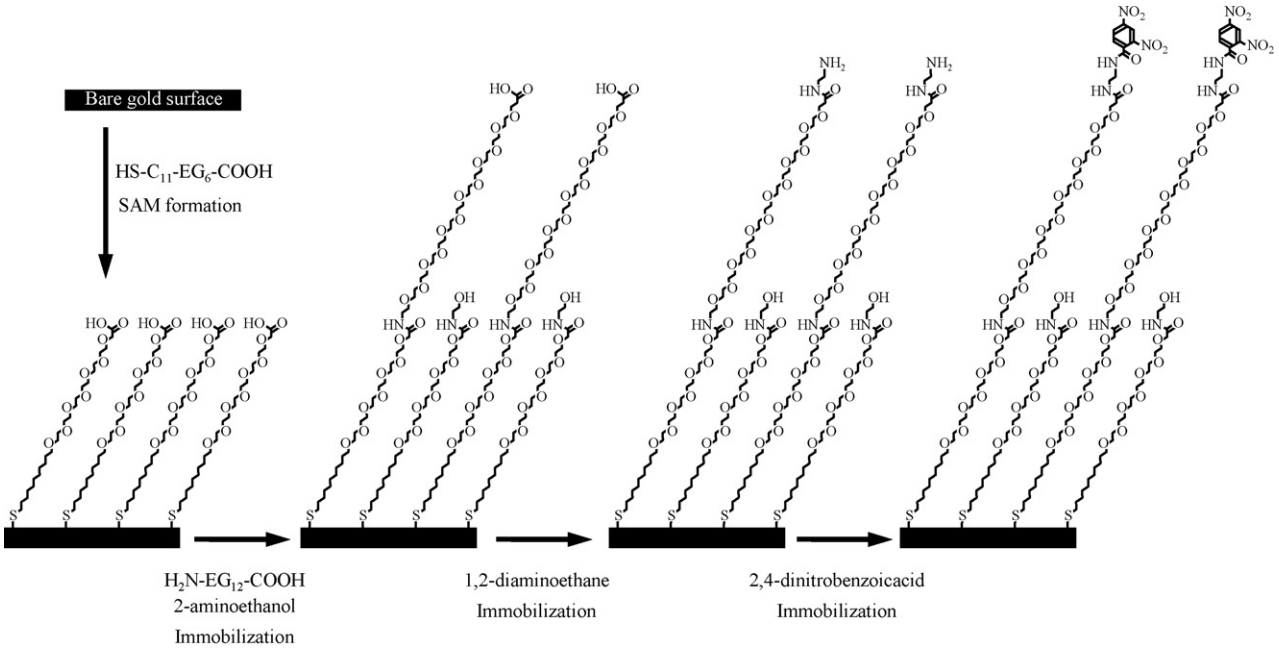
In present study, the novel sensor chip for detection of 2,4-DNT was fabricated by modifying the surface of a SAM formed on a bare gold chip. The fabrication process is illustrated in Scheme 1. Prior to the formation of the SAM with HS-C_{11}-EG_6-COOH, a gold chip was first cleaned in acetone with sonication for 10 min, ethanol for 5 min, and 2-propanol for 2 min each in sequence. Then, the chip was treated with a freshly prepared piranha solution (98% H_2SO_4 and 30% H_2O_2, 7:3 by volume) for 15 min at 60 °C and rinsed thoroughly with Milli-Q water until neutralized. The clean gold chip was immediately immersed in a HS-C_{11}-EG_6-COOH solution (2 mM in ethanol, pH 2.0) for about 24 h to form OEG-SAM on the gold surface. The chip was then successively rinsed with ethanol and dried with nitrogen gas.

