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Highly Sensitive Detection of 2,4,6-Trinitrotoluene (TNT) Using Poly(vinylamine-*co*-*N*-vinylformamide)-Based Surface Plasmon Resonance (SPR) Immunosensor

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In this paper, we describe the fabrication of a novel poly-(vinylamine-*co*-*N*-vinylformamide) (poly-(VAm-*co*-NVF))-based surface plasmon resonance (SPR) sensor chip supported by a self-assembled monolayer (SAM) of alkanethiol and its use in the highly sensitive detection of 2,4,6-trinitrotoluene (TNT), which is a typical explosive. 2,4-dinitrophenyl-glycine (DNP-Gly) was immobilized to amino groups on the side chain of poly-vinylamine (poly-VAm) on the sensor surface. The fabricated surface showed a high response for flow of anti-TNT antibodies. However, it was found that the response included the nonspecific adsorption of anti-TNT antibodies, because unreacted amino groups of the poly-VAm were positively charged and it caused nonspecific adsorption due to electrostatic interaction. Then, the number of amino groups was reduced by making low-hydrolyzed poly-*N*-vinylformamide(poly-NVF), i.e., poly(VAm-*co*-NVF) –poly-NVF is a precursor of poly-VAm, and nonspecific adsorption was controlled. The limit of detection (LOD) of TNT was 28 ppt using the sensor chip fabricated with 23% hydrolyzed poly-(VAm-*co*-NVF) by inhibition assay.

1. Introduction

There is increasing concern about terrorist attacks since the terrorist attacks in the USA on September 11, 2001. Concerning security check at airports, metal detectors, X-ray inspection apparatus, and trained dogs are used. However, there are merits and demerits from the viewpoints of sensitivity, reliability, and cost. A sensor that can detect

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an explosive molecule with cheapness, high sensitivity, high speed, and high reliability is required.

The detection of explosives has also been tried by various methods. In one of the methods, the detection was carried out using a quenching of fluorescence by the nitroaromatic explosives.[1,2] The advantage of the method is that explosives can be detected in air. In another method, the detection was performed by local surface plasmon resonance (SPR) using an in-printed nanostructure of gold nanoparticles.[3] The advantage of the method is ultrahigh sensitivity to TNT. Besides these methods, the electrochemical method by the chemical adsorption between the SiO_2 nanostructure and the nitroaromatic components was developed.[4] The advantage of the method is that the instrument for detection is simple and suitable for constructing inexpensive and portable detectors. Indeed, efforts have been considerably made for the detection of TNT.

We realized an ultrahigh-sensitivity TNT sensor using a SPR sensor and antigen-antibody interaction. The SPR sensor, which has a high sensitivity for refractive index change on a thin gold film surface, can detect the binding of a substance on a surface. The sensor can detect explosive substances with high sensitivity and high selectivity when the appropriate structure for antigen-antibody interaction is fabricated on the surface. Highly sensitive detection of TNT is attained with a low concentration of anti-TNT antibody. On the other hand, when the concentration of the anti-TNT antibody is low, the binding amount of the antibody to the surface becomes low, and then the output of the sensor decreases. Therefore, a high binding capacity of the anti-TNT antibody to the surface is also required. In other words, the surface should have many binding sites for the anti-TNT antibody for the highly sensitive detection of TNT.

For the past few years, we have been developing a highly sensitive SPR immunosensor for TNT.[5–7] However, the number of binding sites of an anti-TNT antibody cannot be increased anymore, because the binding sites are arranged in two dimensions on the SPR sensor surface. Therefore, a dendron-modified SPR sensor surface was fabricated for increasing the number of binding sites –the binding sites were arranged in three dimensions.[8]

In this study, we tried to fabricate a sensor surface with an increased number of binding sites by arranging a TNT analogue in three dimensions using a straight-chain polymer, polyvinylamine (poly-VAm), which has an amino group in the side chain. The TNT analogue can be reacted with the amino group of the poly-VAm immobilized on a Au sensor surface via a self-assembled monolayer (SAM). Poly(vinylamine-*co*-*N*-vinylformamide) (Poly(VAm-*co*-NVF) can be obtained by controlling the hydrolysis rate of poly-*N*-vinylformamide (NVF). We investigated the characteristics of nonspecific adsorption of the poly-VAm and poly-(VAm-*co*-NVF)-based surface, and the Au sensor surface was optimized using low-hydrolyzed poly-NVF.

