Sensors and Materials, Vol. 19, No. 5 (2007) 261–273
MYU Tokyo

S & M 0681

Novel DNP-KLH Protein Conjugate Surface for Sensitive Detection of TNT on SPR Immunosensor

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(Received March 28, 2007; accepted May 1, 2007)

Key words: TNT sensor, SPR sensor, inhibition assay, DNP-KLH protein conjugate

A nanoscale biosensor chip surface for the detection of trinitrotoluene (TNT) was fabricated using dinitrophenylated-keyhole lympet hemocyanin (DNP-KLH) protein conjugate as ligand supported by an underlying 11-amino 1-undecanethiol hydrochloride (AUT) self-assembled monolayer (SAM) and bis sulfo-succinimidyl suberate (BS^3) as crosslinker. Bioactive thin films were fabricated over gold chip via layer-by-layer self-assembly methods. The biomolecular interaction between substrate-specific TNP-glycine-KLH mouse Ig antibody and DNP-KLH conjugate surface was monitored using a surface plasmon resonance-based optical sensor. The quantitation of (TNT) on this bioactive surface was carried out using the solution-based competitive inhibition assay. The DNP-KLH surface biosensor has shown a detection limit of 0.15 ng/ml (150 ppt) and was sensitive up to the 0.005 ng/ml (5 ppt) level for the TNT molecule. This TNT-specific biosensor holds the promise to be one of the most sensitive, fast TNT detecting sensor surfaces under indirect competitive assay format. A 12 s injection pulse of 10 mM glycine-HCl solution was sufficient for the regeneration of the DNP-KLH surface for repeated use. The DNP-KLH sensor platform was checked for its reproducibility and storability.

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1. Introduction

Surface plasmon resonance (SPR) biosensors are optical sensors exploiting refracted evanescent electromagnetic waves over thin gold film for sensing applications. The SPR biosensors find their applications in the detection of chemical and biological analytes. The biophysical analyses of biomolecular interactions are monitored in real-time and without labeling with fluorescent or radioactive dyes.[1] SPR provides a simple, valuable, unmatched platform to probe ligand–receptor interactions,[2,3] protein-protein interactions,[4] protein-DNA interactions,[5,6] DNA-DNA interactions,[7,8] and DNA-drug[9] interactions.

The highly selective and sensitive detection of explosive molecules such as TNT, DNT, and RDX is the central challenge to the scientific community for the assurance of safety in air travel, cargo handling at sea ports, controlling the trafficking of explosives, and checking the rise of global terrorism. Explosives and warfare agents have been produced and used in large amounts before and during World War II, and even now, are the major source of soil and water contamination. Millions of land mines are buried in the field and are still being laid every year, thus creating a very unsafe environment for human society world over. Trinitrotoluene (TNT), the main component of these products serves as an indicator of the existence of these explosives and is a contaminating molecule.

Current physical detection methods are less selective, unreliable and have poor accuracy. Emerging sensor technologies such as ground penetrating radar[10] suffer from low sensitivity, accuracy and false negative results as it is unable to distinguish the patterns obtained from plastic land mines. The upgraded piezo sensors[11] are useful at a high concentration level in spite of inadequacies such as inconsistency, frequent errors and selectivity. Dogs are very effective in detecting explosive substances. However, dogs need extensive training, and a trainer, and are unfit for round-the-clock security protocol. TNT detection by rapid fluorescent quenching[12] with analyte on porous polymer films appears to be promising. In this method, fluorescent attenuation depends on several factors that need careful manipulation. Moreover, the fluorescent sensor membrane becomes quenched quickly by samples containing analyte and loses its efficacy in a very short time. Microcantilever sensors[13] can measure the extremely small forces caused by molecular attachment and offer adaptation to sensing platform suitable for multiplexed sensing. Although cantilever sensors are sensitive, they suffer from an intrinsic selectivity problem. Analytical method based on immunological reactions such as displacement immunoassay[14–16] using fluorescent-labeled antigen is well suited for trace detection of explosives. This method using antigen-antibody reaction requires a fluorescent-labeled antigen, thus limiting its generic nature and is cumbersome for on-site detection of explosive molecules.

