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Flow immunoassay of trinitrophenol based on a surface plasmon resonance sensor using a one-pot immunoreaction with a high molecular weight conjugate

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Abstract

A surface plasmon resonance (SPR) immunosensor based on a competitive immunoreaction for the determination of trinitrophenol (TNP) is described. A goat anti-mouse IgG (1st antibody), which recognizes an Fc moiety of an antibody, was immobilized on a gold film of an SPR sensor chip by physical adsorption. A TNP solution containing a fixed concentration of a mouse anti-TNP monoclonal antibody (2nd antibody) and a TNP-keyhole limpet hemocyanin (KLH) conjugate was incubated in one-pot and introduced into the sensor chip. The TNP-KLH conjugate competes with TNP for binding with the 2nd antibody. The resulting complex of the 2nd antibody with the TNP-KLH conjugate was bound to the 1st antibody, which is immobilized on the sensor chip. The SPR sensor signal based on resonance angle shift is dependent on the concentration of TNP in the incubation solution in the range from 25 ppt to 25 ppb, and the coefficient of variation of the SPR signals for the 25 ppb TNP solution was determined to be 13% ($n = 4$). The experimental results for the adsorption constant of the 1st antibody on the sensor chip and the binding constant of the 1st antibody complex with the 2nd antibody are discussed, together with theoretical considerations.

Keywords: Surface plasmon resonance immunosensor; Trinitrophenol; Competitive immunoreaction

1. Introduction

A great deal of efforts has been devoted to development of a chemical sensor for trinitrotoluene (TNT) and related nitro-aromatic compounds to detect the location of buried landmines, as an alternative to or combined with conventional metal detectors. A number of reports on analytical methods for the determination of TNT and related compounds, such as capillary electrophoresis, fluorescence spectrophotometry, etc., as well as chemical sensors for such explosive compounds [1–20]. Because of its sensitivity and selectivity, a surface plasmon resonance (SPR) immunosensor is would be a promising chemical sensor for such purposes. In our previous papers, an SPR immunosensor for TNT and related compounds based on a competitive immunoassay was reported [21–23]. In a continuation of this research, we reported on attempts to develop an SPR immunosensor by several approaches for enhancing its sensitivity with respect to explosive compounds.

Three approaches can be considered for the SPR determination based on a competitive immunoassay. The first approach involves the immobilization of an antibody on the gold film of an SPR sensor chip. The second is the immobi-

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lization of an antigen (or a conjugate of the target molecule) on a sensor chip and the third is the immobilization of an antibody (1st antibody) that recognizes an Fc moiety of the 2nd antibody on the sensor chip. In our previous paper [24], in an example of the first approach, we utilized a gold binding polypeptide (GBP) and Protein G for the immobilization of an anti-2,4-dichlorophenol antibody on the gold film of the SPR sensor and applied this immobilization technique to the determination of 2,4-dichlorophenol. The immobilization procedures used here led to the orientated immobilization of the antibody, in a stable form, in a high density. Although this procedure is excellent for laboratory use, its use in a field analysis, such as the detection of a landmine, it is complicated and the GBP and Protein G reagents are costly. The second approach is to immobilize the target molecule on the sensor chip. Miura et al. reported on an SPR immunosensor based on this approach for the determination of benzo[*a*]pyrene and other polyaromatic hydrocarbon as well as trinitrophenol (TNP) by using a bovine serum albumin (BSA) conjugate with the target molecule [25–27]. In their method, the BSA conjugate was immobilized on the gold film of the sensor chip by physical adsorption. They pointed out the advantages of this method, in which the sensor chip could be regenerated after the assay by dissociation of the antibody complex with the target molecule-BSA conjugate by a treatment with HCl/glycine solution or a pepsin solution. In our previous paper, a Bisphenol A derivative was immobilized on the gold film by means of an amino-coupling method with the assistance of 2-aminoethanethiol and the immobilization method was used for the determination of Bisphenol A based on the SPR immunosensor [28].