2. Experimental Methods

2.1 *Materials*

PEG6-COOH aromatic dialkanethiol was purchased from Senso Path Technologies (Bozeman, MT, USA) to form SAM with a carboxyl group end. *N*-hydroxysuccinimide

(NHS), 1-ethyl-3-(3'-dimethylaminopropyl) carbodiimide hydrochloride (EDC), and ethanolamine HCl were obtained as an amine coupling kit from GE Healthcare Bio-Sciences (Uppsala, Sweden). *N*-vinylformamide (NVF) and VA-044 (2, 2' -Azobis [2-(2-imidazolin-2-yl) propane] dihydrochloride), which is an initiator of radical polymerization, were purchased from Wako Pure Chemical Industries (Osaka, Japan) to synthesize poly-vinylformamide (poly-NVF), which is a precursor of poly-VAm. A dialysis tube (Spectra/Por MWCO: 3500) was obtained from Spectrum Laboratories (CA, USA) to purify the polymer. A vacuum freeze dryer (FDU-1200) was prepared from Tokyo Rikakikai (Tokyo, Japan) to lyophilize the polymer solution. 2,4-dinitrophenylglycine (DNP-Gly) was purchased as a TNT analogue from Tokyo Chemical Industry (Tokyo, Japan). The mouse anti-TNT monoclonal antibody (anti-TNT antibody) was obtained from Strategic Biosolutions (DE, USA). A TNT aqueous solution of 21.8 ppm was purchased from Chugoku Kayaku (Hiroshima, Japan). Bovine serum albumin (BSA, Wako Pure Chemical Industry), lysozyme (Sigma Aldrich, MO, USA), and rabbit anti-biotin polyclonal antibody (Bethyl Laboratories, TX, USA) were used in order to evaluate nonspecific adsorption. Biacore J (GE Healthcare Bio-sciences) was prepared for the SPR sensor. Zetasizer Nano (Malvern Instruments, Worcestershire, UK) was prepared to measure the zeta potential. Frontier Gold FTIR (PerkinElmer, MA, USA) was prepared to analyze the polymer by Fourier transform infrared spectroscopy. All the aqueous solutions were prepared from Milli-Q water obtained from Milli-Q system (Millipore, MA, USA).

2.2 *Synthesis of poly-VAm*

Poly-VAm cannot be synthesized from a monomer and is obtained from the hydrolysis of poly-NVF, which is made from a monomer.[9] Figure 1 shows the synthesis procedure of poly-VAm. First of all, NVF and VA-044 were dissolved in Milli-Q water, and each of the solutions was degassed for 1.5 h. The solutions were mixed at a molar ratio of NVF:VA-044 = 3000:1. The polymerization was performed for 24 h at 60° C, and poly-NVF was obtained. Next, the poly-NVF was dissolved in 25% ethanol aqueous solution. Then, when acetone was added, a resinlike polymer was precipitated. After that, the resinlike polymer was dissolved in water. After the polymer solution was dried with a vacuum freeze dryer, a spongelike poly-NVF was obtained. Next, the poly-NVF was dissolved in 2 M NaOH solution at 2 wt%. The hydrolysis of poly-NVF was performed for 4 h at 80°C, and a poly-VAm solution was obtained. After that, the solution was neutralized and refined by dialysis. Finally, a spongelike poly-VAm was obtained after lyophilization using a vacuum freeze dryer.

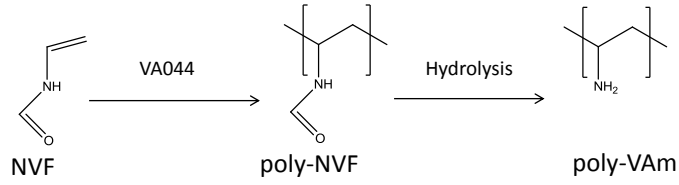

Fig. 1. Synthesis of poly-vinylamine.