On the other hand, the SPR sensor has potential to be useful for on-site detection of explosive substances.[17,18] It appears probable to achieve high sensitivity for the on-site detection of TNT and other chemical substances in trace amount using SPR affinity sensors. In the past few years, we have been concentrating on the development of a highly sensitive SPR immunosensor for TNT and TNP detection using antigen-antibody-based assay methods.[19,20] Rapid, inexpensive, reliable and sensitive sensors for the

detection of TNT and similar compounds are therefore urgently needed. Therefore, in this paper, we report the fabrication of a novel sensor platform using DNP-KLH ligand, immobilized over gold chip by layer-by-layer self-assembly procedures formed by covalent bonds resulting in a very stable, robust, and sensitive sensor surface.

2. Experimental Details

2.1 *Materials*

2.1.1 *Reagent and chemicals*

The following chemicals were used as received from suppliers: 11-amino 1-undecanethiol hydrochloride (AUT, >99% purity) was purchased from Dojindo Laboratory, Kumamoto, Japan, and bis(sulfo succinimidyl) suberate (BS^3) was purchased from Pierce Technology, Rockford, USA. Upon receipt, the products were stored desiccated at 4°C. Anti-TNT monoclonal antibody (TNT Ab), raised in mouse against TNP-glycine-KLH as immunogen, was purchased from Strategic Biosolutions, USA. TNT solution (21.8 ppm) in Milli-Q water was purchased from Chugoku Kayaku Co. Ltd, Japan. DNP-KLH conjugate (molecular ratio: 4 ± 1.0) was manufactured by LSL Co. Ltd, Japan and marketed by Cosmo Bio, Japan. All aqueous solutions were prepared from Milli-Q (18 MΩ-cm resistivity) deionized water obtained from Milli-Q-system (Millipore Corporation, USA). KH_2PO_4 (99% purity) and Na_2PO_4 were purchased from Wako Chemicals, Japan. NaCl and KCl of 99.5% purity were purchased from Kanto Chemicals, Japan. TRIS extrapure was purchased from ICN Biomedicals, Ohio, USA. All other chemicals were of analytical grade purchased either from Sigma or Pierce Technology, Rockford, USA.

2.1.2 *Gold chip*

The SIA Kit from Biacore, Uppsala, Sweden was used for the multistep immobilization of various films on the surface. Initially, the sensor chip Au packet was allowed to equilibrate at room temperature, so that the gold chip can be released from the gel easily and there should not be any condensation on the surface. The sensor is always picked by standard tweezers from the glass and gold side in a way that it should not go beyond 2 mm. This sensor chip was first washed in acetone for 10 min in an ultrasonic cleaner, then in ethanol and 2-propanol for 2 min each. Thereafter, the chip was washed in solution[21] (NH_3: H_2O_2: H_2O (18 MΩ-cm) in 2:2:10 v/v ratio on a hot plate at 90°C for 20 min (Caution: Hydrogen peroxide solution is a strong oxidizing agent. It has an immediate harmful effect on skin; intoxication occurs if inhaled.) Upon removal from this solution, the chip was rinsed with deionized water, followed by drying in high-purity nitrogen gas.

2.2 *Surface chemistries*

2.2.1 *Thiols modification*

The thiols coating of the gold surface was carried out in 1 mM 11-amino 1-undecanethiol hydrochloride (AUT) in ethanol for 18–22 h. This procedure makes a thin coating of self-assembled monolayer (SAM) containing amino group at the other

end. Upon removal of the chip from the thiol/ethanol, the gold substrate was rinsed in excess ethanol, and blown dry in nitrogen gas. This SAM-modified substrate was then used for carrying out the DNP-KLH protein immobilization through BS^3 succinimidyl linker group.

2.2.2 *Covalent attachment of proteins to gold chip*

For the immobilization of proteins, the amino-coated substrates were incubated in 10 mM BS^3 in PBS (pH 7.2) for 30 min, rinsed with Milli-Q water, and dried under nitrogen. Following this, the gold substrates were treated with 200 ppm DNP-KLH in PBS (pH 7.2) protein solutions for 2 h at room temperature. Protein-treated substrates were washed with PBS in a sonicator for 5 min and in Milli-Q water and then nitrogen-dried. BS^3 is a water-soluble ester, having sodium salt at both terminuses; it shows high reactivity towards primary amines. BS^3 possess a charged group at its surface, making it very useful for cell surface protein cross-linking.

