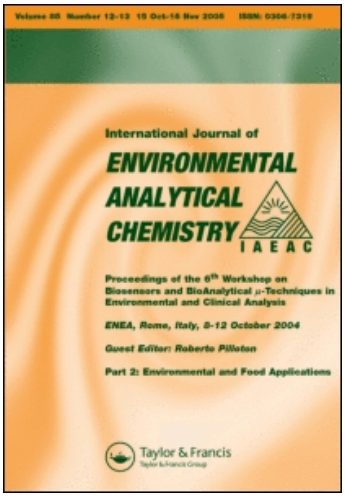

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Fabrication of novel molecular recognition membranes by physical adsorption and self-assembly for surface plasmon resonance detection of TNT

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Recent concern on international terrorism and weapons of mass destruction demands the development of novel analytical methods for identification and quantification of explosive molecules. In this article, we describe the development of high-performance immunosensors for detection of 2,4,6-trinitrotoluene (TNT), a prime component of the landmines and bombs used by terrorist and military forces. The immunosensors were constructed by physical adsorption and self-assembly methods, and their binding interactions with a monoclonal anti-TNT antibody were evaluated for TNT detection using the surface plasmon resonance technique. A home-made 2,4,6-trinitrophenyl-keyhole limpet hemocyanine conjugate was used for physical adsorption. A poly(ethylene glycol) hydrazine hydrochloride thiolate was used in the construction of self-assembled monolayer surface and was immobilized with trinitrophenyl-β-alanine by the amide coupling method. The immunosensors were highly selective, regenerable, rapid, and exhibited remarkable sensitivity down to the parts-per-trillion level for TNT by the indirect competitive inhibition principle.

Keywords: Immunosensor; Explosives; TNT; Surface plasmon resonance; Environmental monitoring

1. Introduction

There is an increasing concern on the safety and security of the global community because of recent issues of international terrorism and weapons of mass destruction. The production, testing, and waste discharge of explosives (for military and terrorist

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activities) result in extensive contamination of the soil and groundwater, which presents severe health risks to humans and ecosystems [1–3]. Thus, the development of novel analytical methods for identification and quantification of explosive molecules is an important analytical issue for environmental monitoring, prevention of terrorism, and homeland security. It is important to produce highly specific and cost-effective sensing methods capable of rapid, accurate, and sensitive detection of explosive molecules. Immunosensors are potential candidates to fulfil these requirements due to their advantages of sensitive and selective antigen–antibody immunoreaction coupled with a suitable signal transducer [4–6]. A number of immunosensors were reported for monitoring explosive molecules based on fluorescence or chemiluminescence detection using labelled biomolecules [7–12]. However, labelling of the biomolecules increases the cost and assay time. Moreover, labelling alters the binding capability of the biomolecules, leading to reduced qualitative and quantitative information on the biochemical interactions. Hence, there is great interest in finding a label-free immunoassay technique for efficient monitoring of explosives molecules.

Surface plasmon resonance (SPR) is a promising option in this regard because it allows label-free measurement of biomolecular interactions. SPR is a surface-sensitive optical technique capable of real-time monitoring of biomolecular interactions on transducer surfaces by exploiting the interfacial refractive index changes associated with any affinity binding interactions without labelling of the reagents [4–6, 13, 14]. In a recent review article, we clearly described the advantages of this simple technique compared with other transducers for the basic research and application in a wide area such as drug discovery, food analysis, and environmental monitoring [14]. The immobilization of a biological sensing element (antigen or antibody) on a transducer surface is of great technological interest in the fabrication of commercially viable immunosensors. A number of immobilization methods have been used for sensor fabrication, including physical adsorption [15, 16], Langmuir–Blodgett deposition [17], self-assembly [18, 19], embedding in polymers or membranes [20], etc. each having its own advantages and limitations. Among them, physical adsorption and self-assembly methods are frequently used. Physical adsorption is the simplest method and has the advantage of immobilizing large amounts of biomolecules on the transducer surface. We have constructed highly effective immunoassays by physical adsorption for detection of several important analytes [21–24]. The self-assembly method favours oriented immobilization of biomolecules on the transducer surfaces. Recently, poly(ethylene glycol) (PEG)-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 without the problem of non-specific adsorption [18].