2.3 *Fabrication of sensor chip surfaces*

SIA Kit Au (GE Healthcare Bioscience), which contains sensor chips with an unmodified gold layer of ca. 50 nm thickness, was used for the immobilization of various reagents on the surface. Figure 2 shows the fabrication procedure of the poly-VAm-based sensor surface. First of all, the sensor chip was cleaned in a mixed solution of Milli-Q water, ammonia solution, and hydrogen peroxide with a 5:1:1 volume ratio at 90 °C for 20 min. After that, the sensor chip was immersed in 1 mM PEG6-COOH aromatic dialkanethiol (in ethanol) for 24 h at 18°C to form a self-assembled monolayer (SAM) with a carboxyl terminal group. Next, the sensor chip was immersed in a mixed solution of 0.4 M EDC (in water) and 0.1 M NHS (in water) with a 1:1 volume ratio for 60 min; the carboxyl terminal groups of the SAM were converted to NHS esters. Then, the chip was immersed in 50 mg/ml poly-VAm solution (in water) for 60 min; amino groups of poly-VAm reacted with NHS esters and poly-VAm immobilized on the surface. After the reaction, the chip was immersed in ethanolamine HCl for 15 min to block unreacted NHS esters. In parallel with the reactions, a mixed solution of 0.1 M NHS (in DMF), 0.4 M EDC (in water), and 10 mM DNP-Gly (in DMF) with a 1:1:1 volume ratio was

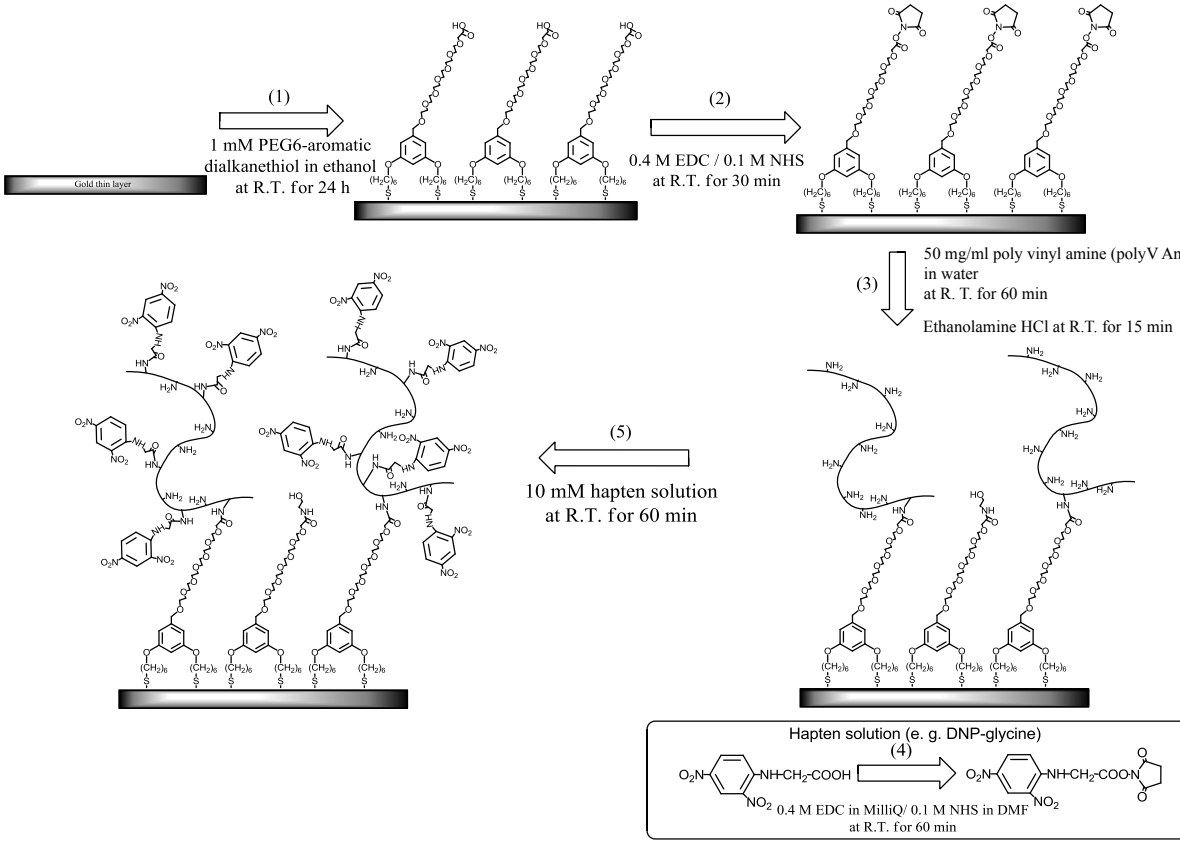

Fig. 2. Fabrication procedure of the polymer-based sensor surface.