2.2.3 *Quenching of unreactive ester sites*

Quenching for the remaining reactive ester sites was carried out by incubating the derivatized substrates in 50 mM Tris (pH 7.5) for 15 min at room temperature. After being rinsed with Milli-Q water, the substrates were blown dry under nitrogen and used for SPR studies or stored at 4°C in desiccators for subsequent use.

2.3 *SPR interaction analyses*

SPR measurements were carried out using Biacore X, Uppsala Sweden. The Biacore X SPR system is equipped with a manual injection port to allow the introduction of sample to the sensor chip. The loop volume of the SPR flow cell is 100 µl. Temperature in the flow cell was controlled by an internal thermostat at 25°C. The immobilization of the molecular matrix over the sensor surface results in the change in refractive index, which is measured through the relative change in resonance units (RU, where 1000 RU is equal to 0.1 degree shift in resonance angle). Flow rate of 10 µl/min was maintained for most of the experiments. All SPR experiments were conducted at a constant temperature of 25°C. Glycine-HCl solution (10 mM; pH 2.0) was used for the regeneration of protein-derivatized surfaces after each binding step. The biomolecular interaction experiment was carried out in PBST buffer (PBS plus 0.05% v/v Tween 20) pH 7.2. For further investigations of protein-protein interactions, PBST was used as both the sample and carrier buffer. For competitive inhibition assay, TNT Ab (1 µg/ml) was mixed with analyte (TNT) at a given concentration and allowed to reach equilibrium in 15 min. Protein solution (50 µl) was flowed over the sensor surface at a flow rate of 10 µl /min.

2.4 *Competitive assay principle and procedure*

Figure 1 shows the principle of competitive immunoreaction as previously given.[22,23] An increase in resonance angle shift occurs when TNT Ab binds to DNP-KLH solid-phase conjugate on gold sensor chip ($\Delta\theta_0$). When incubated mixture of antibody and analyte is flown over the conjugate surface; antibody unreacted with analyte will be able to bind to the sensor surface, and hence, a decrease in angle shift ($\Delta\theta$) is recorded over

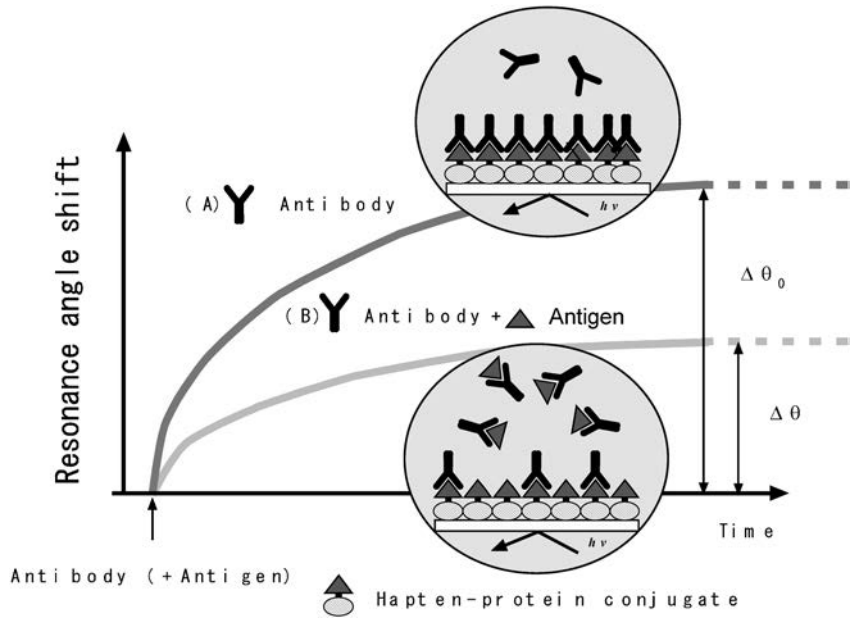

Fig. 1. Schematic representation of indirect competitive assay format. The hapten-protein conjugate was immobilized on gold surface and an antibody solution was introduced over conjugate surface through flow cell for antibody-conjuagte immunoreaction. (A) Immunoreaction in the absence of analyte; resonance angle shift of $\Delta\theta_0$ was observed corresponding to amount of antibody attached. (B) SPR resonance angle shift in presence of analyte, $\Delta\theta$, inhibited response because some of antibody has already attached to analyte, thereby making fewer antibody available for immunoreaction on conjugate surface.