For modification of the SAM surface, an amine-coupling procedure was employed. The carboxyl group in HS-C_{11}-EG_6-COOH on the surface was activated by submerging the chip into a equivalent mixture solution of 0.4 M EDC and 0.1 M NHS in Milli-Q water (NHS/EDC solution) for 2 h at RT. Immobilization was then conducted by immersing the NHS-activated chip in a mixture (1:9 volume ratio) of 10 mM H_2N-EG_{12}-COOH in 25 mM borate buffer (pH 8.5) and 10 mM 2-aminoethanol in 25 mM borate buffer (pH 8.5) for 1 h. After the surface was rinsed with Milli-Q water, the chip was soaked in a 1 M 2-aminoethanol–HCl solution (pH 8.5) for 10 min to block any NHS-ester that remained on the surface. Then, the chip was mounted onto the BIAcore J system. The flow rate of solutions was maintained at 30 μL min^{-1}, and the working temperature was kept at 25 °C. This system allows two flow cells to be measured simultaneously. First, the NHS/EDC solution was flowed over both of the flow cells for 20 min. For flow cell 1 (the measurement channel), 0.1 M 1,2-diaminoethane in borate buffer (pH 8.5) was injected for 20 min over the surface in order to convert the terminal COOH group of immobilized H_2N-EG_{12}-COOH into an NH_2 group for termination by DNBA, the 2,4-DNT analog. The surface of flow cell 2 was treated with 0.1 M 2-aminoethanol for 20 min, and the terminal carboxyl group was converted into an OH group that has no reactivity to NHS-activated DNBA. Therefore, flow cell 2 can be used as a reference channel. Then, the sensor chip was undocked, and NHS–DNBA solution prepared as described in Section 2.3 (25-fold diluted solution with 25 mM borate buffer, pH 8.5) was spotted onto the surface of the chip. The reaction was allowed to proceed for 3 h, rinsed with Milli-Q water, and then 1 M 2-aminoethanol in 25 mM borate buffer (pH 8.5) was spotted onto the surface. Finally, the chip was rinsed thoroughly with Milli-Q water and remounted on the BIAcore J system for SPR measurements.

The sensor surface for FTIR-RAS measurement was fabricated by a slightly modified procedure. Namely, a surface immobilized with H_2N-EG_{12}-COOH and 2-aminoethanol was immersed in NHS/EDC solution and 0.1 M 1,2-diaminoethane in turns. Therefore, a uniform surface is produced after the termination with DNBA and is hence appropriate for RA spectrometry.

2.9. Surface characterization by FTIR-RAS

The surface of the fabricated OEG-based sensor chip was evaluated using FTIR-RAS, with a liquid nitrogen-cooled mercury–cadmium–telluride detector. The incident angle of



Scheme 1. Schematic illustration of the sensor chip fabrication.

the infrared beam was 82°, and the spectrum was recorded with an average of 1000 scans with a 4 cm^{-1} resolution. Here, a bare gold surface was used to obtain the reference spectrum.

2.10. Binding of anti-DNPh-KLH-400 antibody to the fabricated sensor surface and utility of the sensor surface

The prepared sensor surface was evaluated in terms of reusability and repulsion of non-specific adsorption. The reusability was studied by repeating sequential injections of 10 μg mL^{-1} anti-DNPh-KLH-400 antibody and 5 mM NaOH for 30 cycles. Non-specific adsorptions by proteins were investigated using BSA and rabbit IgG. Each protein was diluted with PBS and injected into the SPR sensor.

2.11. Indirect competitive SPR measurement for 2,4-DNT

For highly sensitive detection for 2,4-DNT, we employed the indirect competitive principle effective for detecting low-molecular weight compounds. The schematic diagram of the indirect inhibition SPR measurement is shown in Fig. 1. When the sensor chip is exposed to an anti-DNPh-KLH-400 antibody solution alone (no 2,4-DNT in the measurement sample), the resonance angle shift will increase rapidly and strongly, whereas when a measurement sample containing 2,4-DNT was injected, 2,4-DNT in the solution inhibits binding of anti-DNPh-KLH-400 antibody to the sensor surface and consequently the SPR angle shift will decrease.

