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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),

100 μg ml^{-1} human IgG, and 100 μg ml^{-1} rabbit anti-mouse IgG (Jackson Immuno Research Laboratories, USA) solutions diluted with running buffer were injected into the SPR instrument. Each protein solution was injected three times.

2.3.3. Determination of kinetic parameters and affinity constants

The three kinds of EG 17 (8:2) sensor chips with TNP-glycine, DNP-glycine, and DNP-acetic acid terminals were used for determination of kinetic parameters and affinity constants. TNT antibody solutions of 0.5, 1, 2, 5, 10, 20, and 40 μg ml^{-1} (ppm) were injected into the SPR instrument for 3 min and observe natural dissociation under running buffer solution for 2 min. Obtained sensorgrams were analyzed using BIAevaluation software (ver. 3.2, BIAcore) to find out kinetic parameters. Considering bivalency of antibody and mass transport effect (Myszka, 1997) in this interaction condition, "Bivalent with mass transfer" fitting model was chosen. Association and dissociation rate constants k_a, k_d were determined using "Global fitting" algorithm from the given 7 sensorgrams simultaneously. The equilibrium constants (K_A) were calculated form the equation, $K_A = k_a/k_d$.

2.3.4. TNT detection

The three kinds of EG 17 (8:2) sensor chips with TNP-glycine, DNP-glycine, and DNP-acetic acid terminals, were used for SPR interaction analysis for TNT detection. Five hundred nanograms per milliliter (ppb) of TNT antibody stock solutions were mixed with varying concentration of TNT solution (0, 20, 100, and 200 pg ml^{-1}; 1, 2, 10, and 20 ng ml^{-1}) in 1:1 volume ratio and incubated at room temperature for about 15 min before injection (final concentration of TNT antibody in each sample solution was 250 ng ml^{-1}, and that of TNT was 0, 10, 50, 100, and 500 pg ml^{-1}; 1, 5, and 10 ng ml^{-1}, respectively). Three hundred microliters of each sample solution (5 min injection at 60 μl min^{-1} flow rate) was injected three times and the median of the data were used for calibration. Each sample solution was prepared with PBST running buffer.

2.3.5. TNT detection with secondary antibody signal amplification

Rabbit anti-mouse IgG (Jackson Immuno Research Laboratories) was used for secondary antibody enhancement. Fifty nanograms per milliliter (ppb) of TNT antibody stock solutions were mixed with varying concentration of TNT solution (0, 20, 100, and 200 pg ml^{-1}; 1, 2, 10, and 20 ng ml^{-1}) in 1:1 volume ratio and incubated at room temperature for about 15 min before injection (final concentration of TNT antibody in each sample solution was 25 ng ml^{-1}, and that of TNT was 0, 10, 50, 100, and 500 pg ml^{-1}; 1, 5, and 10 ng ml^{-1}, respectively). Three hundred microliters of each sample solution (5 min injection at 60 μl min^{-1} flow rate) was injected. Immediately after finishing sample injection, 300 μl of 100 μg ml^{-1} secondary antibody solution was injected for signal enhancement. Each sample was analyzed three times and the median of the data were used for calibration. Each sample solution was prepared with PBST running buffer.

2.3.6. Regeneration condition

The surface-bound TNT antibody was dissociated with the mixed solution of 3.13 mM NaOH, 30% (v/v) acetonitrile, and 0.05% (v/v) Tween 20 (1 min injection at 60 μl min^{-1} flow rate). When the surface was not completely regenerated, the mixed solution with higher concentration of NaOH (6.25 or 12.5 mM) was used.