The third approach is to immobilize an antibody (1st antibody) that recognizes the Fc moiety of another antibody (2nd antibody) for a target molecule. The immobilization of an antibody for a target molecule with a high orientation on a solid phase is required for the selective and sensitive binding to the target molecule or its conjugate, when an immunoreaction between the target molecule or its conjugate in an aqueous solution and the antibody adsorbed on the solid matrix is conducted. While, the inherent selectivity between the antibody and target molecule or its conjugate is maintained for the immunoreaction in a homogenous aqueous phase. If an antibody (1st antibody), that recognizes an Fc moiety of the objective antibody (2nd antibody), is immobilized on the SPR sensor chip, even though the immobilization of the 1st antibody is not oriented perfectly, a selective SPR determination of the target molecule is possible by the use of a competitive immunoassay, in which the target molecule and its conjugate competitively bind to the 2nd antibody by incubating in a one-pot reactor and the incubated solution is introduced into the SPR sensor chip. If a conjugate with a molecular weight much higher than that of the 2nd antibody is utilized, a large SPR signal would result, because the sensitivity of the SPR sensor is dependent on the magnitude of the induced mass change on the sensor chip. In this paper, we wish to report on the determination of trinitrophenol (TNP) as a model of TNT based on the third approach, i.e. the use of a high molecular weight conjugate, keyhole limpet hemocyanin.

2. Experimental

2.1. Preparation of reagent solution

A phosphate buffer solution was prepared by dissolving 0.2 g KH_2PO_4, 1.14 g Na_2HPO_4, 8.0 g NaCl and 0.2 g KCl in 1.0 L of deionized water for the preparation of antibody solutions, etc. Goat anti-mouse IgG, an Fc affinity purified polyclonal antibody (abbreviated as the 1st antibody) was purchased from Chimcon International Inc. (USA), and a 2400 ppm solution was diluted to an appropriate concentration with the above the phosphate buffer. The mouse anti-TNP monoclonal antibody (abbreviated as the 2nd antibody) was purchased from PharMingen Inc. (USA), and a 500 ppm solution was diluted to an appropriate concentration with above phosphate buffer solution. The 2,4,6-trinitrophenyl-KLH (keyhole limpet hemocyanin) conjugate (abbreviated as TNP-KLH conjugate) was obtained from Biosearch Technologies, Inc. (USA) and was dissolved in the phosphate buffer to prepare a stock solution. 2,4,6-Trinitrophenol (TNP) and the other reagents were purchased from commercial source and were used without further purification. A polymer solution of poly(methacryloyloxyethyl phosphorylcholine) with a hydrophobic moiety, donated by the Nippon Yushi Co. Ltd. (Japan) was used as a blocking solution.

A sample solution containing 25–50 ppm of the 1st antibody, 100–200 ppm of TNP-KLH conjugate and 25 ppt–25 ppb of TNP and 0.05% Tween 20 was incubated at 25 °C for periods of 5 min to 120 min.

2.2. Apparatus

An SPR system was constructed from an SPR sensor with a flow-cell and a syringe pump (Cavro, 3000X) and an injector (Rheodyne, 7125) with a sample loop (100 μL). The SPR sensor used in this work was developed by some of the authors. The SPR sensor is a dual type, with reference and sample channels. Detailed information on the performance of the SPR sensor has been reported elsewhere [29]. The flow-cell was assembled on the SPR sensor by attaching a silicon sheet with a thickness of 0.5 mm with two grooves (3 mm × 14 mm) between the gold film deposited on a cover glass (sensor chip) and a prism of the SPR sensor. The sensor chip (16 mm × 16 mm × 0.15 mm^t) was obtained from Eliotech Co. (Japan). Matching oil with a reflective index of 1.5616 was coated between the backside of the cover glass of the sensor chip and the prism. The SPR sensor was placed in a thermostated incubator at 25 ± 0.1 °C.

2.3. Protocol

The sensor chip was sonicated for 20 min in acetone and then soaked in a piranha solution (conc. H_2SO_4/30% H_2O_2 = 1/1, v/v) for 15 min. After washing with deinonized water and drying, the sensor chip was then placed in the SRP sensor. A 100 μL aliquot of 100 ppm 1st antibody solution was introduced into the sample channel of the sensor chip from the injector at a flow rate of 20 μL/min by a carrier solution from the syringe pump in order to immobilize the 1st antibody on the sensor chip. The phosphate buffer was used as the carrier solution. After 30 min, 100 μL of the blocking solution was injected into the sensor chip by the carrier solution at a flow rate of 20 μL/min for 30 min. Subsequently, 100 μL of a solution containing the 2nd antibody, TNP and the TNP-KLH conjugate, prepared prior to the experiment, was injected into the sensor chip at the same flow rate of 20 μL/min for 30 min. The phosphate buffer solution was also pumped into the reference channel at a flow rate of 20 μL/min. During the three injections of each of the solutions, the resonance curves of the SPR sensor from the sample and reference channels were monitored by means of a computer and at the same time, the resonance angle from the sample channel subtracted from the reference channel was monitored by the computer.