reacted for 60 min; the activated DNP-Gly solution was prepared. Finally, the chip was immersed in the activated DNP-Gly solution for 60 min to combine the amino group of poly-VAm and the carboxyl group of DNP-Gly. The sensor chip that has binding sites of an anti-TNT antibody on polymer was completed.

2.4 *Instrument and conditions for SPR*

Biacore J was used for the SPR measurement. The measurements were performed using the flow cell equipped in Biacore J. HBS-T (10 mM 2-[4[-(2-hydroxyethyl)-1-piperazinyl]-ethane sulfonic acid, 150 mM NaCl, 0.05% Tween 20: pH 7.4) was used as a running buffer solution. The measurements were conducted at a constant temperature of 25°C. The flow rate of the solution was 30 or 10 μl/min. When the antibody solution is allowed to flow over the DNP-Gly immobilized on the chip surface, the anti-TNT antibody is bound to the DNP-Gly. The SPR sensor can measure a change in refractive index. The SPR response, which is defined as "resonance unit (RU)", increased with an increase in the amount of antibody bound on the surface. A resonance angle shift of 0.1º is defined as 1,000 RU and is equivalent to a change in mass of 1 ng/mm2 on the surface.[10] In most of the experiments, dissociation of surface-bound antibodies for regeneration was completed when 50 mM NaOH or 3 M NaCl was allowed to flow over the surface.

2.5 *TNT detection using the inhibition assay*

TNT detection was carried out by inhibition assay.[11] An antibody solution of known concentration was mixed with a TNT solution and incubated at room temperature for about 30 min. An anti-TNT antibody was interacted by specific binding with TNT during incubation. After that, when the mixed solution was allowed to flow over the chip, the anti-TNT antibodies that did not react with TNT were bound to the DNP-Gly on the chip. The sensor response was obtained by the binding of anti-TNT antibodies to the DNP-Gly on the chip. The TNT concentration of a sample solution is determined from the ratio between a response of an antibody solution without TNT and that with TNT.

3. Results and Discussion

3.1 *Binding capacity of polyVAm-immobilized surface*

The antibody solution of 25 ppm concentration (in HBS-T) was allowed to flow over the poly-VAm-immobilized surface for 2 min. Figure 3 shows the sensor response. The solid line shows the SPR sensor response on the poly-VAm-based surface, and the response was 11,187 RU. The dashed line shows the SPR sensor response of a surface-immobilized DNP-Gly via ethylenediamine (EDA) instead of poly-VAm. EDA is a low-molecular-weight compound and has an amino group at both ends. The response on the EDA-based surface was 1,698 RU. The poly-VAm-based surface shows about 6.6-fold higher binding response than the EDA (nonpolymer)-based surface. This result suggests that the amount of sensor response depends on the density of the surface-bound DNP-Gly. It also supported the success of the fabrication of the three-dimensional structure on the sensor surface.

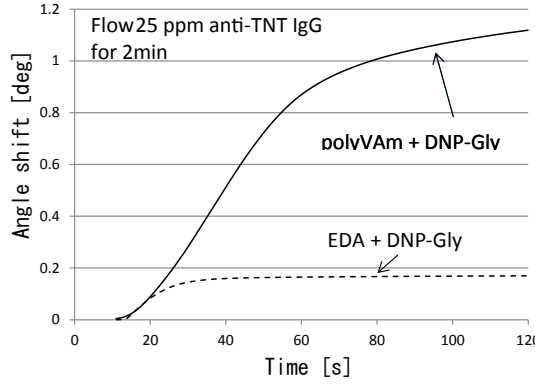

Fig. 3. SPR sensorgram: polyVAm: three-dimensional, EDA: two-dimensional.