the system. The decrease in angle shift increases with analyte concentration (TNT). Thus, the measured binding response is inversely proportional to the concentration of free TNT in the solution. Therefore, a correlation between concentration of TNT in solution and binding response can be established. This correlation is utilized for the determination of concentration of TNT in mixed solution.

3. Results and Discussion

The commercial TNT antibody used in this study was raised using TNP-Glycine-KLH as immunogen;[24] KLH is high-molecular-mass protein, a good carrier molecule and considered to be generating a strong immunogenicity.[25] To elicit antibodies to a small molecule like TNT, it is necessary to link this molecule through a spacer arm to an immunogenic carrier protein like KLH to stimulate the mammalian immune system[26,27] Anti-TNT antibody is a macromolecule; this helps the SPR biosensor technique in amplifying the detection signal for the antigen.[28] DNP-KLH protein matrix immobilized solid-phase immunoassay for TNT analyte was first tested for its loading capacity against

anti-TNT antibody.

Figure 2 shows the SPR binding response (RU) against the varying concentration of anti-TNT antibody over the DNP-KLH prepared sensor surface. The graph shows an exponential rise initially at a lower antibody concentration followed by a gradual increase in response at a higher antibody concentration range of 40–100 ppm. Concentrated TNT antibody (2 ppm) shows the binding response over 1200 RU, while 10 ppm TNT antibody displays a response over 2000 RU, which is relatively high on this sensor surface. Thus, this binding curve underlines the high loading capacity of the sensor surface for anti-TNT antibody. It indicates that the density of molecular binding sites per unit area of sensor surface is high and is paving the way for increased loading of antibody. The higher the conjugate's ability to hold antibody, the lower would be the required concentration of working antibody, allowing the immunoassay to be less costly for practical applications.

Solid-phase immunoassay relies on the immobilization of analyte or analog derivative on the sensor surface. In this study, DNP-KLH protein conjugate was used as a solid phase matrix for the detection of TNT under the principle of indirect competitive assay.[22,23] Figure 3 shows the SPR sensorgram of competitive inhibition assay performed using equilibrated 500 ppb (0. 5 μg/ml) anti-TNT antibody concentration and TNT as an analyte in varying concentration. Incubated antibody and antigen mixture was flowed after 15 min of unstirred immunoreaction in incubator at 25°C.

Immunocycle A shows the sensor response when 0.5 μg/ml anti-TNT antibody in the

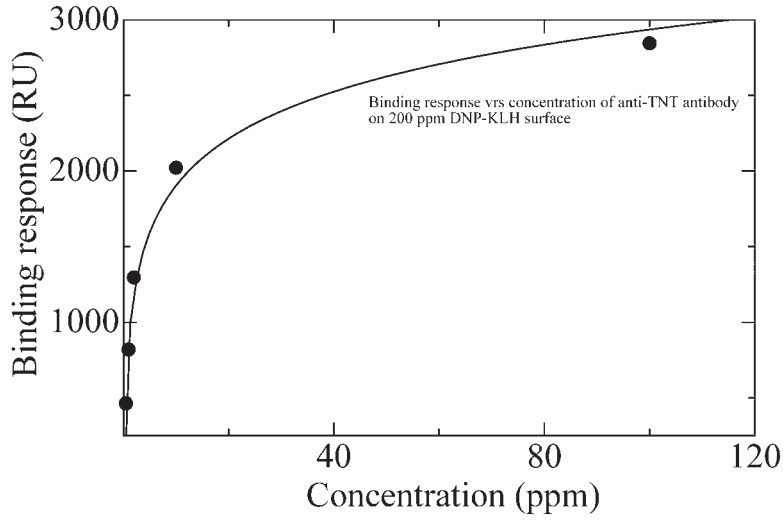

Fig. 2. Binding response of SPR signal on DNP-KLH sensor surface against increasing concentration of anti-TNT antibody. Dots are experimental data points, while the line is a guide for the eye.