The assay was performed as follows. A 2,4-DNT solution with a concentration in the range 0.02–2000 ng mL^{-1} was mixed with an equal volume of anti-DNPh-KLH-400 antibody solution (20 μg mL^{-1} in PBS) and incubated for 20 min at RT. Then the mixture was flowed over the sensor surface for 3 min, and the binding between the anti-DNPh-KLH-400 antibody and the immobilized 2,4-DNT analog was monitored in real time. After a measurement, the sensor chip was regenerated by injecting 5 mM NaOH for 1 min. The flow rate of the sensor was maintained at 30 μL min^{-1}, and all assays in the SPR system were carried out at 25 °C. Each measurement was recorded with the background response by injecting the mixture through a reference flow cell (flow cell 2), that had no 2,4-DNT analog on the sensor surface.

2.12. Data analysis

Experimental values obtained from indirect competitive assays were converted into inhibition values ($\%B/B_0$) by using following equation:

$$\frac{B}{B_0}(\%) = 100 \times \frac{B}{B_0}$$

In ic-ELISA, B is the absorbance value for each standard and B_0 is the absorbance value resulting from a zero dose standard (blank value). In SPR measurement, the resonance angle shift corresponds to absorbance values for ic-ELISA.

Curve fitting of standard curves was performed by use of following equation [27]:

$$Y = \frac{100}{1 + (x/c)^b}$$

where c is the midpoint, b is the slope of the curve, and x is the standard concentration.

The limit of detection (LOD) was calculated as three times the standard deviation of a blank value, following IUPAC rules.

3. Results and discussion

3.1. Association properties of anti-DNPh-KLH-400 antibody

First, the association properties of the antibody raised against nitroaromatic compounds were evaluated by the ic-ELISA data. In this experiment, DNPh–OVA was used as a coating antigen–protein conjugate because the use of immunizing hapten (DNPh) is expected to give us accurate information about the polyclonal character.

The midpoints (IC_{50}) and molar cross-reactivities are listed in Table 1. TNPh-Gly and TNT showed a cross-reactivity of less than 6%, when that of 2,4-DNT was set as 100%. These results show that anti-DNPh-KLH-400 antibody can discriminate between dinitrophenyl and trinitrophenyl compounds. 2,6-DNT showed about 14% cross-reactivity, suggesting that this antibody discriminated the position of the nitro group and recognized dinitrophenyl compounds. 1,3-DNB and 2ADNT showed cross-reactivities around 50%, which indicates their moderate recognition of the same position on the benzene ring with 2,4-DNT. On the other hand, 2,4-DNPh-Gly showed high cross-reactivity of more than 1000%. This clearly indicates that the NH moiety of the 1-position of the benzene ring, which has the same structure of DNPh, strongly contributes to the recognition of this antibody. Actually, the cross-reactivity of DNBA which has a COOH group in the position was extremely low (1%).

3.2. Adoption of coating hapten suitable for high sensitivity

In polyclonal antibody-based competitive assays, the use of a heterologous competitor significantly affects the assay sensitivity [28–30]. In this strategy, a coating hapten that binds to the antibody with moderately low affinity will result in enhanced sensitivity. In Section 3.1, the NH moiety of the DNPh was suggested to provoke strong binding of the anti-DNPh-KLH-400 antibody. On the other hand, DNBA showed a certain reactivity with an IC_{50} value 14.2 μM, a value that is not considered very low. Therefore, DNBA–OVA was synthesized, and we evaluated whether this candidate hapten is suitable for sensitive indirect competitive assay or not.