3.2 *Characteristics of nonspecific adsorption on poly-VAm-based surface*

An anti-TNT antibody has two antigen binding sites that bind specifically with TNT or a TNT analogue. The concentration of TNT is determined from the interaction between the immobilized TNT analogue and the anti-TNT antibody on the sensor surface –the anti-TNT antibody should be able to adsorb onto the surface only using the binding sites. Hence, if nonspecific adsorption of the anti-TNT antibody occurs, it is difficult to determine the TNT concentration, particularly if the concentration is low. Therefore, we evaluated the characteristics of nonspecific adsorption on the poly-VAm-based surface.

The amount of adsorption to the surface was measured by SPR, when 25 ppm anti-TNT antibody solution and 25 ppm anti-biotin antibody solution were allowed to flow over the chip for 2 min. The result is shown in Fig. 4. The response of the anti-TNT antibody was 14685 RU. The response of the anti-biotin antibody was 4834 RU. The anti-biotin antibody cannot be bound specifically on the surface because biotin does not exist in the sensor chip surface. Therefore, this result shows that nonspecific adsorption occurred considerably on the poly-VAm-based surface. Thus, it was suggested that the response of the anti-TNT antibody (Fig. 4) included the response of nonspecific adsorption.

The amounts of adsorption of BSA and lysozyme to the surface were measured by SPR because hydrophobic interaction and electrostatic interaction are known as the mechanisms of nonspecific adsorption.[12] BSA and lysozyme are proteins and cannot be bound specifically to the sensor surface. BSA is charged negatively (–20.2 mV) and lysozyme is charged positively (+22.3mV) in a solution of pH 7.0, according to the results of zeta potential measurement. Figure 5 shows the SPR sensor responses when solutions of 1,000 ppm BSA and lysozyme were allowed to flow over the chip for 2 min. The response of lysozyme was 377 RU. The response of BSA was 5,018 RU. Although BSA tended to adsorb on the sensor chip from this result, it turned out that lysozyme barely adsorbs on the chip. It was suggested that the nonspecific adsorption was caused by the electrostatic interaction because the surface was charged positively by protonated

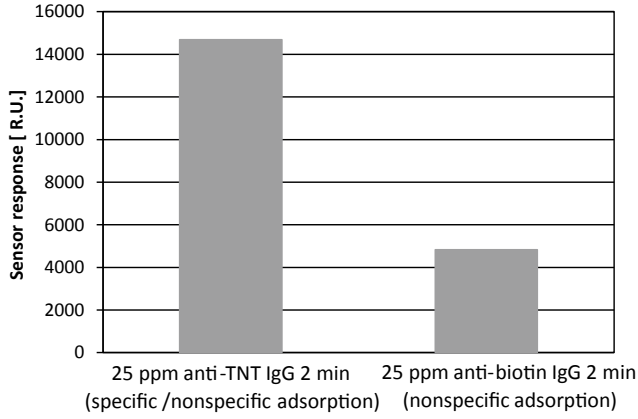

Fig. 4. Adsorption of anti-TNT IgG and anti-biotin IgG.

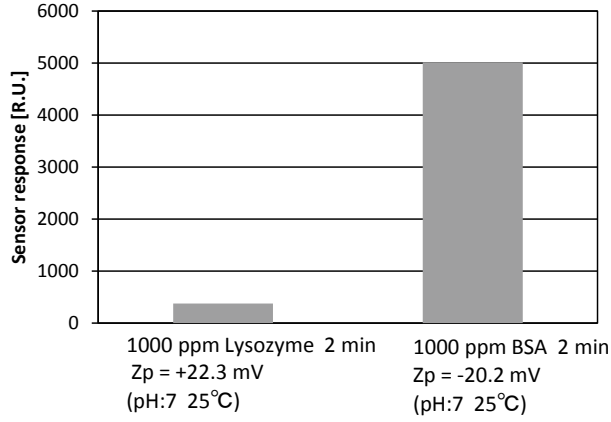

Fig. 5. Adsorption of lysozyme and BSA.

amino groups, which did not react with the activated DNP-Gly.