3. Theoretical considerations of the present SPR immunoassay

Fig. 1 shows a typical sensor response obtained when the three solutions were sequentially injected, as described in the experimental section. When 100 ppm of the 1st antibody solution was injected ((a) in Fig. 1), the angle shift was increased by about 0.035°, indicating the successful immobilization of the 1st antibody on the sensor chip. When the blocking solution was injected ((b) in Fig. 1), a small angle shift was observed, indicating that any unmodified gold film of the sensor chip was blocked, thus preventing the adsorption of any extraneous substances in the subsequent injected solution. When a solution containing 25 ppm of the 2nd antibody, 250 ppb of TNP and 100 ppm of TNP-KLH conjugate was injected ((c) in Fig. 1), the angle shift was increased by about 0.025°, indicating the binding of the 2nd antibody species to the 1st antibody on the sensor chip.

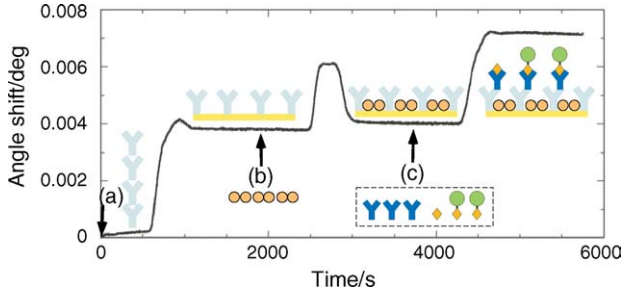

Fig. 1. Typical sensor response to the 1st antibody, blocking reagent and the incubation solution: (a) 100 ppm 1st antibody solution (100 μL), (b) blocking solution (100 μL) and (c) incubation solution containing 25 ppm 2nd antibody, 250 ppb TNP and 100 ppm TNP-KLH (100 μL). Carrier solution: phosphate buffer, pH 7.2, flow rate 20 μL/s.

The overall SPR immunoassay consisted of three steps: (1) an immobilization step for the 1st antibody onto the sensor chip, (2) an incubation step for the 2nd antibody, the TNP-KLH conjugate and TNP and (3) a competitive binding step of the 2nd antibody species in the incubated solution with the 1st antibody immobilized on the sensor chip. The chemical equilibrium in the incubation solution and the competitive binding are considered in this section. The immobilization step is described in Section 4.

The concentrations of the second antibody species, the free antibody, C_{Ab2}, the antibody-TNP complex, $C_{Ab2\text{-}Ag}$, and the 2nd anti-body-TNP-KLH conjugate complex, $C_{Ab2\text{-}AgC}$, can be calculated from the following binding constants and the mass balance concerning with the 2nd antibody, TNP and the TNP-KLH conjugate:

$$\mathrm{Ab2} + \mathrm{Ag} \rightarrow \mathrm{Ab2\text{-}Ag}$$

$$K_1 = \frac{C_{\mathrm{Ab2\text{-}Ag}}}{C_{\mathrm{Ab2}} C_{\mathrm{Ag}}} \tag{1}$$

$$\mathrm{Ab2} + \mathrm{AgC} \rightarrow \mathrm{Ab2\text{-}AgC}$$

$$K_2 = \frac{C_{\mathrm{Ab2\text{-}AgC}}}{C_{\mathrm{Ab2}} C_{\mathrm{AgC}}} \tag{2}$$

where K_1 and K_2 are the binding constants of the 2nd antibody-TNP complex and the 2nd antibody-TNP-KLH conjugate complex, respectively.

$$C^{\mathrm{T}}_{\mathrm{Ab2}} = C_{\mathrm{Ab2}} + C_{\mathrm{Ab2\text{-}Ag}} + C_{\mathrm{Ab2\text{-}AgC}} \tag{3}$$

$$C^{\mathrm{T}}_{\mathrm{AgC}} = C_{\mathrm{AgC}} + C_{\mathrm{Ab2\text{-}AgC}} \tag{4}$$

$$C^{\mathrm{T}}_{\mathrm{Ag}} = C_{\mathrm{Ag}} + C_{\mathrm{Ab2\text{-}Ag}} \tag{5}$$

where $C^{\mathrm{T}}_{\mathrm{Ab2}}$, $C^{\mathrm{T}}_{\mathrm{AgC}}$ and $C^{\mathrm{T}}_{\mathrm{Ag}}$ are the total concentrations of the 2nd antibody, the TNP-KLH conjugate and TNP, respectively.