In the present work, we have demonstrated the development of high-performance SPR immunosensors for detection of 2,4,6-trinitrotoluene (TNT), a prime component of the landmines and bombs used by the military and terrorist forces. We constructed two different immunosensor surfaces by physical adsorption and self-assembly methods. Both immunosensors were evaluated for detection of TNT based on the principle of indirect competitive immunoreaction without labelling the reagents. This detection principle is simple and offers tremendous promise for highly sensitive detection of low-molecular-weight analytes like TNT compared with other detection principles such as sandwich, displacement, and direct immunoassays [14, 25–27].

2. Experimental

2.1 *Chemicals*

Monoclonal anti-TNT antibody was purchased from Strategic Biosolutions (Ramona, CA). 2,4,6-Trinitrophenyl–keyhole limpet hemocyanine (TNPh–KLH) conjugate was prepared in one of our laboratories. PEG-6-hydrazine alkanethiol was purchased from Sensopath Technologies (Bozeman, MT). Trinitrophenyl-β-alanine (TNPh-β-alanine) was received from Research Organics (Cleveland, OH). TNT solution was obtained from Chugoku Kayaku (Hiroshima, Japan). BSA and pepsin were obtained from Sigma (St. Louis, MO). All reagents were of analytical grade and were obtained from Sigma and Wako Chemicals (Osaka, Japan). Deionized water ($\rho = 18\,\mathrm{M}\Omega$) was used throughout the experiment. All experimental solutions were prepared with phosphate-buffered saline (PBS, 0.1 M, pH 7.2) containing 1% ethanol. Tris buffer (pH 8.0) was used during the immobilization of TNPh-β-alanine. Pepsin solution (0.2 M) was prepared with glycine with the pH adjusted to 2.0 using 1 M HCl. Throughout the experiment, the room temperature was maintained at $25 \pm 1^{\circ}\mathrm{C}$.

2.2 *Instrumentation*

The principle of the SPR immunosensor and the present instrumentation setup are illustrated in figure 1. In SPR immunosensor, a plane polarized light is directed through a glass prism to the gold/solution dielectric interface over a wide range of incident angles, and the intensity of the resulting reflected light is measured with a detector.