3.3 *Control of nonspecific adsorption on the poly-(VAm-*co*-NVF)-based surface*

The characteristic of nonspecific adsorption on the poly-VAm-based surface was evaluated, when the quantity of the unreacted amino group was reduced. It was expected that the nonspecific adsorption decreases by lowering the hydrolysis of the poly-NVF. The poly-NVF (synthesized at § 2.2: spongelike poly-NVF) was dissolved in 2 M

sodium hydroxide at 2wt%. The hydrolysis was performed for 5, 10, 20, 30, 60, 120, and 240 min at 50°C, and 240 min at 80°C. Figure 6 shows the result analyzed by FTIR (ATR) to calculate each hydrolysis rate of the obtained polymer: poly-(vinylamine-*co*-*N*-vinylformamide), poly-(VAm-*co*-NVF). The C=O stretching peak of the amide linkage of 1650 cm^{-1} decreases as hydrolysis progresses. In contrast, the N-H angle peak of primary amine of 1600 cm^{-1} increases. Figure 7 shows the result of the hydrolysis rate, which was calculated from those peak intensities.

The zeta potential of poly-(VAm-*co*-NVF) depends on the quantity of protonated amino groups. Thus, it depends on the hydrolysis rates of poly-NVF. Figure 8 shows

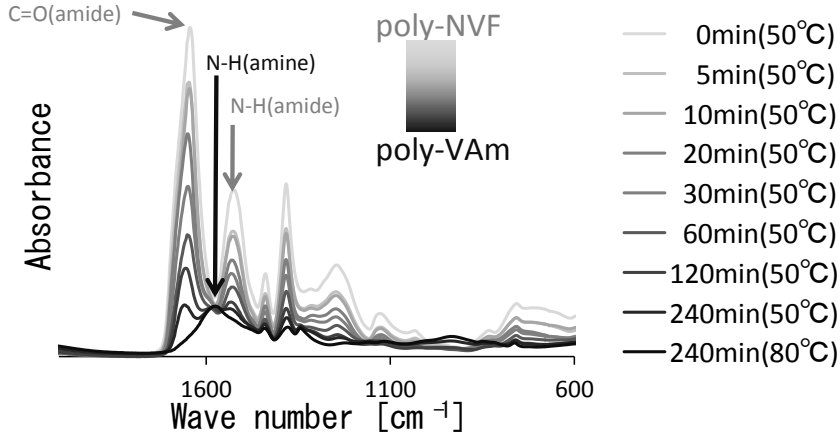

Fig. 6. FT-IR spectra of polyVAm, which changed the hydrolysis condition of poly-NVF.

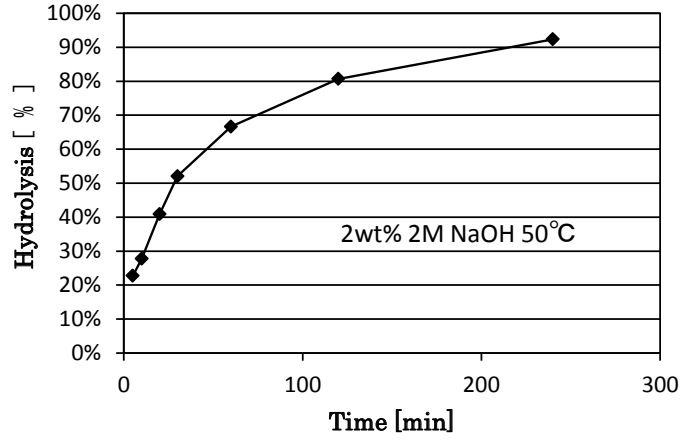

Fig. 7. Time conversion for hydrolysis.