3. Results and discussion

3.1. Characteristics of the fabricated sensor surfaces

In this work, we fabricated novel sensor surfaces to suppress non-specific protein adsorption using OEG containing linker. Since OEG group has also hydrophilic property, it can improve the hydrophilicity of the sensor surface. Given that antigen-antibody immunoreactions take place in aqueous phase, reactivity between antibody and immobilized hapten is also expected to improve. Hydroxyl terminated OEG chain is particularly known to suppress protein non-specific binding most effectively (Silin et al., 1997) and play a role on a protein non-fouling background (Yu et al., 2005; Kyo et al., 2005). According to prior works, gold chips were soaked in mixed solutions of long-chain alkane-OEG-thiol with active terminal groups, such as carboxyl groups and amino groups, and short-chain alkane-OEG-thiol with hydroxyl terminals to form mixed SAMs by co-adsorption. Optimal ratio of them was also examined by varying the ratio of the mixed solutions of SAM reagents (Yu et al., 2005; Larsson et al., 2006; Subramanian et al., 2006).

However, as longer-chain thiol has higher energy of adsorption, displacement of adsorbed shorter-chain thiol on the surface by the longer-chain thiol may occur when the Au sensor chip was immersed for a long period (Xing et al., 2005). In this study, a gold-coated SPR chip was firstly modified with aromatic alkanedithiol to form single component SAMs and then mixed solutions of OEG linker reagents were attached to the SAMs with various ratios instead of making mixed SAMs. Aromatic alkanedithiol was used as a SAM reagent because it has two sulfhydryl groups and an aromatic ring in one molecule and therefore form robust SAMs on Au surface. Repeatability and durability of the sensor chip was also expected to improve. PEG_6-COOH aromatic alkanedithiol was used as the SAM-forming reagent. The compound also has 6 ethylene glycol (EG) functional groups in one molecule.

Mono-*N*-*t*-boc-amido-dPEG$_{11}$-amine ($H_2N(C_2H_4O)_{11}C_2H_4NHCOOC(CH_3)_3$) was used as a linker to combine TNT analogues with SAMs on the sensor surface to react with carboxyl groups of them. Since the compound has two amino terminals at both ends, but one end is protected by Boc group, it makes step-by-step reaction possible while preventing dimerization (Mitchell et al., 2005, 2006). The compound has 11 EG functional groups in one molecule and the total number of the EG functional groups on the surface is 17. Amino-dPEG$_4$ alcohol ($H_2N(C_2H_4O)_3C_2H_4OH$) was used as a protein non-fouling background in this study. Carboxyl groups of the aromatic alkanedithiol SAMs on the surface were reacted with mixed solutions of $H_2N(C_2H_4O)_{11}C_2H_4NHCOOC(CH_3)_3$ and $H_2N(C_2H_4O)_3C_2H_4OH$ at varying ratios (10:0, 8:2, 5:5, and 2:8) and optimal ratio was examined in the following experiment. Three kinds of TNT analogues, namely TNP-glycine, DNP-glycine, and DNP-acetic acid, were used as immobilized ligands. The three TNT analogues can bind to TNT antibody and be called as haptens. The fabrication process is schematically illustrated in Fig. 1.

Since longer reaction time compared to usual on-line immobilization procedure (Johnsson et al., 1991) was taken, blocking process was not required for the fabrication process (see Section 2.2). In fact, when 1000 μg ml^{-1} BSA, 100 μg ml^{-1} human IgG, and 100 μg ml^{-1} anti-mouse IgG were flowed over the fabricated surfaces, even a slight increase of sensor response was not observed on EG 17 (10:0, 8:2, 5:5, and 2:8) surfaces (see Section 3.2 and Fig. 2). It means none of empty sites and amine-reactive NHS esters had not remained on the surfaces by the fabrication procedure. The sensor responses on EG 0 and EG 3 surfaces gradually decrease to zero as PBS running buffer flows (data not shown). It also means that the