Fig. 2 shows the standard curve for 2,4-DNT using ic-ELISA. The data are shown as mean values ± standard deviation (S.D.). The LODs were 240 pg mL^{-1} for the DNPh–OVA system and 18 pg mL^{-1} for the DNBA–OVA system. The apparent affinity constants (Kd_{app}) calculated by Seligman's method [31] were 3.7×10^{-7}

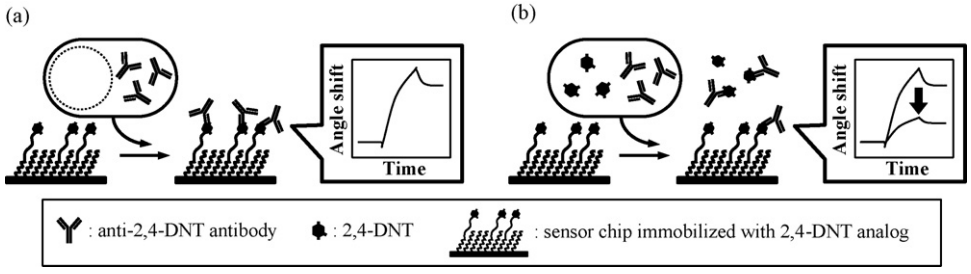
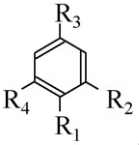


Fig. 1. The principle of indirect competitive SPR measurement for detection of 2,4-DNT. Sensor response to a measurement sample (a) without and (b) with 2,4-DNT.

Table 1
Cross-reactivity of anti-DNPh-KLH-400 antibody for nitroaromatic compounds



Compound	Abbreviation	R_1	R_2	R_3	R_4	IC_{50} (M)	CR (%)
2,4-Dinitrotoluene	2,4-DNT	CH_3	NO_2	NO_2	H	1.14×10^{-7}	100
2,4-Dinitrophenyl-glycine	2,4-DNPh-Gly	$NH–CH_2–COOH$	NO_2	NO_2	H	8.82×10^{-9}	1293
2-Amino-4,6-dinitrotoluene	2ADNT	CH_3	NO_2	NO_2	NH_2	1.79×10^{-7}	64
1,3-Dinitrobenzene	1,3-DNB	H	NO_2	NO_2	H	2.42×10^{-7}	47
2,4,6-Trinitrophenyl-glycine	TNPh-Gly	$NH–CH_2–COOH$	NO_2	NO_2	NO_2	2.04×10^{-6}	6
2,4,6-Trinitrotoluene	TNT	CH_3	NO_2	NO_2	NO_2	2.04×10^{-6}	6
2,6-Dinitrotoluene	2,6-DNT	CH_3	NO_2	H	NO_2	2.31×10^{-5}	5
4-Amino-2,4-dinitrotoluene	4ADNT	CH_3	NO_2	NH_2	NO_2	1.05×10^{-5}	1
2,4-Dinitrobenzoic acid	DNBA	COOH	NO_2	NO_2	H	1.42×10^{-5}	1

and 1.2×10^{-8} M, respectively. From the result, DNBA is found to be a suitable coating antigen for developing a sensitive immunosensor based on an indirect competition format.

3.3. Surface characterization by FTIR-RAS

IR spectroscopy is a useful technique that provides structural and morphological information about fabricated functional surfaces on gold substrates. The RA spectrum of the fabricated OEG-based sensor surface is shown in Fig. 3. The results showed good agreement with previous studies using surfaces with immobilized nitroaromatic compounds [32,33]. The peaks around 1520 and at 1354 cm^{-1} were assigned to asymmetric and symmetric vibrations of the aromatic NO_2 group, respectively. The amide I peak (C=O), which resulted from the amine-coupling procedure during the fabrication process, is found around 1669 cm^{-1}. Another C=O stretching band and a C–OH stretching band appeared at 1746 and 1075 cm^{-1}, respectively. For the OEG moiety, with which this study is concerned, a strong peak at 1115 cm^{-1} and a peak at 1235 cm^{-1} were observed as the bands of C–O–C stretching and twisting, respectively. In addition, both of them indicate the presence of a helical OEG chain, which is resistant to non-specific adsorption [34,35]. Furthermore, the peak at 1115 cm^{-1} was increased after the immobilization of H_2N-EG_{12}-COOH and 2-aminoethanol on the surface, where SAMs of HS-C_{11}-EG_6-COOH were formed (data not shown), suggesting the OEG chain of the complicated surface contains a helical OEG chain that possibly reduces non-specific adsorption by proteins.