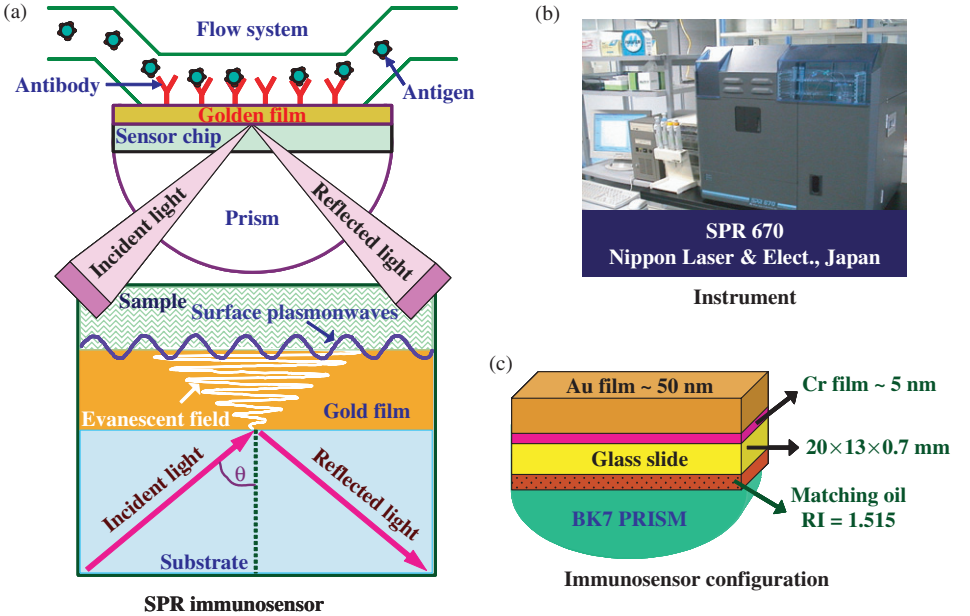

Figure 1. (a) Schematic view of the surface plasmon resonance immunoassay principle, (b) SPR instrument used in the present study, and (c) immunosensor configuration.

At a certain incident light wavelength and angles, a minimum in reflectivity is observed at which the light waves are coupled to the oscillations of surface plasmons at the gold/solution interface. The wavelength at which resonant excitation occurs depends on the refractive index of the analytes in close proximity to the SPR surface. The relationship between the SPR signal and the refractive index and/or thickness changes enable SPR to measure accurately the adsorption of the molecules on the metal surface and their eventual interactions with specific ligands.

We used the SPR 670 instrument manufactured by Nippon Laser and Electronics (Nagoya, Japan) in the present immunoassay study. The SPR instrument was equipped with an automatic flow injection system to allow the introduction of solutions to the gold surface. This contains an internal thermostat for temperature compensation. The instrument contains an inbuilt buffer-degassing mechanism. The gold chip was attached to the prism of the SPR instrument using a refractive index matching liquid. The gold chips for SPR measurements were prepared in our laboratory using BK7 type microscopic glass slides (size = $20 \times 13 \times 0.7$ mm) by high-vacuum sputtering of 5 nm of chromium (adhesive promoting) followed by 50 nm of high-purity gold on the well-cleaned glass slides.

2.3 *Preparation of TNPh–KLH conjugate*

TNPh–KLH conjugate was prepared in one of our laboratories as follows; 2,4,6-trinitrobenzene sulfonate sodium salt (1 mg mL^{-1} H_2O) was reacted with 1 mL of 480 mM $NaHCO_3$ solution (pH 8.5) containing 10 mg KLH for 2 h at 40°C. After the reaction, the conjugate mixture was dialysed in H_2O at 4°C for 2 days. The TNPh–KLH conjugate thus produced was then lyophilized and stored at −20°C.

2.4 *Immunosensor fabrication*

In the present work, two different methods such as physical adsorption and self-assembly were used to prepare the immunosensors. Figure 2 shows a simple scheme of the immunosensor fabrication by (a) physical adsorption and (b) self-assembly methods. A TNPh–KLH conjugate prepared by our group was used for the construction of the immunosensor by physical adsorption. For self-assembly, PEG-NH_2 thiolate was used as a functional monolayer linker molecule. A TNT derivative (TNPh-β-alanine) was attached over the PEG-NH_2 SAM surface by the amide coupling method. A detailed description on the immunosensor fabrication by these methods is given in section 3.

2.5 *Immunoassay principles*

In general, four different principles such as direct, sandwich, displacement, and indirect competitive inhibition were used in immunoassays depending on the nature of the target analyte, analytical sample, instrumental sensitivity, and application [14]. In direct immunoassays, antibodies are immobilized on the sensor surface and subjected to the binding interaction with the analyte of interest. The change in resonance angle due to the binding interaction between the analyte and the antibody is directly proportional