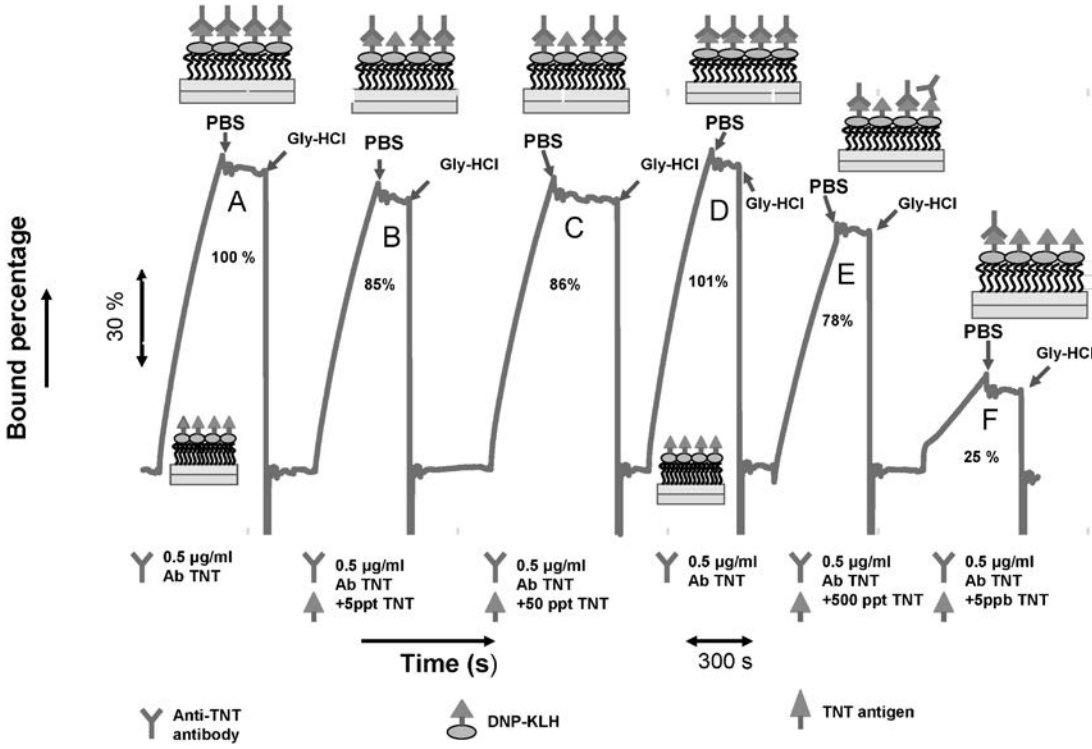

Fig. 3. SPR sensorgram of competitive inhibition assay performed using 0.5 µg/ml anti-TNT antibody concentration and TNT as an analyte in varying concentrations.

absence of antigen was injected over the sensor surface for 5 min at a flow rate of 10 µl/m. In cycle B, a mixture of 0. 5 µg/ml anti-TNT antibody and 5 ppt TNT analyte was flowed at the same duration in the flow cell; binding response is decreased and measured to 85% of previous response with no analyte. Bound percentage was calculated considering only antibody response as 100% ($\Delta\theta_0$ is angle change in terms of RU for antibody in the absence of analyte). Bound percentage for inhibited response was calculated as ($\Delta\theta/\Delta\theta_0 \times 100$), where $\Delta\theta$ is angle change in terms of RU for antibody-antigen mixed sample. In the next cycle C, when the analyte concentration was raised to 50 ppt, the immunocycle shows inhibited response to almost the same level as that with 5 ppt. This is usually seen with a mixed sample having the ppt-level TNT concentration (0.5–50 ppt TNT) in the present assay system. This may be attributable to competitive binding of TNT-antibody complex and free TNT antibody on sensor surface.