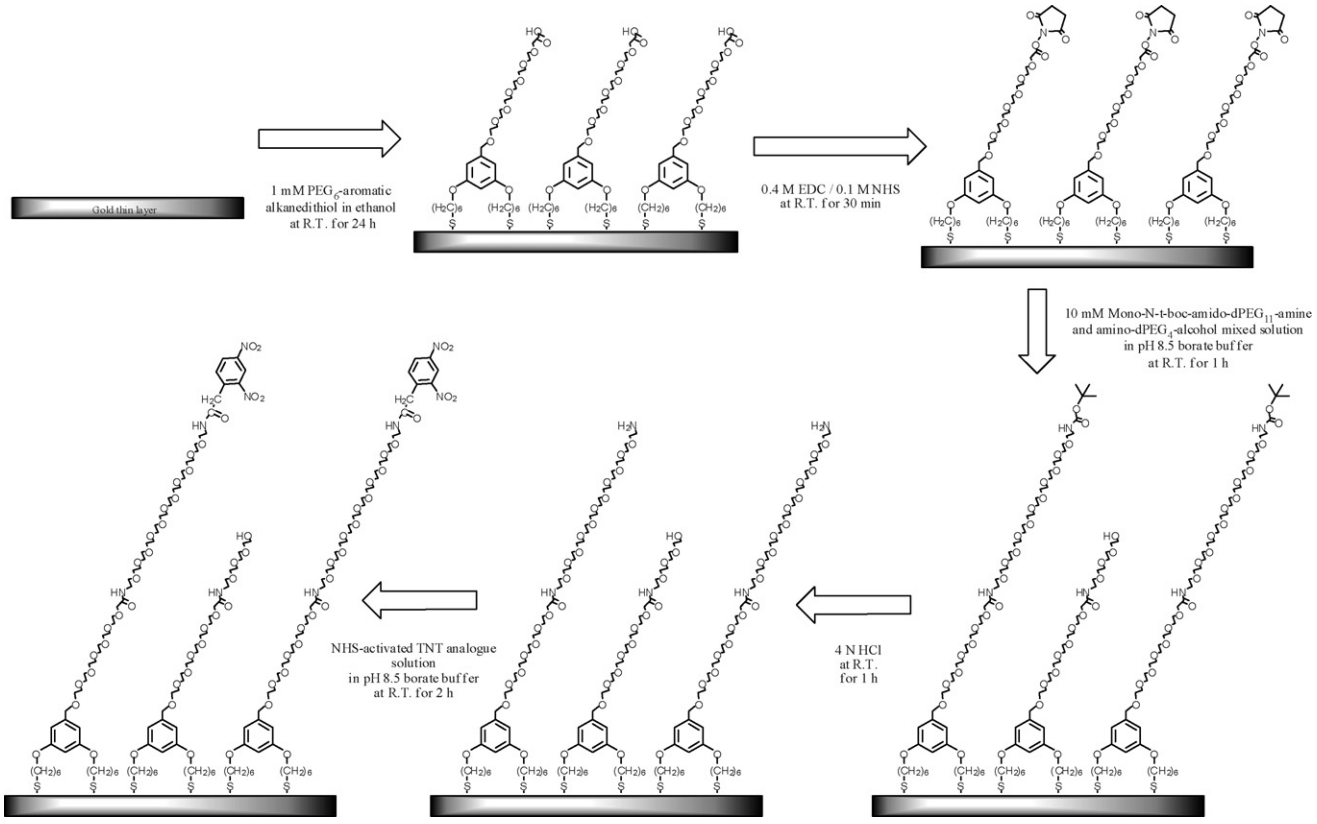

Fig. 1. Fabrication process of sensor chip surface.

sensor responses are caused by non-specific adsorption of proteins (if NHS-activated groups remained on the surfaces, amino functional groups on the proteins form stable amido bonds through amine coupling reaction and the obtained sensor signal did not decrease).

As described above, the novel surface fabrication process in this study only requires immersing Au sensor chip in the reagent solutions in turn without complicated and troublesome organic synthesis.