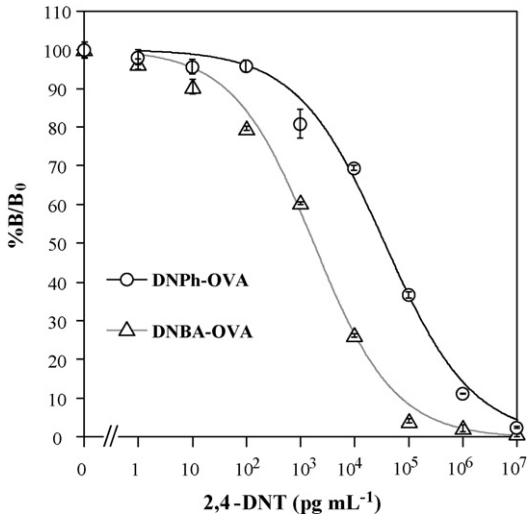


Fig. 2. Ic-ELISAs for 2,4-DNT using DNPh–OVA and DNBA–OVA as coating antibody–protein conjugates.

3.4. Utility of fabricated SAM surface

The OEG-based sensor surface has shown excellent properties that reduce the undesired non-specific adsorption of proteins such as serum albumin and immunoglobulins irrelevant to the specific immunoreaction [36]. This character enhances the reliability of the sensor response and is expected to extend the life span of the sensor chip. Furthermore, this type of sensor surface has also been shown to produce constant baselines and to be stable under repeated surface regenerations and long-term storage [37]. In the present study, we prepared the OEG-based sensor chip for 2,4-DNT detection based on a novel fabrication procedure, and the resistance to non-specific protein adsorption and stability under repeated surface regenerations were investigated.

For the non-specific adsorption study, BSA and rabbit IgG were employed. The adsorption character of BSA has been extensively researched, and the use of rabbit IgG allows us to not only evaluate non-specific adsorption but also to assess whether the binding of the anti-DNPh-KLH antibody to the surface is specific or not. The result is shown in Fig. 4. While the injection of the anti-DNPh-KLH-400 antibody, corresponding to a final concentration of antibody in indirect competitive SPR measurement, produced a strong SPR signal (39 mdeg), each protein produced small responses (less than 0.6 mdeg) even at higher concentrations. These results

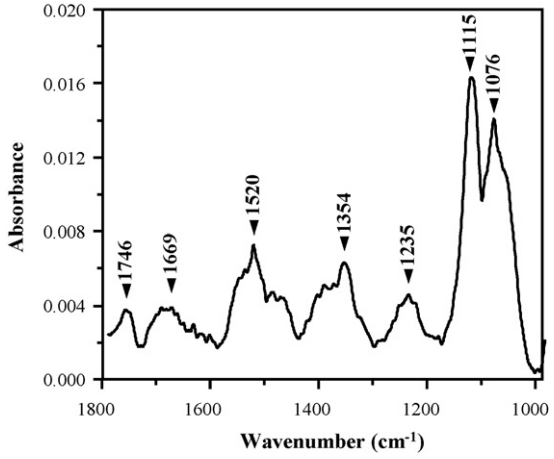


Fig. 3. FTIR spectrum of the fabricated OEG-based sensor surface on a gold surface.

