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

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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

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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.