3.2. Evaluation of repellent ability against protein non-specific adsorption of OEG containing sensor surfaces

We conducted experiments to evaluate how effectively the newly fabricated sensor chip surfaces reduce non-specific adsorption of proteins. The three kinds of proteins, BSA, human IgG, and rabbit anti-mouse IgG, were flowed over the six kinds of sensor chip surfaces: EG 17 (10:0, 8:2, 5:5, and 2:8) chips, EG 3 chip, and EG 0 chip. BSA and human IgG was chosen because their adsorption characteristics have been well studied on a variety of surfaces (Silin et al., 1997), and they have opposite net charges. Furthermore, human IgG was chosen considering for future on-site analysis by operator because it was sourced from human fluid. Anti-mouse IgG was selected considering that it can be used as the secondary antibody for signal amplification because the monoclonal TNT antibody used in this study was raised in mouse.

Sixty microliters of high concentration of these protein solutions, 1000 $\mu g\,ml^{-1}$ BSA, 100 $\mu g\,ml^{-1}$ human IgG, and 100 $\mu g\,ml^{-1}$ anti-mouse IgG, were flowed over the sensor chip surfaces. The result is shown in Fig. 2(a). The vertical axis shows the sensor response value. 1 RU is determined as 0.0001° of resonance angle shift and is equivalent to the change in the mass of the 1 $pg\,mm^{-2}$ on the sensor surface (Subramanian et al., 2006). The error bar shows the value of the standard deviation of the data. It is found that the non-specific adsorption can be suppressed effectively as the surfaces have longer EG chain. Among the EG 17 chips, the 8:2 chip reduced non-specific adsorption more effectively than the 10:0 chip. However, the difference was hardly seen with the 8:2, 5:5, and 2:8 chip, and the amount of protein non-specific adsorption on the 8:2 chip was also negligible. Therefore, the 8:2 chip was determined to be optimal considering sensor response.

Fig. 2(b) shows SPR sensorgrams obtained by injection of the three kinds of proteins over the DNP-glycine immobilized EG 17 (8:2) chip surface. The sensor response increases during injection, but goes back to nearly zero when injection is over. It means that protein adsorption on the surface was almost completely suppressed.

3.3. Signal change due to OEG moiety

In order to examine the relationship between sensor response and OEG moiety length, the same concentration of TNT antibody solutions (20 $\mu g\,ml^{-1}$) were flowed over the three kinds of DNP-acetic acid immobilized surfaces (1 min at 60 $\mu g\,ml^{-1}$ flow rate). The sensor response of EG 17 (8:2) chip, 2250 RU, was about two times higher than that of EG 0 chip, 1200 RU. EG 17 (8:2) chip, 2250 RU, also shows higher response than that of EG 3 chip, 1635 RU (see supplemental information Supplementary data, Fig. S2). The fact clearly indicates that signal changes deeply depend on the length of OEG chain; it can be explained that the antibody can easily approach TNT analogues on the surfaces.