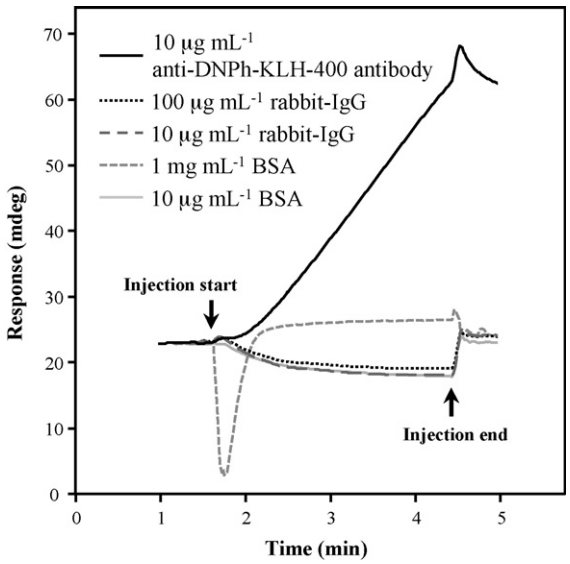


Fig. 4. Responses of the fabricated sensor surface to different proteins.

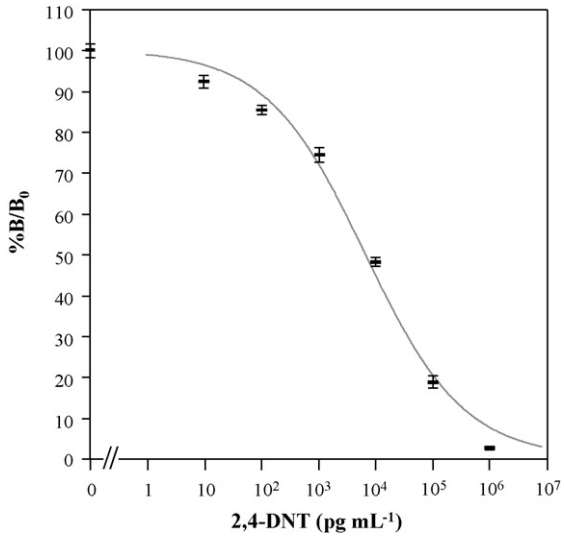


Fig. 6. Standard calibration curve of 2,4-DNT obtained from indirect competitive SPR measurement.

indicate that the anti-DNPh-KLH-400 antibody bound specifically to DNBA immobilized on the sensor surface and that the OEG-based sensor surface effectively repelled non-specific adsorption by proteins.

Fig. 5 shows SPR responses obtained from repeated binding-regeneration cycles using the fabricated sensor chip. The sensor responses in cycle 10 (38.3 mdeg), 20 (38.4 mdeg), and 30 (39.9 mdeg) were comparable to that of the initial cycle (39.2 mdeg) and the base line was highly stable throughout the experiment. Thus, a single OEG-based sensor chip could be used effectively for at least 30 cycles without any noticeable loss of sensor performance.

From these results, the newly fabricated OEG-based surface is shown to posses excellent specificity for a certain antibody and surface stability. These features are highly advantageous compared to other immunosensors reported for nitro aromatic explosives including our previous study using the surface fabricated by physical adsorption [17].

3.5. Detection of 2,4-DNT by indirect competitive immunoassay

Detection of 2,4-DNT was carried out by indirect competitive SPR measurement with the successively fabricated sensor surface. Standard solutions of 2,4-DNT were equivalently mixed with anti-DNPh-KLH-400 antibody to make assay mixtures and incubated. The mixtures were then allowed to flow over the DNBA-terminated sensor surface. Fig. 6 shows the result. The data are presented as mean values ± S.D. from triplicate measurements. As observed in ic-ELISA study, the signal decreased with the increase in 2,4-DNT concentration, and which indicates that 2,4-DNT in the mixture inhibited the binding between the anti-DNPh-KLH-400 antibody and the DNBA immobilized on the OEG-SAM. The LOD for 2,4-DNT was 20 pg mL^{-1}, and the linear dynamic range was found to be between 1 and 100 ng mL^{-1}. This sensitivity is comparable to most of the other immunoassays demonstrated for 2,4-DNT and other explosives [38]. The sensor performance using practical samples, such as gas-phase 2,4-DNT generated from TNT, is under investigation. The prototype of gas-phase sampling system has been developed in our previous research [39].