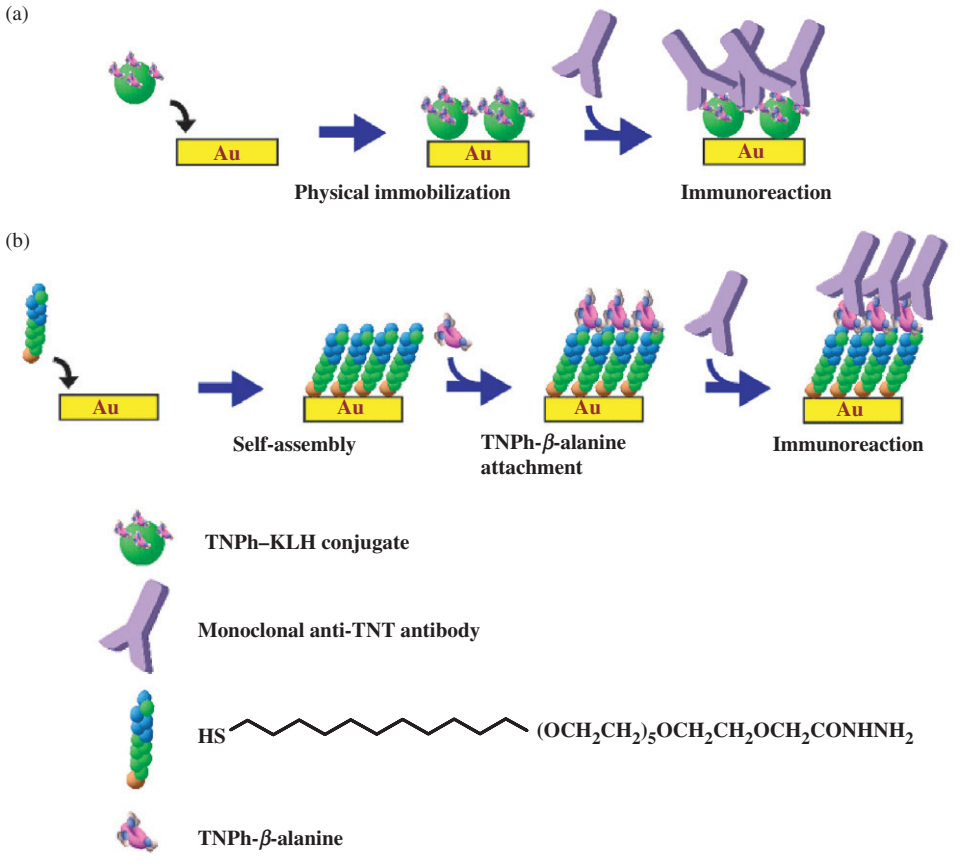

Figure 2. Schematic view of immunosensor fabrication by (a) physical adsorption and (b) self-assembly methods.

to the concentration of analyte. The sandwich assay generally consists of two recognition steps. In the first step, an antibody immobilized on a transducer surface is allowed for binding with an analyte of interest. In the second step, a secondary antibody is allowed to flow over the sensor surface to bind with the previously captured analyte. In the displacement assay format, an excess of labelled analyte is introduced over an antibody-immobilized surface to occupy all the binding sites of the antibody. Upon introduction of an unlabelled analyte, displacement of the labelled analyte occurs. In general, in these immunoassay formats, the biomolecules need to be labelled and are measured by fluorescence or chemiluminescence methods. However, in an indirect competitive inhibition assay, the reagents do not need to be labelled, and the method is highly sensitive for low-molecular-weight analytes [25–27]. This principle generally involves immobilizing the low-molecular-weight analyte (antigen) on the sensor surface. The analyte is then mixed with the respective antibody and introduced over the analyte-immobilized surface. The concentration of the antibody is kept constant so that the response variations are proportional to the amount of analyte mixed with the antibody. An increase in the resonance angle occurs when the antibody binds with the conjugate immobilized on the surface. However, when an equilibrium

mixture of antibody and analyte is allowed to flow over the conjugate, only the unbound antibody in the equilibrium mixture can be available for binding to the conjugate surface, and hence a decrease in the resonance angle is observed. The measured binding response is, therefore, inversely proportional to the concentration of analyte in solution.