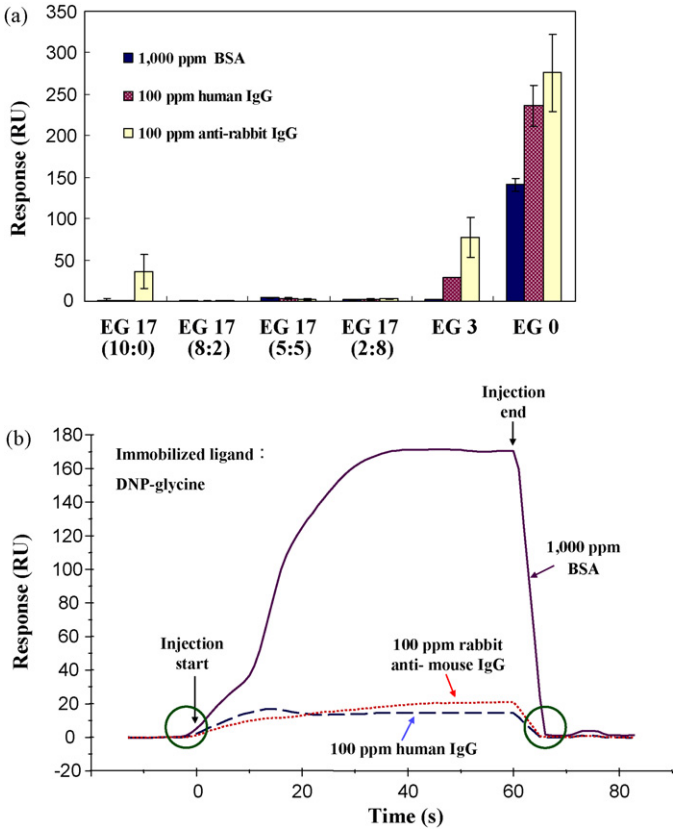

Fig. 2. (a) Non-specific binding of proteins for different sensor surfaces. (b) Overlaid SPR sensorgrams obtained by injections of three kinds of proteins (BSA, human IgG and rabbit anti-mouse IgG) over the DNP-glycine immobilized EG 17 (8:2) chip surfaces.

3.4. Principle of indirect competitive assay

In indirect competitive assay format, small molecular antigen (or its analogue) is immobilized on a sensor surface and large molecular antibody is flowed over the surface as an analyte because large molecular weight of antibody (MW ca. 150,000) is expected to gain larger signal than small molecules like explosives and therefore is more sensitive. The principle of indirect competitive assay has been described in numerous papers (Miura et al., 2007; Shankaran et al., 2006; Singh et al., 2007; Mitchell et al., 2006). Simply put, antibodies are initially mixed with their antigens at a certain concentration. When the mixture is flowed over the surface, a lower resonance angle shift compared to antibody solutions in the absence of the antigens is observed. It can be thought that a part of the antibodies has reacted with their antigens to become inactive, and the amount of free antibody molecules is reduced. The reduced extent of signal is inversely proportional to the amount of antigen molecules in a sample solution. Thus the amount of the antigen can be determined indirectly.

3.5. The influence of the hapten structure for the measurement

3.5.1. Evaluation of affinity constants of the hapten compounds to the antibody

The commercial TNT antibody (Strategic Biosolutions, USA) used in this work was described to be raised in mouse against TNP-glycine-KLH conjugate as an immunogen (Zeck et al., 1999). Therefore, the affinity strength of the TNT analogues is speculated from structural similarity to the hapten part of the immunogen (TNP-glycine) to be in the order: TNP-glycine > DNP-glycine > DNP-acetic acid.

The strength of affinity constants (K_A), determined in Section 2.3.3, was ranked in the order: TNP-glycine (ca. $3.0 \times 10^7\ M^{-1}$) > DNP-glycine (ca. $2.0 \times 10^7\ M^{-1}$) > DNP-acetic acid (ca. $1.0 \times 10^7\ M^{-1}$); this is in agreement with the former speculation.

3.5.2. Consideration of the sensitivity of TNT detection using three kinds of hapten

TNT was measured by indirect competitive assay according to the procedure described in Section 2.3.4. In the indirect competitive assay format, the response of reference sample must be accurate. However, slight decreases of sensor response were observed even with the same surface using the same concentration of antibody as the number of immunocycle grows. It may be due to