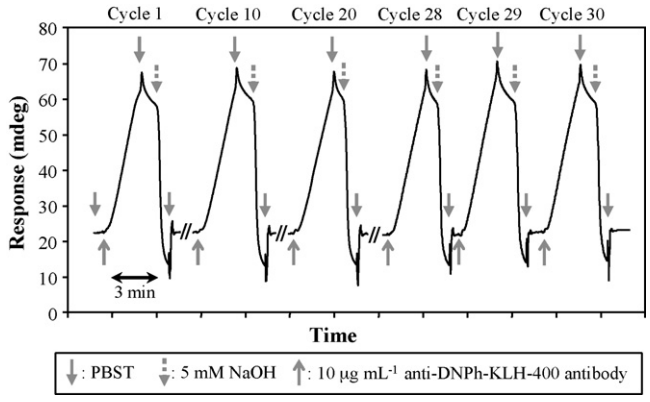


Fig. 5. Stability of the fabricated sensor surface during repeated binding-regeneration cycles.

4. Conclusion

This paper describes the development of an SPR immunosensor to detect 2,4-DNT, the chemical signature of TNT, based on a novel surface fabrication method for direct immobilization of 2,4-DNT analog via a self-assembled OEG-monolayer. The prepared anti-DNPh-KLH-400 antibody specifically discriminated the characteristic structure of 2,4-DNT, and bound to 2,4-DNT with high avidity. From the cross-reactivity data, DNBA was chosen and immobilized onto a gold surface via an OEG linker. This sensor surface showed good baseline stability, resistance to regeneration, and ability to repel adsorption of non-specific proteins. These surface features make the immunsoensor an reliable tool for 2,4-DNT detection. In the indirect competitive SPR measurement using the anti-DNPh-KLH-400 antibody and the OEG-based sensor surface, the immunosensor exhibited high sensitivity for detection of 2,4-DNT with a limit of detection of 20 pg mL^{-1} and a linear dynamic range of 1–100 ng mL^{-1}. These experimental results show that the proposed fabrication technique has the potential to be utilized for design of sensor surfaces and the development of sensitive immunosensors.

Acknowledgement

The authors acknowledge gratefully the Japan Science and Technology Agency of Japan for financial support through the CREST project.