From Eqs. (1)–(5), an equation concerning with the concentration of the free 2nd antibody, C_{Ab2}, is derived.

$$\begin{aligned} &K_1K_2C^3_{\mathrm{Ab2}} + (K_1K_2(C^{\mathrm{T}}_{\mathrm{Ag}} + C^{\mathrm{T}}_{\mathrm{AgC}} - C^{\mathrm{T}}_{\mathrm{Ab2}}) + K_1 + K_2)C^2_{\mathrm{Ab2}} \\ &+ (K_1C^{\mathrm{T}}_{\mathrm{Ag}} + K_2C^{\mathrm{T}}_{\mathrm{AgC}} - (K_1 + K_2)C^{\mathrm{T}}_{\mathrm{Ab2}} + 1)C_{\mathrm{Ab2}} \\ &- C^{\mathrm{T}}_{\mathrm{Ab2}} = 0 \end{aligned} \tag{6}$$

A suitable root for C_{Ab2} can be obtained by numerical calculation using Eq. (6) by assuming the appropriate binding constants K_1 and K_2 and assuming that the values of the total concentrations of the second antibody, $C^{\mathrm{T}}_{\mathrm{Ab2}}$, the conjugate, $C^{\mathrm{T}}_{\mathrm{AgC}}$, and TNP, $C^{\mathrm{T}}_{\mathrm{Ag}}$, are constant.

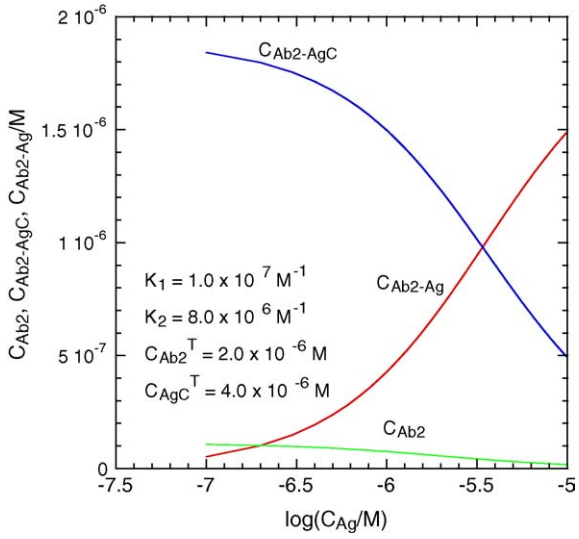

Fig. 2. Concentration of 2nd antibody, 2nd antibody complex with antigen and 2nd antibody complex with the conjugate in the incubation solution as a function of the concentration of the antigen calculated from Eqs. (4) to (6), assuming $K_1 = 1.0 \times 10^7$, $K_2 = 0.8 \times 10^7$ and $C^{\mathrm{T}}_{\mathrm{Ab2}}, = 2.0 \times 10^{-6}$ M, $C^{\mathrm{T}}_{\mathrm{AgC}} = 4.0 \times 10^{-6}$ M.

The concentrations of the 2nd antibody-TNP complex, $C_{\mathrm{Ab2\text{-}Ag}}$, and the 2nd antibody-TNP-KLH conjugate complex, $C_{\mathrm{Ab2\text{-}AgC}}$, were calculated from C_{Ab2} and Eqs. (4) and (5). In Fig. 2, the calculated C_{Ab2}, $C_{\mathrm{Ab2\text{-}Ag}}$ and $C_{\mathrm{Ab2\text{-}AbC}}$ are plotted as a function of the total concentration of TNP when the following values are assumed: $K_1 = 1.0 \times 10^7$, $K_2 = 0.8 \times 10^7$ and $C^{\mathrm{T}}_{\mathrm{Ab2}} = 2.0 \times 10^{-6}$ M, $C^{\mathrm{T}}_{\mathrm{AgC}} = 4.0 \times 10^{-6}$ M. As can be seen from Fig. 2, the concentration of the 2nd antibody-TNP-KLH conjugate complex gradually decreases with increasing concentrations of TNP, while the concentration of the 2nd antibody-TNP complex gradually increases with increasing concentrations of TNP. This indicates that the 2nd antibody in the incubation solution binds competitively to the TNP-KLH conjugate and TNP. In other words, the 2nd antibody preferentially binds to TNP with increasing concentrations of TNP in the incubation solution.