3. Results and discussion

3.1 *Immunosensor fabrication by physical adsorption*

Fabrication of a stable and highly effective sensing surface is an important aspect in immunoassays. For physical adsorption, a home-made TNPh–KLH conjugate was allowed to flow over the SPR gold surface for 10 min. Figure 3 shows the SPR response observed for the flow of 200 μg mL^{-1} TNPh–KLH conjugate over the gold surface. A smooth increase in the resonance angle indicates the adsorption of the conjugate on the gold surface. The immobilized conjugate biofilm was highly stable, as can be seen from the SPR response, which remained almost stable after completion of the injection and during the flow of the carrier buffer (PBS) solution. In immunoassays, non-specific adsorption of proteins or irrelevant compounds on the sensor surface is an important issue which causes error in the measurement. In our study, we have avoided this problem by the injection of an excess of BSA over the conjugate immobilized surface to occupy any of the adsorption sites left available on the gold surface. In the case of a self-assembly method, the flow of BSA is not necessary because SAMs (in particular, PEG based SAMs) are highly effective in minimizing the non-specific adsorption.

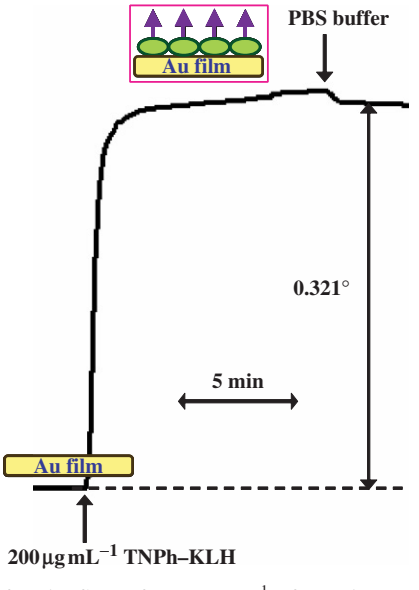

Figure 3. Response transient for the flow of 200 μg mL^{-1} of TNPh–KLH conjugate over an SPR gold surface. Carrier solution: PBS buffer; flow duration: 10 min.

3.2 *Immunosensor fabrication by self-assembly*

In the self-assembly method, methanol was used as a carrier solution during the formation of SAM of PEG-NH_2 on the gold surface. In figure 4, curve (a) corresponds to the flow of 1 mM PEG-NH_2 in methanol over the gold surface. As can be seen, the resonance angle increased smoothly with time during the flow of the PEG-NH_2 over the gold surface, which indicates the formation of a well-ordered monolayer on the gold surface. It is expected that the PEG-NH_2 self-assembles on the gold surface with an amine end-group which could be used to attach TNPh-β-alanine. After the formation of the PEG-NH_2 SAM on the gold surface, the carrier solution was changed to 10 mM Tris buffer (pH 8.0) for immobilization of TNPh-β-alanine. Prior to the injection, the TNPh-β-alanine was activated with *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride) (EDC) and *N*-hydroxysuccinimide (NHS) for effective immobilization on the amine-terminated thiolate (PEG-NH_2) monolayer surface. Here, 2.5 $\mu g\,mL^{-1}$ of TNPh-β-alanine was mixed with 1000 $\mu g\,mL^{-1}$ each of EDC and NHS, and the mixture was allowed to flow over the PEG-NH_2 SAM surface for 14 min. Curve (b) in figure 4 indicates the immobilization of TNPh-β-alanine over the self-assembled surface. The increase in the resonance angle indicates the immobilization of TNPh-β-alanine on the SAM surface, which was confirmed by its specific interaction with M-TNT Ab.