After each injection of antibody solution, the sensor surface was regenerated very cleanly with 12 s (2 µl) injection of 10 mM Glycine-HCl. Regeneration is a major advantage of the DNP-KLH surface; it is very fast, clean, and does not affect the sensor

surface in the long run. In cycle D, when 0. 5 μg/ml anti-TNT antibody was injected again to see the surface behaviour, we found that the surface shows full binding response ~100% as in cycle A. The binding response of the sensor against the flow of mixture of antibody and 500 ppt TNT is shown in curve E; it shows an inhibition of almost 22% corresponding to 78 bound percentage. In order to assess the range of this immunoassay, 5 ppb TNT solution mixture was flowed subsequently and 25% binding response was recorded. These immuno-curves show that the antibody has a very high affinity for the TNT antigen; thus, it is very useful for the detection of TNT antigen specifically at lower concentration level in the sample. The complete immunocycle including regeneration cycle takes about 6 min (~5 min for sample flow and ~1 min for the regeneration cycle), which is quite short, making this sensor surface suitable for on-site detection.

DNP-KLH-based immunoassay is showing the lowest detection limit of 150 ppt, as shown in Fig. 4, which is arrived at when considering the usual 5% standard deviation in the SPR response signal for the single-measurement cycle; we have taken 15% inhibition as the lowest detection point for analyte concentration. Data is analyzed using least-square curve fitting with an appropriate function representing sigmoidal behaviour.

Figure 4 shows that a low analyte concentration (0.5–50 ppt) is inhibiting the antibody binding to the sensor surface, thereby making it sensitive enough to record trace amounts of TNT on the SPR sensor. The lowest detection limit (defined as three standard deviations above blank) for TNT has been reported to be 100 ppb by Sakai *et al.*[29] using anti-TNP antibody in (5–40 μg/ml) concentration range under label-free, competitive inhibition assay over TNP-OVA surface using Spreeta™ SPR sensor (Texas

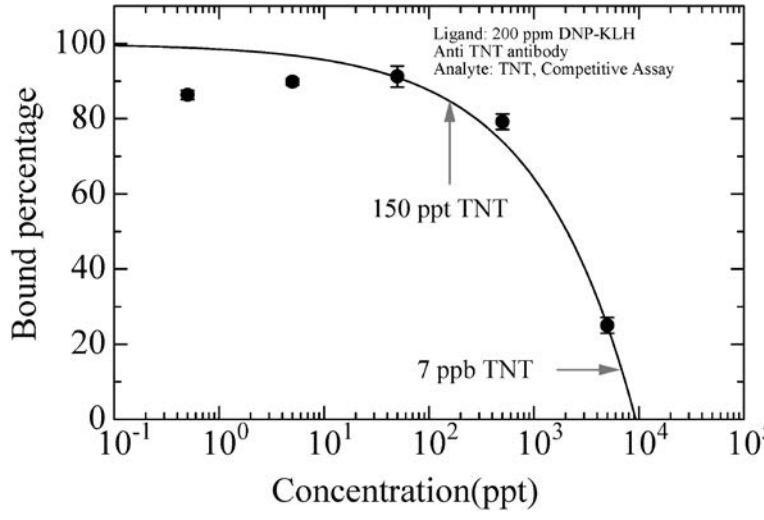

Fig. 4. Bound percentage of anti-TNT antibody versus TNT concentration under indirect competitive inhibition format. Dots are experimental data points, while the curve shows the least-square fit using a suitable function.

Instruments, Inc., Texas, USA). Larsson and coworkers[30] have used a novel competitive assay format based on displacement of bound antibodies and had reported the detection limit in the 1–10 ppb range for TNT depending upon TNT analogues immobilized on the biochip surface using SPR and QCM biosensors. Competitive fluorescent displacement assay[31] has achieved a lower detection limit of 500 ppt in PBS, which is improved to 50 ppt for TNT dissolved in artificial seawater. A chemiluminescence-based flow-injection immunosensor[32] designed for field analysis has reached the detection limit (IC_{20}) below 100 ppt (0.1 μg/l) for 2,4,6-trinitrotoluene (TNT). The DNP-KLH sensor surface being reported in this study has demonstrated the detection limit to 150 ppt (0.15 ng/ml) for TNT molecule under competitive inhibition assay. This assay can be used for sensing up to 7 ppb concentration of TNT. To further enhance the assay range, a high-ppm antibody can be used at the cost of detection limit.