When the incubation solution is introduced into the sensor chip immobilized with the 1st antibody, the 2nd antibody species binds competitively to the 1st antibody on the sensor chip, because the 1st antibody has an affinity for the Fc moiety of the 2nd antibody. The surface concentration of the 2nd antibody species can be estimated from the following binding reactions by assuming a Langmuir isotherm equilibrium:

$$\underline{\mathrm{Ab1}} + \mathrm{Ab2} \rightarrow \underline{\mathrm{Ab1\text{-}Ab2}}$$

$$K_3 = \frac{[\underline{\mathrm{Ab1\text{-}Ab2}}]}{[\underline{\mathrm{Ab1}}]\, C_{\mathrm{Ab2}}} \tag{7}$$

$$\underline{\mathrm{Ab1}} + \mathrm{Ab2\text{-}AgC} \rightarrow \underline{\mathrm{Ab1\text{-}Ab2\text{-}AgC}}$$

$$K_4 = \frac{[\underline{\mathrm{Ab1\text{-}Ab2\text{-}AgC}}]}{[\underline{\mathrm{Ab1}}]\, C_{\mathrm{Ab2\text{-}AgC}}} \tag{8}$$

$$\underline{\mathrm{Ab1}} + \mathrm{Ab2\text{-}Ag} \rightarrow \underline{\mathrm{Ab1\text{-}Ab2\text{-}Ag}}$$

$$K_5 = \frac{[\underline{\mathrm{Ab1\text{-}Ab2\text{-}Ag}}]}{[\underline{\mathrm{Ab1}}]\, C_{\mathrm{Ab2\text{-}Ag}}} \tag{9}$$

where the chemical species with underlines denote those adsorbed on the sensor chip and chemical species in brackets denote the surface concentrations in nmol mm^{-2}.

From the mass balance of the 1st antibody on the surface of the sensor chip, the following equation holds:

$$[\underline{\mathrm{Ab1}}]^{\mathrm{T}} = [\underline{\mathrm{Ab1\text{-}Ab2}}] + [\underline{\mathrm{Ab1\text{-}Ab2\text{-}AgC}}] + [\underline{\mathrm{Ab1\text{-}Ab2\text{-}Ag}}] \tag{10}$$

where $[\mathrm{Ab1}]^{\mathrm{T}}$ is the total surface concentration of the 1st antibody on the sensor chip.

As a first approximation, it is possible to consider that the binding constants of the complexes formed on the 1st antibody layer with the 2nd antibody species do not differ substantially among the 2nd antibody species. Namely, it is reasonable to assume that $K_3 = K_4 = K_5$. This is because the nature of the binding of the Fc moieties of the 2nd antibody species does not change, even when the 2nd species is bound to TNP and the TNP-KLH conjugate.

If the binding of the 2nd antibody species with the 1st antibody on the sensor chip can be assumed to obey the Langmuir isotherm equation, the following equations hold:

$$\frac{[\underline{\mathrm{Ab1\text{-}Ab2}}]}{[\underline{\mathrm{Ab1}}]^{\mathrm{T}}} = \frac{K_3 C_{\mathrm{Ab2}}}{1 + K_3 C_{\mathrm{Ab2}} + K_4 C_{\mathrm{Ab2\text{-}AbC}} + K_5 C_{\mathrm{Ab2\text{-}Ag}}} \tag{11}$$

$$\frac{[\underline{\mathrm{Ab1\text{-}Ab2\text{-}AgC}}]}{[\underline{\mathrm{Ab1}}]^{\mathrm{T}}} = \frac{K_4 C_{\mathrm{Ab2\text{-}AgC}}}{1 + K_3 C_{\mathrm{Ab2}} + K_4 C_{\mathrm{Ab2\text{-}AbC}} + K_5 C_{\mathrm{Ab2\text{-}Ag}}} \tag{12}$$