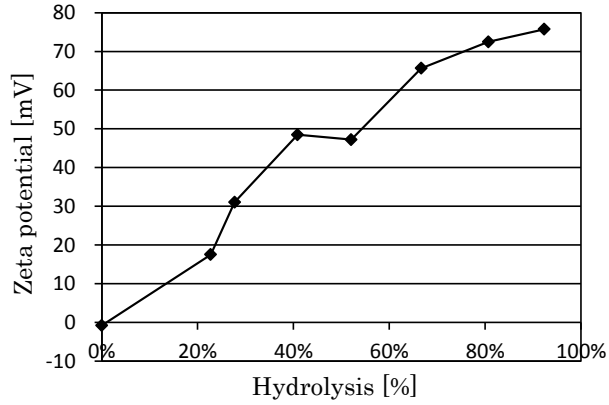

Fig. 8. Hydrolysis dependence of zeta potential.

the result of measuring the 10 mg/ml poly-(VAm-*co*-NVF) solution at pH 7.0 and 25°C. First, the poly-(VAm-*co*-NVF) with barely 100% hydrolysis rate was charged positively (+75.7 mV). Therefore, it is explained that lysozyme was barely adsorbed on the surface although BSA was easily adsorbed. Then, the zeta potential was decreased and approached to 0 mV, when the hydrolysis rate is lowered because the number of protonated amino groups was decreased. Moreover, the zeta potential of poly-NVF was about 0 mV. Thus, nonspecific adsorption was reduced by using poly-(VAm-*co*-NVF) with a low hydrolysis rate.

Figure 9 shows dependences of specific/nonspecific adsorption of anti-TNT antibody and nonspecific adsorption of anti-biotin antibody on each hydrolysis rate of poly-(VAm-*co*-NVF) used in the sensor surface. There are two considerations. First, we focused on the adsorption of the anti-TNT antibody. The amounts of bound anti-TNT antibody were almost unchanged over the 41% hydrolysis rate. Therefore, it is probable that DNP-Gly was immobilized with about 30–40% of the amino group on poly-VAm (100% hydrolyzed poly-NVF). However, the amounts of bound anti-TNT antibody decreased at 28% and 23% hydrolysis rates. It is likely that the amount of DNP-Gly immobilized on poly-(VAm-*co*-NVF) was decreased, because the number of amino groups was low at the low hydrolysis rate. In contrast, since the reaction between the DNP-Gly and the amino group was saturated, the amount of bound anti-TNT antibody was also saturated over the 41% hydrolysis rate. Thus, the unreacted amino groups existed at least when poly-(VAm-*co*-NVF) at the hydrolysis rates of 41%, 52%, 67%, 81%, and 92% was used.

Secondly, we focused on the nonspecific adsorption of the anti-biotin antibody. Figure 9 shows that the amount of nonspecific adsorption of the anti-biotin antibody decreased when poly-(VAm-*co*-NVF) at the low hydrolysis rate was used. Figure 10 shows the adsorption ratios of the anti-biotin antibody and anti-TNT antibody. The ratio is equivalent to the quantity of nonspecific adsorption. The amount of nonspecific adsorption was low, when the low hydrolysis rate was used. Therefore, the surface with

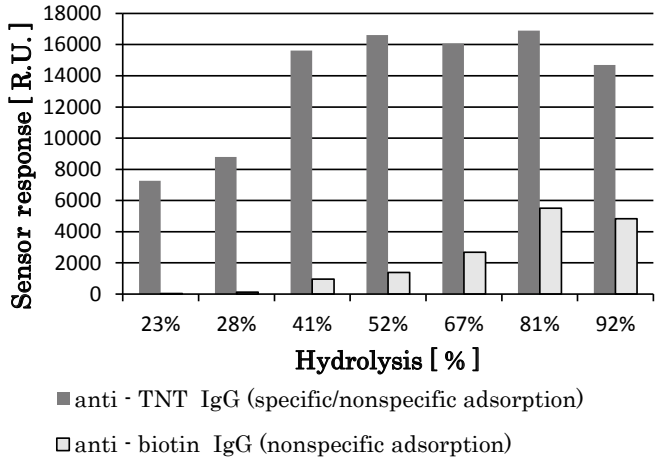

Fig. 9. Hydrolysis dependence of specific/nonspecific adsorption.