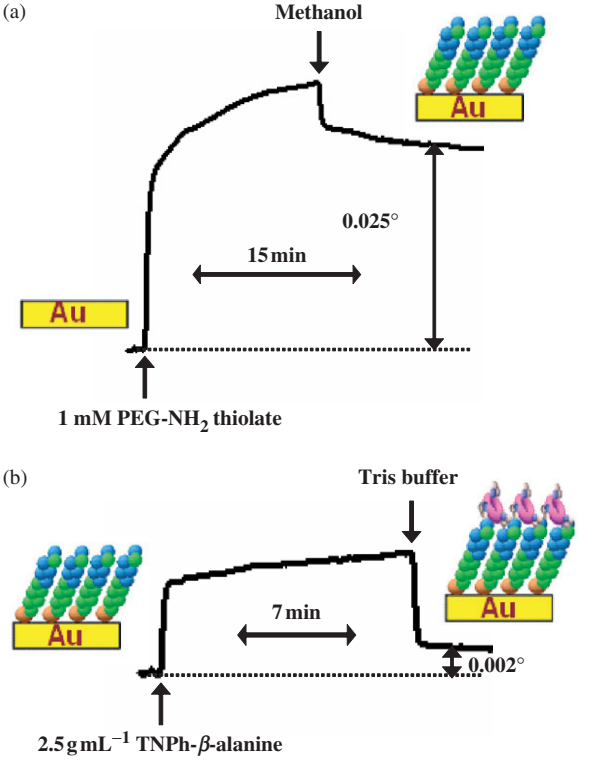

Figure 4. (a) SPR response for the flow of 1 mM PEG-NH_2 thiolate in methanol over an activated gold surface. Carrier solution: methanol, flow duration 14 min. (b) SPR response for the immobilization of TNPh-β-alanine via amide coupling reaction. 2.5 $\mu g\,mL^{-1}$ of TNPh-β-alanine was flowed over the PEG-NH_2 SAM surface. Carrier solution: 10 mM Tris buffer (pH 8.0); flow duration: 14 min.

3.3 *Immunoreaction*

The immunosensor surfaces fabricated by physical adsorption and self-assembly were examined for their binding interaction with M-TNT Ab. Figure 5 depicts the immunoreaction of a TNPh–KLH conjugate immobilized surface with different concentrations of the M-TNT Ab. The increase in the resonance angle during the flow of the antibody indicates the binding interaction between TNPh–KLH conjugate and M-TNT Ab. It can also be noted that the resonance angle shift increases with increasing concentration of the antibody. After each immunoreaction, the conjugate surface had been regenerated by the flow of $5\,\mu g\,mL^{-1}$ of pepsin solution (pH 2.0, glycine–HCl buffer). The pepsin solution dissociates the antigen–antibody immunocomplex while keeping the activity of the conjugate, which can be evidenced from the identical baseline level before the sample injection and after regeneration. The TNPh-β-alanine immobilized SAM surface showed an almost similar binding interaction with M-TNT Ab. The results indicate that both the immunosensor surfaces are highly interactive with M-TNT Ab with a good reproducibility and stability. In fact, the immunosensor prepared by the self-assembly method showed very high stability, retaining its original response on multiple analysis by surface regeneration for more than 100 cycles.

3.4 *Detection of TNT by indirect competitive immunoassay*

For the detection of TNT by an indirect inhibition assay format, a known concentration of antibody (M-TNT Ab) is mixed with TNT solution and injected