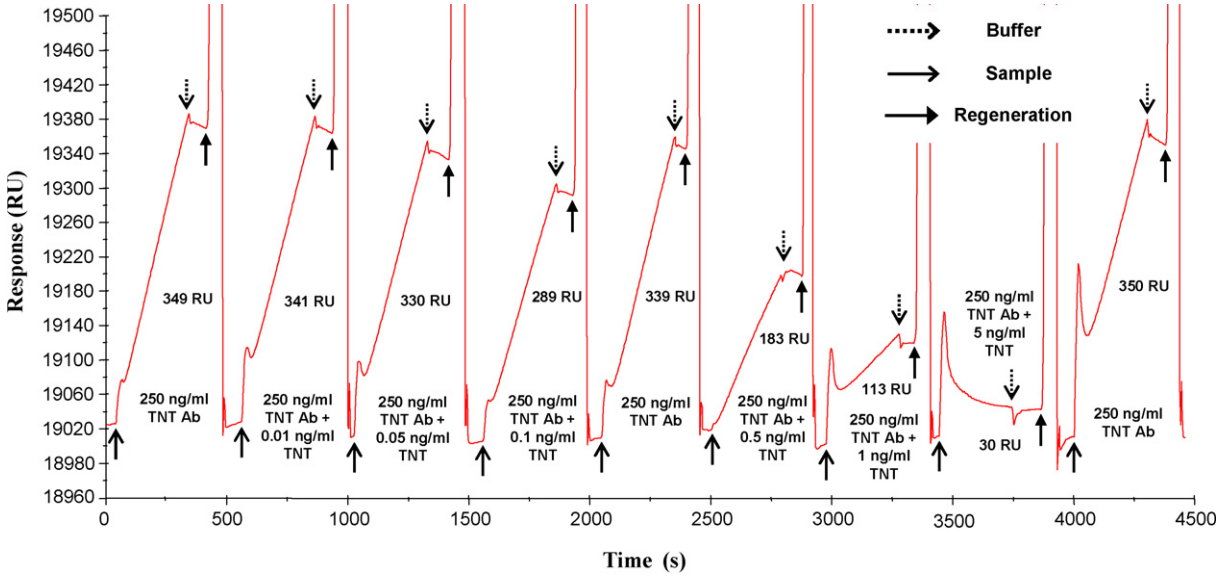

Fig. 3. SPR sensorgram of indirect competitive assay using 250 ng ml^{-1} TNT antibody for detection of varying concentration of TNT on DNP-acetic acid immobilized EG 17 (8:2) surface.

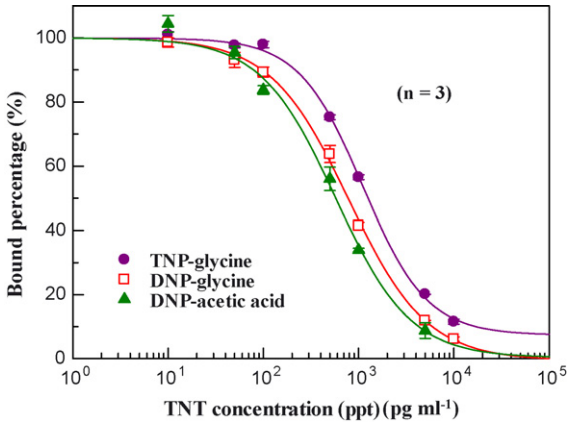

Fig. 4. Calibration curves of indirect competitive assay for three kinds of haptens immobilized sensor surfaces using 250 ng ml^{-1} concentration of TNT antibody. R.S.D.s of the data are shown using error bars.

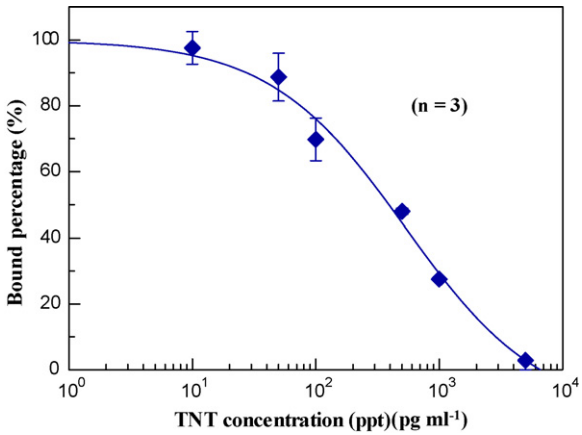

Fig. 5. Calibration curve of indirect competitive assay for DNP-acetic acid immobilized sensor chip using 25 ng ml^{-1} concentration of TNT antibody followed by secondary antibody signal amplification. R.S.D.s of the data are shown using error bars.