3.1 *Regeneration and stability*

Regeneration is a process that allows the sensor surface to be ready to perform the next immunocycle. Therefore, a quick, clean and matrix-friendly regeneration is suitably chosen and employed for a particular surface. Figure 5 shows that the DNP-KLH protein conjugate surface is quickly regenerated with only a 10 mM glycine-HCl pulse of 12 s (2 μl). This regeneration is so clean and quick, and the baseline is very stable, enabling the repeated use of the surface for many immunocycles. The dotted line shows the SPR response on the same flow channel after 100 regenerative cycles over 20 days, which

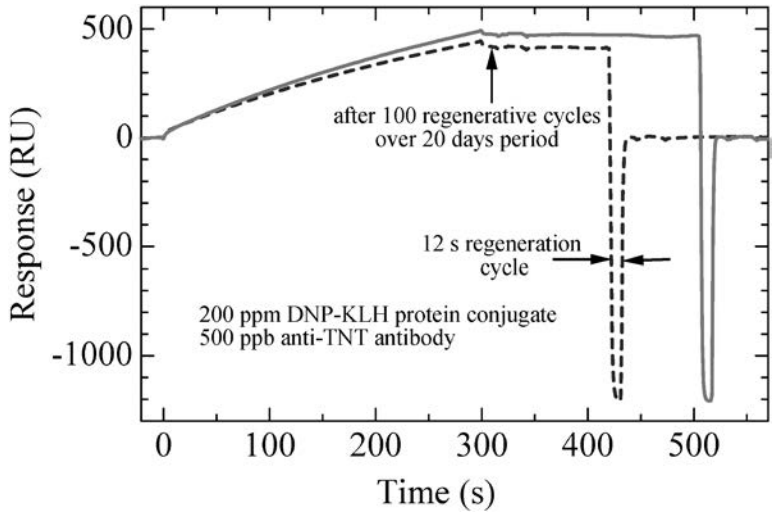

Fig. 5. SPR sensorgram showing regeneration response of sensor surface against repeated use over 20 days, which is around 10% at the end of 100 cycles.

indicates that the sensor surface is stable, robust and does not deteriorate over many weeks of repeated use.

Quick regeneration and longer stability are the advantages of the DNP-based sensor surfaces and is an improvement of our earlier work.[19,20] The DNP-KLH surface can be reused more than 100 times without deteriorating its performance, opening the way for low-cost applications. In this assay, we also used only 0.5 μg/ml concentrated antibody for our immunocycle, which is also small compared to what is used by many researchers.[29,30,32]

3.2 *Storability and reproducibility*

In order to assess the stability of the sensor matrix and its life with the view of long-term storability, the sensor chip was stored at 4–8°C in a refrigerator for over 6 months, and its performance was evaluated with regards to the detection limit and sensitivity. The sensor chip has shown good consistency and a stable response, suggesting that the protein conjugate did not deteriorate during the 6-month period if kept under dry and cool conditions.

Figure 6 shows the sensor performance after 6 months of its fabrication. It shows that the detection limit of the sensor deteriorated to 240 ppt from 150 ppt (Fig. 4), however, its working range increased to the 15 ppb level. This implies that there is a slight degradation in activity of the immobilized matrix over the long period of storage at 4–8°C.

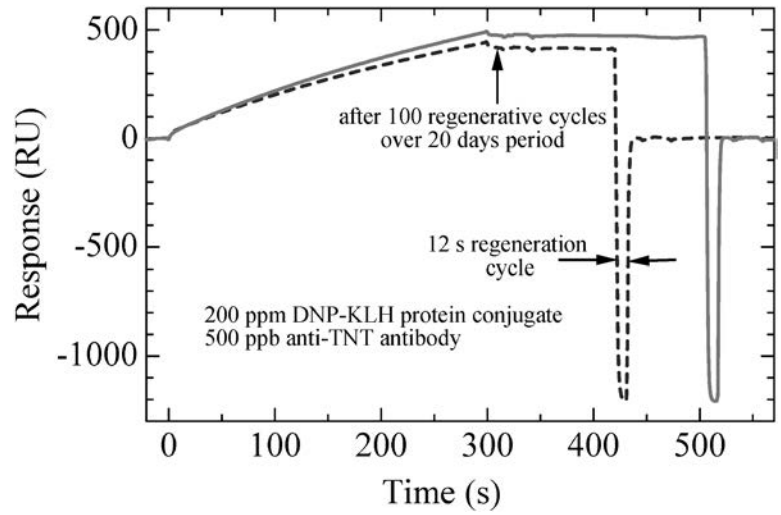

Fig. 6. Bound percentage of anti-TNT antibody versus concentration under competitive inhibition assay performed after 6 months of sensor chip storage. Inset shows the sensorgram after storage. Dots are experimental data points, while the curve is a least-square fit using a suitable function.

