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# Development of an oligo(ethylene glycol)-based SPR immunosensor for TNT detection

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ABSTRACT

This paper describes the development of novel biosensor surfaces supported by robust self-assembled monolayers (SAMs) of aromatic alkanedithiol and oligo(ethylene glycol) (OEG) linker for highly sensitive surface plasmon resonance (SPR) detection of 2,4,6-trinitrotoluene (TNT). Aromatic alkanedithiol SAMs were firstly formed on Au sensor surface and TNT analogues were immobilized on it through OEG chain. Two kinds of OEG containing amine compounds, where $H_2N(C_2H_4O)_{11}C_2H_4NHCOOC(CH_3)_3$ served as a linker to react with carboxyl groups of TNT analogues while $H_2N(C_2H_4O)_3C_2H_4OH$ served as a protein non-fouling background, were covalently bound to carboxyl terminal groups of SAMs with a certain ratio. Optimal ratio of them was also examined. Three kinds of TNT analogues, namely TNP-glycine, DNP-glycine, and DNP-acetic acid were used as immobilized ligands. Highly sensitive TNT detection by indirect competitive assay was conducted on the fabricated sensor surfaces; we examined how structural variations of them affect sensitivity in order to choose optimal hapten as well to improve sensitivity. The DNP-acetic acid immobilized surface, which had the lowest affinity to the TNT antibody among the three, showed the best limit of detection (LOD) value (ca. 80 ppt (pg $ml^{-1}$)). On the other hand, the TNP-glycine immobilized surface, which had the highest affinity, showed the worst LOD value (ca. 220 ppt). The LOD got lower to ca. 50 ppt by the use of the secondary antibody on the DNP-acetic acid immobilized surface. The sensor surfaces are durable for more than 100 times repeated use without any noticeable deterioration by their chemical stability and rather mild regeneration condition.



## 1. Introduction

Reliable explosive detectors are highly demanded to forestall terrorism. The phenomena of terrorism which is unpredictable can happen anywhere at anytime. The merits for detectors that attract our attention are small, portable, and field deployable to make on-site detection possible, but simultaneously can detect explosives rapidly with high sensitivity and specificity.

From these viewpoints, we have focused on development of a surface plasmon resonance (SPR)-based immunosensor for highly sensitive detection of explosives (Miura et al., 2007; Shankaran et al., 2006). However, there exists a concern for non-specific adsorption of proteins. It is supposed to be one of the major causes of false detection when on-site test is conducted; SPR cannot distinguish specific binding from non-specific adsorption. Considering the importance of explosive detection, cutting down false detection by eliminating or suppressing non-specific adsorption to the maximum extent possible is an important step to be tackled with the utmost priority.

The indirect competitive assay format is generally adopted to enhance the sensitivity for detection of small molecules such as 2,4,6-trinitrotoluene (TNT) (Miura et al., 2007; Shankaran et al., 2006; Mitchell et al., 2005; Yu et al., 2005). Contrary to the direct detection format, hapten–protein conjugates are usually immobilized on a sensor surface and a premixed solution of antibodies and their antigens at a certain concentration is flowed over the surface, so that the unoccupied antibodies could bind to the surface. However, full investigation has not been conducted to know how affinity strength between immobilized hapten and its correspondent antibody affect sensitivity in the indirect competitive assay format.

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In this study, therefore, we used oligo(ethylene glycol) (OEG), which is known to resist protein adsorption by its flexibility and hydrophilic property (Siegel et al., 1997; Lahiri et al., 1999; Silin et al., 1997), containing self-assembled monolayers (SAMs) for immobilization of ligand. TNT was chosen as the target substance in this study because it is one of the main components of explosives. Three kinds of TNT analogues were used as immobilized ligands; we examined how structural variations of the three TNT analogues with different affinity to its correspondent antibody affect sensitivity of TNT detection in order to choose optimal hapten for immobilization as well to improve sensitivity.

## 2. Experimental details

### 2.1. Reagent and chemicals

The following chemicals were used without further purification: mouse anti-TNT monoclonal antibody (TNT antibody) was obtained from Strategic Biosolutions, USA. 21.8 μg ml$^{-1}$ of TNT solution in Milli Q water was purchased from Chugoku Kayaku Co. Ltd., Japan. 2,4,6-Trinitrophenyl glycine (TNP-glycine) was purchased from Research Organics, USA. 2,4-Dinitrophenyl glycine (DNP-glycine) and 2,4-dinitrophenyl acetic acid was purchased from Tokyo Chemical Industry, Japan. $PEG_6$-COOH aromatic alkanedithiol was purchased from Sensopath Technologies, USA. 11-Amino-1-undecanethiol hydrochloride (AUT > 99% purity) was purchased from Dojindo Laboratory, Japan. 4,4′-Dithiodibutyric acid (DDA) was purchased from Moritex, Japan. Amino-$dPEG_4$ alcohol and mono-*N*-*t*-boc-amido-$dPEG_{11}$ amine was obtained from Quanta Biodesign, USA. *N*-*t*-Boc-4,7,10-trioxa-1,13-tridecanediamine was obtained from Sigma–Aldrich, USA. 1-Ethyl-3-(3′-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxy succinimide (NHS) was purchased from Biacore, Sweden. All other chemicals were purchased either from Tokyo Chemical Industry, Japan or Wako Pure Chemical Industries, Japan. All aqueous solutions were prepared from Milli Q deionized water obtained from Milli-Q-system (Millipore Corporation, USA).