degradation of sensor surface or antibodies in buffered aqueous solution. Since we focus on highly sensitive detection with ppt level, the difference must be carefully considered. In this respect, 250 ng ml^{-1} TNT antibody solutions with varying concentration of TNT were measured in sequence to obtain calibration curve; 0 (reference), 10, 50, and 100 pg ml^{-1}, 0 (reference), and 500 pg ml^{-1}, 1, and 5 ng ml^{-1}, 0 (reference), and 10 ng ml^{-1}. The observed sensorgram on DNP-acetic acid immobilized EG (8:2) chip surface was given in Fig. 3. The cycle was repeated for three times to obtain full calibration curve. Five hundred nanograms per milliliter stock solution of TNT antibody was freshly prepared for each cycle.

Fig. 4 shows calibration curves obtained by least square curve fitting. The vertical axis shows bound percentage which means relative percentage of sensor response of TNT containing antibody solution to antibody solution in the absence of TNT ($\Delta\theta/\Delta\theta_0 \times 100$; $\Delta\theta_0$ is sensor response (RU) for 250 ng ml^{-1} antibody solution without TNT, $\Delta\theta$ is sensor response (RU) for mixed solution of 250 ng ml^{-1} antibody and certain concentration of TNT). Two hundred fifty nanograms per milliliter antibody solutions without TNT, which work as the reference, were measured for three times in a cycle of measurement (Fig. 3). Bound percentage was calculated using the median value of the three measurements. Discrepancies of obtained data from multiple experiments were very small (relative standard deviations (R.S.D.s) of the data were less than 4% throughout the experimental data). This is thought to be due to the fact that the non-specific adsorption of proteins can be substantially suppressed by the sensor surfaces. Since the R.S.D.s of 250 ng ml^{-1} antibody solutions without TNT (reference) of each surface were less than 3%, the limit of detection (LOD) values were determined as the TNT concentration yielding 10% inhibition (ca. 3 R.S.D.s).

According to Fig. 4, inhibition occurs frequently on the surface of DNP-acetic acid, DNP-glycine, and TNP-glycine in the order. The DNP-acetic acid immobilized surface, which has the lowest affinity to the antibody among the three, showed the best LOD (ca. 80 ppt). On the contrary, the TNP-glycine immobilized surface, which has the highest affinity to the antibody, showed the worst LOD among the three (ca. 220 ppt). DNP-glycine immobilized sensor chip, which has intermediate affinity, showed ca. 100 ppt. The sensitivity of TNT detection is inversely related to the affinity strength of the hapten to the antibody.

For interpretation of the results, it is assumed that dissociation of TNT antibodies from preformed TNT-antibody complexes and rebinding of the dissociated TNT antibodies to surface bound haptens occurs more frequently on the TNP-glycine immobilized surface which has higher affinity than the other two (DNP-glycine and DNP-acetic acid). It is also assumed that mere binding of free antibodies to surface bound haptens occurs more frequently on the TNP-glycine immobilized surface. As a result, inhibition occurred less frequently and LOD got worse on the TNP-glycine immobilized surface. Therefore, affinity of an immobilized hapten to its corresponding antibody is desired to be as weak as possible for highly sensitive detection using indirect competitive assay. However, if affinity of the hapten to the antibody is too weak, adequate sensor response for SPR interaction analysis cannot be obtained, so it is necessary to consider balance between affinity and sensor response.