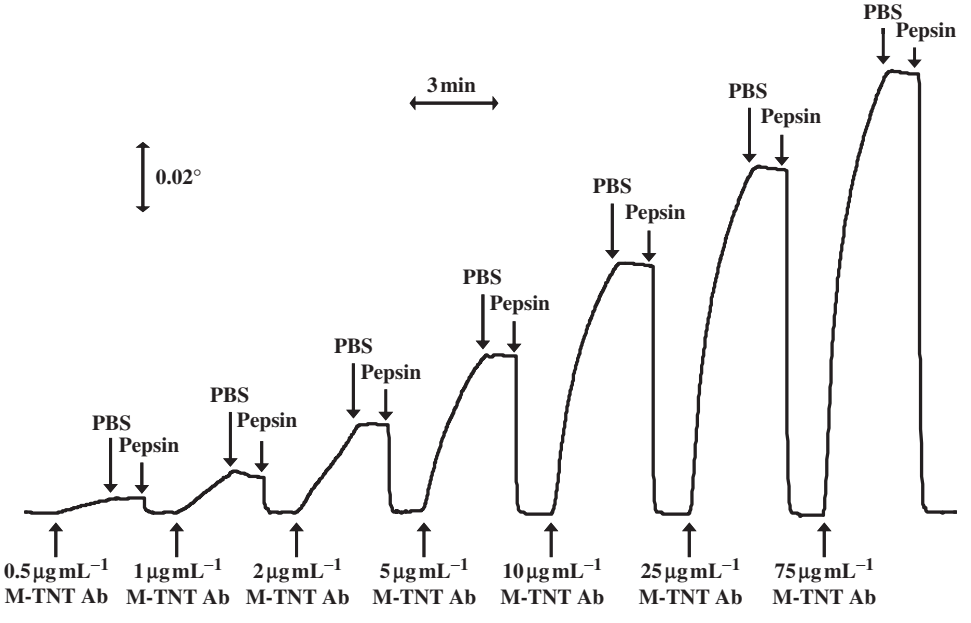

Figure 5. SPR response for the immunoreaction of TNPh–KLH conjugate with different concentrations of M-TNT Ab. Carrier solution: PBS buffer; flow duration: 2 min. The immunosurface was regenerated by the injection of pepsin solution.

over the immunosensor surface. It is expected that the TNT in solution inhibits the binding of the M-TNT Ab to the immobilized TNT on the surface, and the level of inhibition is proportional to the concentration of TNT present in solution. We prepared standard TNT solutions in the concentration range from 0.01 pg mL^{-1} to 1000 ng mL^{-1} in PBS solution, each containing 10 µg mL^{-1} of M-TNT Ab. The mixtures were allowed to incubate for about 10 min before injection over the sensor surface. After each competitive immunoreaction, the surface was rinsed with a running PBS buffer and then regenerated by the injection of pepsin solution. It was observed that the resonance angle shift decreased progressively with increasing concentration of TNT. Figure 6 depicts the sensorgram (overlaid) obtained for the detection of TNT using the immunoreaction between physically immobilized TNPh–KLH conjugate and M-TNT Ab. Curve (a) corresponds to the immunoreaction of M-TNT Ab with TNPh–KLH conjugate in the absence of TNT. Curves (b) and (c) correspond to the immunoreaction in the presence of 0.01 and 1 ng mL^{-1} TNT. The response time is only 36 s, and a single immunocycle could be done within 2 min including surface regeneration enabling rapid detection of TNT. From the results of the indirect immunoassay studies, we observed a linear detection range between 8 parts per trillion and 400 parts per billion with a physically immobilized surface, while a concentration range from 9 parts per trillion to 30 parts per billion was observed with the