References

[1] J. Yinon, Trends Anal. Chem. 21 (2002) 292.
[2] S. Singh, J. Hazard. Mater. 144 (2007) 15.
[3] A. Fainberg, Science 255 (1992) 1531.
[4] T.F. Jenkins, D.C. Leggett, P.H. Miyares, M.E. Walsh, T.A. Ranney, J.H. Cragin, V. George, Talanta 54 (2001) 501.
[5] T. Naddo, Y. Che, W. Zhang, K. Balakrishnan, X. Yang, M. Yen, J. Zhao, J.S. Moore, L. Zang, J. Am. Chem. Soc. 129 (2007) 6978.
[6] G.K. Kannan, A.T. Nimal, U. Mittal, R.D.S. Yadava, J.C. Kapoor, Sens. Actuators B 101 (2004) 328.
[7] R.G. Bozic, A.C. West, R. Levicky, Sens. Actuators B 133 (2008) 509.
[8] J.M. Sylvia, J.A. Janni, J.D. Klein, K.M. Spencer, Anal. Chem. 72 (2000) 5834.
[9] A. Halasz, C. Groom, E. Zhou, L. Paquet, C. Beaulieu, S. Deschamps, A. Corriveau, S. Thiboutot, G. Ampleman, C. Dubois, J. Hawari, J. Chromatogr. A 963 (2002) 411.
[10] T. Borch, R. Gerlach, J. Chromatogr. A 1022 (2004) 83.
[11] G.P. Anderson, J.D. Lamar, P.T. Charles, Environ. Sci. Technol. 41 (2007) 2888.
[12] R. Wilson, C. Clavering, A. Hutchinson, Analyst 128 (2003) 480.
[13] J. Homola, Chem. Rev. 108 (2008) 462.
[14] S. Dong, X. Chen, Rev. Mol. Biotechnol. 82 (2002) 303.
[15] S.J. Kim, K.V. Gobi, H. Iwasaka, H. Tanaka, N. Miura, Biosens. Bioelectron. 23 (2007) 701.
[16] E. Mauriz, A. Calle, L.M. Lechuga, J. Quintana, A. Montoya, J.J. Manclus, Anal. Chim. Acta 561 (2006) 40.
[17] D.R. Shankaran, K. Matsumoto, K. Toko, N. Miura, Sens. Actuators B 114 (2006) 71.
[18] B.-K. Oh, B.S. Chun, K.-W. Park, W. Lee, W.H. Lee, J.-W. Choi, Mater. Sci. Eng. C 24 (2004) 65.
[19] E. Mauriz, A. Calle, A. Abad, A. Montoya, A. Hildebrandt, D. Barceló, L.M. Lechuga, Biosens. Bioelectron. 21 (2006) 2129.
[20] S. Cosnier, Biosens. Bioelectron. 14 (1999) 443.
[21] T. Kawaguchi, D.R. Shankaran, S.J. Kim, K.V. Gobi, K. Matsumoto, K. Toko, N. Miura, Talanta 72 (2007) 554.
[22] X. Lou, L. He, Sens. Actuators B 129 (2008) 225.
[23] A.D. Taylor, J. Ladd, S. Etheridge, J. Deeds, S. Hall, S. Jiang, Sens. Actuators B 130 (2008) 120.
[24] N. Miura, M. Sasaki, K.V. Gobi, C. Kataoka, Y. Shoyama, Biosens. Bioelectron. 18 (2003) 953.
[25] T. Sakai, A. Torimaru, K. Shinahara, N. Miura, T. Imato, K. Toko, K. Matsumoto, Sens. Mater. 15 (2003) 439.
[26] E.W. Weiler, M.H. Zenk, Phytochemistry 15 (1976) 1537.
[27] H. Holthues, U. Pfeifer-Fukumura, I. Sound, W. Baumann, J. Immunol. Methods 304 (2005) 68.
[28] Y. Liang, X.J. Liu, Y. Liu, X.Y. Yu, M.T. Fan, Anal. Chim. Acta 615 (2008) 174.
[29] Y.J. Kim, Y.A. Cho, H.S. Lee, Y.T. Lee, Anal. Chim. Acta 494 (2003) 29.
[30] R. Galve, F. Sancheg-Baeza, F. Camps, M.P. Marco, Anal. Chim. Acta 452 (2002) 191.
[31] S.T. Seligman, J. Immunol. Methods 168 (1994) 101.
[32] A. Larsson, J. Angbrant, J. Ekeroth, P. Mansson, B. Liedberg, Sens. Actuators B 113 (2006) 730.
[33] H. Chen, H. Cheng, J. Lee, J.-H. Kim, M.H. Hyun, K. Koh, Talanta 76 (2008) 49.
[34] P. Harder, M. Grunze, R. Dahint, G.M. Whitesides, P.E. Laibinis, J. Phys. Chem. B 102 (1998) 426.
[35] R. Valiokas, S. Svedhem, M. Östblom, S.C.T. Svensson, B. Liedberg, J. Phys. Chem. B 105 (2001) 5459.
[36] Y. Mizuta, T. Onodera, P. Singh, K. Matsumoto, N. Miura, K. Toko, Biosens. Bioelectron. 24 (2008) 191.
[37] K. Jans, K. Bonroy, R.D. Palma, G. Reekmans, H. Jans, W. Laureyn, M. Smet, G. Borghs, G. Maes, Langmuir 24 (2008) 3949.
[38] R.G. Smith, N. D'Souza, S. Nicklin, Analyst 133 (2008) 571.
[39] T. Onodera, K. Miyahara, M. Iwakura, K. Hayashi, N. Miura, K. Matsumoto, K. Toko, IEEJ Trans. SM 126 (2006) 621.