3.6. Signal amplification and enhancement of sensitivity by secondary antibody

In indirect competitive assay format, LOD gets lower as the antibody concentration decreases (Mitchell et al., 2005; Gobi et al., 2007; Yu et al., 2005); it is necessary to use as low concentration of antibody as possible for highly sensitive detection. Thus, we chose 25 ng ml^{-1} of TNT antibody in order to achieve further sensitive detection. However, the sensor response of 25 ng ml^{-1} TNT antibody was only around 30 RU (300 μl was injected at 60 μl/min flow rate), which was too small sensor response for the sensitive detection. Therefore, signal amplification is required to be done to obtain enough sensor response (Matsumoto et al., 2005; Mitchell et al., 2005, 2006). We used secondary antibody (rabbit anti-mouse IgG) that recognizes the bound primary antibody as the signal amplification agent because we had made sure that the fabricated sensor chip surfaces can substantially reduce protein non-specific adsorption (see Section 3.2). Immediately after the 25 ng ml^{-1} of TNT antibody injection was over, 100 μg ml^{-1} rabbit anti-mouse IgG (300 μl at 60 μl/min flow rate) was flowed over the surface and enough sensor response of ca. 350 RU was observed.

Considering the previous result in Section 3.5.2, the DNP-acetic acid immobilized sensor chip, which showed the highest sensitivity among the three, was used for the experiment. As the TNT antibody used in this study was raised in mouse, anti-mouse IgG was used as the secondary antibody. Fig. 5 shows calibration curves obtained by the experiment. Since the R.S.D. of 25 ng ml^{-1} of TNT antibody solution without TNT (reference) was ca. 5%, the LOD was determined as TNT concentration at 85% for bound percentage. The determined LOD was ca. 50 ppt.

3.7. Regeneration condition

In SPR immunoassay, dissociation of the bound antibody from the surface matrix is required for the next immunocycle. A mild regeneration condition is required for the retention of activity of the sensor surface to the antibody. According to the former reports, the strong binding between antibody and conjugates like TNP-glycine, TNP-OVA, and TNP-BSA requires strong acid or alkaline buffer for regeneration (Matsumoto et al., 2005; Shankaran et al., 2006). Therefore, the sensor chip cannot withstand repeated use because of its deactivation even after a few dozen immunocycles. The cause of such a strong binding between antibody and immobilized conjugate may be attributed to the fact that the commercial TNT antibody used in this study was raised against TNP-glycine-KLH as immunogen (Zeck et al., 1999) and, as a consequence, the TNT antibody has strong affinity to the TNP-glycine-KLH conjugate and may also recognize the KLH protein part.

On the other hand, the newly fabricated sensor surfaces were durable for more than 100 times repeated use with a slight deterioration. It is because that the surfaces do not have protein part and therefore require rather milder condition for regeneration (3.13 mM NaOH, 30% (v/v) acetonitrile and 0.05% (v/v) Tween 20) than TNP–protein conjugate-based sensor surface. The use of the fabricated surfaces could reduce maintenance work and cost. This is a very desirable characteristic in considering practical application.

4. Conclusions

This work includes fabrication of novel SPR sensor surfaces supported by robust SAMs of aromatic alkanedithiol and OEG linker for highly sensitive detection of TNT with SPR immunosensor. The fabricated surfaces show remarkable protein repellent ability while maintain affinity of immobilized haptens to the antibody; repeatable and reliable TNT detection is possible. In order to examine what characteristics are demanded for immobilized ligands for sensitive detection, we used three kinds of TNT analogues, namely TNP-glycine, DNP-glycine, and DNP-acetic acid as immobilized ligands. Indirect competitive assay for TNT detection was conducted on the fabricated surfaces using 250 ng ml^{-1} of TNT antibody. The best limit of detection of ca. 80 ppt was obtained on the DNP-acetic acid immobilized surface, which had the lowest affinity to TNT antibody among the three. The LOD got lower to ca. 50 ppt using further lower concentration of TNT antibody (25 ng ml^{-1}) followed by secondary antibody signal amplification. 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.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2008.03.042.

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