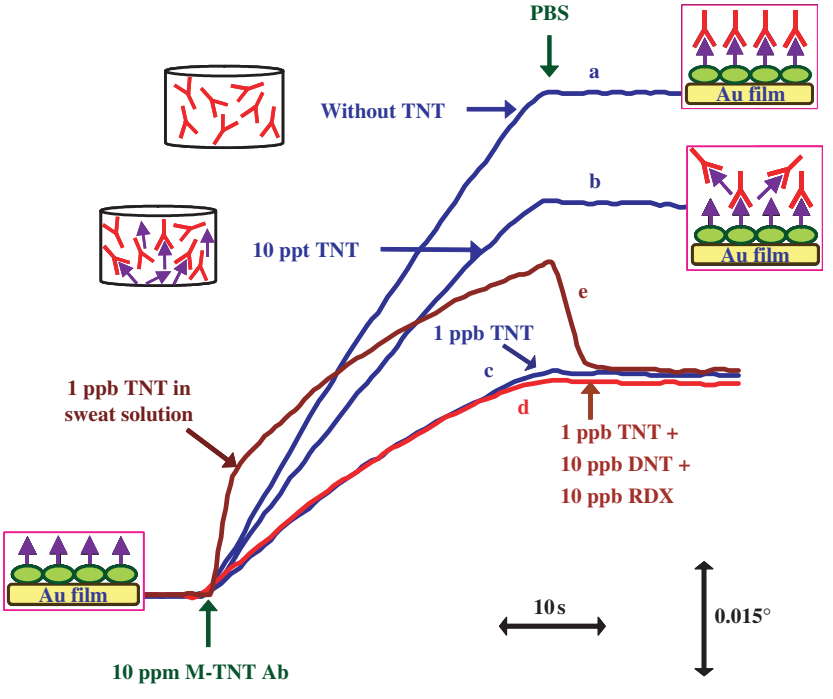

Figure 6. SPR response for the immunoreaction between TNPh–KLH conjugate and M-TNT Ab in the absence (a) and in the presence of 0.01 ng mL^{-1} (b) and 1 ng mL^{-1} TNT (c). Curve (d) corresponds to the flow of 1 ng mL^{-1} of TNT containing 10 ng mL^{-1} each of RDX and 2,4-DNT, and curve (e) corresponds to the flow of 1 ng mL^{-1} of TNT in the sweat ingredients. Carrier solution: PBS buffer; flow duration: 36 s. The immunosurface was regenerated by the injection of pepsin solution.

self-assembled immunosurface ($S/N = 3$). Both the physically adsorbed and self-assembled surfaces showed almost similar lower detection limits with different detection ranges. The difference in the sensitivity pattern is possibly due to the difference in the surface morphology between two immunosurfaces. It is important to note that the sensitivity and the assay speed are highly comparable with the other label-based immunosensors reported for TNT with different sensor configuration and sensing principles [7–12].

The specificity of the immunoassay was evaluated by measuring the cross-reactivity from other related nitroaromatic compounds. Curve (d) in figure 6 corresponds to the immunoreaction for the detection of $1\,\mathrm{ng\,mL^{-1}}$ of TNT in the presence of a tenfold excess of RDX and 2,4-DNT, which showed no appreciable change in the resonance angle shift. We observed that most of the related nitroaromatic compounds showed much less cross-reactivity for detection of TNT which indicate good specificity of the immunoassay. In aviation security, baggage screening is a routine process, and the most likely interference is sweat from the human body. Hence, we checked the interference effect from a sweat solution (NaCl (0.65%) + urea (0.08%) + lactic acid (0.03%)) in the detection of TNT in our system. In figure 6, curve (e) corresponds to the injection of $1\,\mathrm{ng\,mL^{-1}}$ of TNT solution containing the sweat ingredients. The response curve indicates that the presence of the sweat solution did not alter the signal response except the bulk effect. The bulk effect is possibly due to the difference in the refractive index between the PBS and the sweat solution. Thus, the proposed system could be employed in aviation security after optimization and miniaturization.

4. Conclusions

In this work, we constructed two different immunosensors by physical adsorption and self-assembly methods for detection of TNT by the SPR technique. Both immunosensors showed good specificity and sensitivity for TNT detection with good reproducibility. The immunosensor fabricated by self-assembly showed remarkable stability retaining its original response for more than 100 cycles. Moreover, the present label-free, real-time immunosensors offer a number of advantages with respect to analytical speed, throughput, reliability, and flexibility compared with other methods. The results suggest that our immunosensors provide exciting opportunities for on-site screening of TNT in aviation security and environmental monitoring. However, development of the portable systems for commercial applications is yet to be achieved and is under progress in our laboratory.

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