Results obtained as shown in Figs. 3 and 4 were checked for its reproducibility on subsequently prepared sensor chips, and the reproducibility was found to be established. There was a variation in the baseline level, but the sensitivity and detection limit were found to be unchanged on the DNP-KLH sensor surface.

3.3 *Association properties of TNT Ab to nitro-aromatic compounds*

The association of TNT Ab with various nitro-aromatic compounds was investigated by indirect competitive inhibition assay on the SPR sensor. The cross-reactivity study of the compounds structurally similar to TNT needs to be carried out to evaluate the specificity of assay to the target TNT analyte. The cross-reactivity was judged at the concentration of analogs yielding 50% inhibition compared to the blank analyte.

The indirect inhibition response with dinitrotoluene (DNT), *o*-nitrotoluene (*o*-NT), trinitrophenol (TNP) and *m*-dinitro benzene (*m*-DNB) was examined and shown in Table 1. The IC_{50} was defined as the concentration of mixed analyte that yields 50% inhibition compared to only TNT Ab (no analyte) experimental step. Molar cross-reactivities were related to TNT (100); for example, all molar cross-reactivities are determined in relation to TNT. The molar cross-reactivity is calculated using IC_{50} of each derivative according to the following formula, $CR = (IC^*_{50}/ IC_{50}) \times 100$, where CR is molar cross-reactivity (%), IC^*_{50} is the IC_{50} of standard TNT, and IC_{50} is the IC_{50} of derivatives.

Molar cross-reactivity with DNT is found to be 0.026 with this immunoassay, which is negligible and does not pose any threat as an interfering species at low concentration. Similarly, with *o*-NT and *m*-DNB, the molar cross-reactivities are around 0.004 and 0.015, respectively. This implies that TNT detection using this assay is highly specific, and close analogs do not interfere with assay performance at lower concentration. TNP as interfering species has shown a higher inhibition response, but the fact is that TNP is not a derivative of TNT.

4. Conclusions

In this study, we report on the fabrication of a novel DNP-KLH protein conjugate-based sensor surface on gold chip by self-assembled monolayers. The self-assembled monolyers were fabricated by immersing the gold chip in various solutions of thiols

Table 1
Cross-reactivity of the nitroaromatic analogs in the TNT biosensor assay.

Analyte	IC_{50} (ng/ml)	Molar cross-reactivity (%)
TNT	4	100
DNT	15000	0.026
*o*NT	100000	0.004
TNP	20	20
*m*DNB	27000	0.015

and crosslinker before immobilizing the protein on a sensor chip. The fabricated sensor surface was tested for its performance over SPR machines. DNP-KLH based surfaces have shown many advantagous characteristics such as stability, storability, ease of regeneration and detection limit. The sensor has shown a detection limit of 150 ppt (0.150 ng/ml) for TNT analyte under competitive inhibition assay format, where relatively low 0.5 μg/ml TNT antibody was used for each immunocycle, making the assay cheaper for practical applications. The detection limit can be further reduced using surface matrix manipulation and changing the assay procedure in use. This immunosensor has shown excellent regeneration ability after 100 regeneration cycles over 20 days. The sensor's storability is also found to be more than 6 months but at the expense of detection limit. One complete immunocycle on this sensor surface takes approximately 6 min, which is sufficiently short, making it suitable for the on-site analysis of explosive substances.

Acknowledgements

Financial support from the Japan Society for the Promotion of Science (JSPS) to Praveen Singh (P. S.), in the form of a JSPS-PDF for foreign researchers, is gratefully acknowledged. P. S. is also thankful to ICAR, New Delhi, INDIA for granting a study leave for this collaborative research work. This work was partially supported by the Japan Science and Technology Agency through CREST.

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