### 2.2. Fabrication of sensor chip

#### 2.2.1. Fabrication of EG 17 chip

Sensor chips were fabricated using SIA Kit Au (Biacore, Sweden) which contains sensor chips covered with 50 nm thickness of unmodified gold. The sensor chip was cleaned in acetone with ultrasonic cleaner for 10 min, and washed with ethanol and 2-propanol for 2 min each in sequence. Then, the chip was washed in mixed solution of Milli Q water, ammonia solution and hydrogen peroxide with 5:1:1 volume ratio at 90 °C for 20 min (Park et al., 2004). After rinsing with Milli Q water three times, the chip was immersed in 1 mM $PEG_6$-COOH aromatic alkanedithiol in ethanol solution for about 24 h and self-assembled monolayers were formed.

The chip was cleaned in ethanol with an ultrasonic cleaner for 5 min. The SAM-modified chip was immersed in the mixed solution (1:1 volume ratio) of 0.4 M EDC (in water) and 0.1 M NHS (in water) for 30 min and the carboxyl terminal groups of SAMs were converted to *N*-hydroxy succinimide esters. NHS ester has high reactivity towards primary amine to form stable covalent amide bond. Four chips were prepared with the same procedure as described above. After rinsing with Milli Q water and nitrogen blown dry, the obtained chips were immersed in the mixed solutions of 10 mM mono-*N*-*t*-boc-amido-$dPEG_{11}$ amine in 10 mM Borate 8.5 buffer (10 mM dissodium tetraborate pH 8.5, 1 M NaCl (Biacore, Sweden)) and 10 mM amino-$dPEG_4$-alcohol (in 10 mM Borate 8.5 buffer) with a certain volume ratio, namely 10:0, 8:2, 5:5, and 2:8 for about 1 h. As amino terminal groups of mono-*N*-*t*-boc-amido-$dPEG_{11}$ amine and amino-$dPEG_4$-alcohol react with NHS esters of SAMs terminals to form robust amide bonds, the four different kinds of sensor chips were obtained. Hereafter, the sensor chips are called "EG 17 10:0, 8:2, 5:5, and 2:8 chip", respectively. After being washed with Milli Q water three times and nitrogen drying of the surfaces, the chips were immersed in 4N-HCl for 1 h to deprotect tert-butoxycarbonyl (Boc) groups of terminal amino groups.

For the immobilization of TNT analogues using amine coupling reaction, carboxyl groups of each analogue were activated with EDC and NHS solution. Fifty microliters of 10 mM solutions of TNT analogue in *N*,*N*-dimethyl formamide (DMF) was mixed with 50 μl of 0.4 M EDC (in water) and 50 μl of 0.1 M NHS (in DMF) in a microtube for about 1 h.

A small amount of triethylamine diluted with DMF was added to the reactant solution to adjust pH to nearly 8.5. The sensor chips, after treated with 4N-HCl, were immersed in the solution for about 2 h for the reaction between amine terminals of sensor surface and NHS esters of TNT analogue. TNP-glycine, DNP-glycine, and DNP-acetic acid was used as immobilized TNT analogues.

Chemical structures of TNT, TNT analogues, SAM reagent, and OEG linker reagents used in this study are shown in supplemental information (Supplementary data, Fig. S1): (a) TNT and the analogues (TNP-glycine, DNP-glycine, and DNP-acetic acid), (b) $PEG_6$-COOH aromatic alkanedithiol and OEG linker reagents (amino-$dPEG_4$ alcohol and mono-*N*-*t*-boc-amido-$dPEG_{11}$ amine).

#### 2.2.2. Fabrication of EG 3 sensor chip

After the cleaning of gold sensor chip surface, as mentioned in Section 2.2.1, Au sensor chip was immersed in 1 mM DDA (in ethanol) solution for about 24 h to form SAMs. Carboxyl terminal groups of SAM were activated with mixed solution of EDC and NHS for 30 min and then the chip was immersed in 10 mM *N*-*t*-boc-4,7,10-trioxa-1,13-tridecanediamine solution in DMF for about 1 h. After washed with Milli Q water for three times and nitrogen drying, the chip was immersed in 4N-HCl for 1 h and then in the NHS-activated TNT analogue solution for about 2 h. NHS esterification process of TNT analogues was the same as mentioned in Section 2.2.1.

#### 2.2.3. Fabrication of EG 0 sensor chip

After the cleaning of gold sensor chip surface, as mentioned in Section 2.2.1, Au sensor chip was immersed in 1 mM AUT (in ethanol) solution for about 24 h to form amino-terminated SAMs. After washed with ethanol three times and nitrogen drying, the chip was immersed in the NHS-esterified TNT analogue solution for about 2 h. NHS esterification process of TNT analogues was the same as mentioned in Section 2.2.1.

### 2.3. Interaction analyses using SPR sensor

#### 2.3.1. Instrument and conditions

Interaction analyses were done using BIAcore SPR sensor system (Biacore J). All measurements were done at 25 °C. PBST (100 mM phospate buffered saline, 150 mM NaCl, 0.05% (v/v) Tween 20, pH 7.2) was used as running buffer and flowed through the system at 60 μl/min flow rate.

#### 2.3.2. Evaluation of the fabricated sensor chips against non-specific adsorption of proteins

DNP-glycine immobilized EG 17 sensor chips (10:0, 8:2, 5:5, and 2:8), EG 3 sensor chip, and EG 0 sensor chip (total 6 chips) was used for evaluation of protein non-specific adsorption. One thousand micrograms per milliliter BSA (Bovine Serum Albumin),