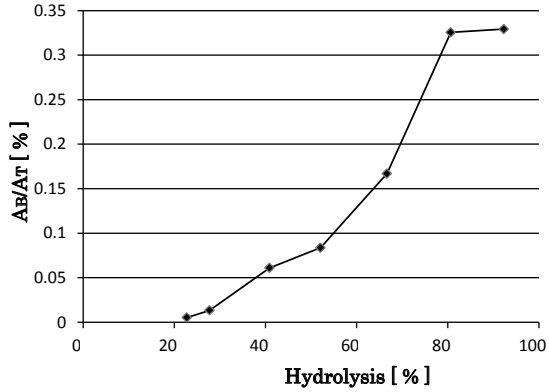

Fig. 10. Ratio of nonspecific adsorption in all the adsorption: AB: adsorption of anti-biotin IgG (nonspecific adsorption), AT: adsorption of anti-TNT IgG (specific/nonspecific adsorption).

the low nonspecific adsorption was realized using a low hydrolysis rate of poly-(VAm-*co*-NVF), because the amount of protonated amino groups was reduced.

3.4 *Highly sensitive detection of TNT*

The sensor chip surface was fabricated using 23% hydrolyzed poly-NVF (VAm-*co*-NVF), and TNT detection was performed by inhibition assay. Figure 11 shows the response curve obtained by the inhibition assay for TNT. The vertical axis indicates bound percentage ($\Delta\theta/\Delta\theta_0$), which is the relative sensor response between the response of the anti-TNT antibody solution with TNT ($\Delta\theta$) and that without TNT ($\Delta\theta_0$). $\Delta\theta_0$ is the

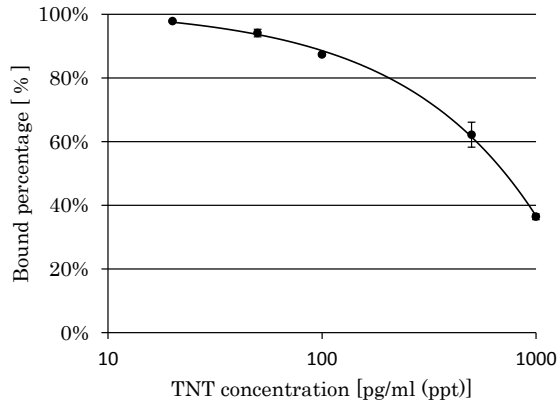

Fig. 11. Response characteristic to TNT: Calibration curves obtained by inhibition assay using 100 ng/ml (100 ppb) anti-TNT antibody. The error bar shows the SD of the data.

sensor response (RU) when the solution of 100 ppb antibody without TNT is allowed to flow for 12 min. $\Delta\theta$ is the sensor response (RU) when a mixed solution of 100 ppb antibody and one of serially diluted TNT solutions is allowed to flow for 12 min. The mixed solution was incubated for 30 min before injection. The percentage is lower at higher TNT concentration, because the anti-TNT antibody bound to TNT cannot be bound to DNP-Gly on the surface. The reference data was averaged from responses before and after a cycle of measurements of the TNT-containing antibody solution. The bound percentage was calculated using the average of three cycle measurements. The error bar shows the standard deviation (SD) of the data. The limit of detection (LOD) was determined to be about 28 ppt by considering 3 SDs of the lowest concentration.

Thus far, we have realized the highly sensitive sensor for TNT using the poly-(VAm-*co*-NVF). It is expected that a higher sensitivity for the detection of TNT is attained by increasing the binding capacity of the anti-TNT antibody from optimizing the lengths of the polymer chain and TNT analogue on the sensor surface.

4. Conclusions

We have fabricated the SPR sensor surface using synthesized poly-VAm for the detection of TNT. Although the surface showed the high capability to bind to the anti-TNT antibody, the capability included not only specific adsorption but also nonspecific adsorption. The adsorption characteristics of BSA and lysozyme to the poly-VAm-immobilized surface showed that the sensor surface was positively charged. It was speculated that the cause of electrification was unreacted amino groups of poly-VAm. The number of unreacted amino groups was decreased by poly-(VAm-*co*-NVF), which was obtained by the low hydrolysis of poly-NVF for reducing the electrification. As a result, it was found that nonspecific adsorption can be controlled by poly-NVF with low

hydrolysis. Then, the response characteristics for TNT were measured using the sensor chip, which was fabricated using 23% hydrolyzed poly-NVF, i.e., poly-(VAm-*co*-NVF). Highly sensitive detection was realized and the limit of detection was 28 ppt.

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