$$\frac{[\underline{\mathrm{Ab1\text{-}Ab2\text{-}Ag}}]}{[\underline{\mathrm{Ab1}}]^{\mathrm{T}}} = \frac{K_5 C_{\mathrm{Ab2\text{-}Ag}}}{1 + K_3 C_{\mathrm{Ab2}} + K_4 C_{\mathrm{Ab2\text{-}AbC}} + K_5 C_{\mathrm{Ab2\text{-}Ag}}} \tag{13}$$

where C_{Ab2}, $C_{\mathrm{Ab2\text{-}Ab}}$ and $C_{\mathrm{Ab2\text{-}AbC}}$ are the concentrations of the 2nd antibody, the 2nd antibody-TNP complex and the 2nd antibody-TNP-KLH conjugate complex, respectively, in the incubation solution. The surface concentrations of the 2nd species are calculated from Eqs. (10)–(13) by using the concentrations of the 2nd species shown in Fig. 2 and assuming the binding constant, $K_3 = 6.8 \times 10^8$ M^{-1}. This value was obtained experimentally and is discussed in the next section. Fig. 3 shows the surface concentrations of the 2nd antibody species. As can be easily estimated from the concentration of the 2nd antibody species in the incubation

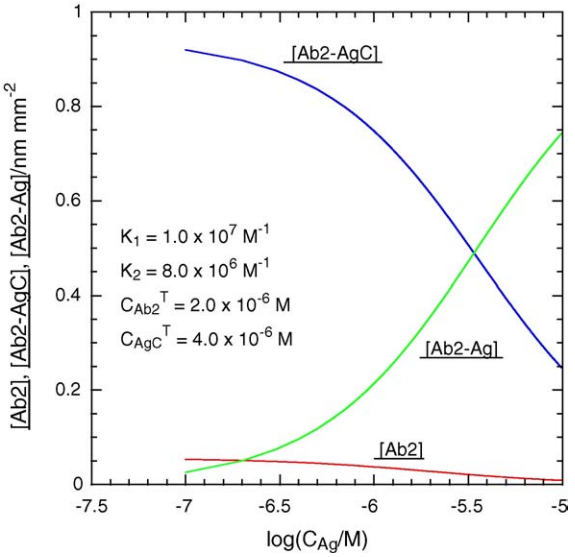

Fig. 3. Surface concentration of 2nd antibody, 2nd antibody complex with antigen and 2nd antibody complex with conjugate on the 1st antibody layer as a function of the concentration of the antigen calculated from Eqs. (11) to (13), assuming $K_3 = K_4 = K_5 = 6.8 \times 10^8\ \mathrm{M}^{-1}$. The values for the other parameters are the same as in Fig. 2.

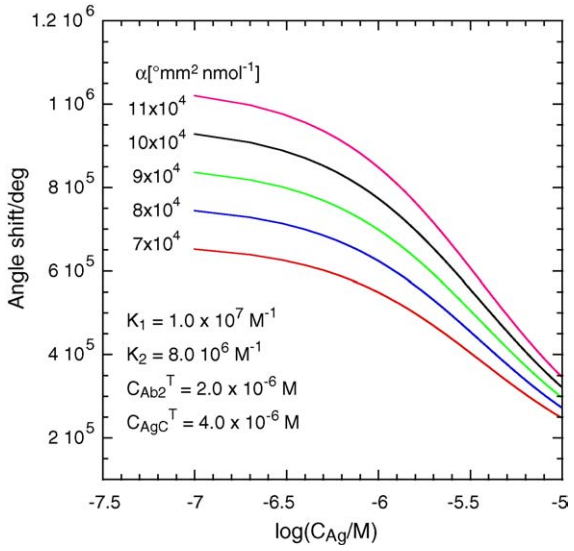

Fig. 4. Relationship between angle shift of SPR sensor and concentration of antigen in the incubated solution calculated from Eq. (14) assuming $\alpha_{\mathrm{Ab2}} = 1.5 \times 10^4$, $\alpha_{\mathrm{Ab2\text{-}Ag}} = 1.5 \times 10^4$ and $\alpha_{\mathrm{Ab2\text{-}AgC}} = 7 \times 10^4$–$11 \times 10^4$ [$^\circ$ mm^2 nmol^{-1}].

solution, the dependency of the surface concentrations of the 2nd antibody species bound to the 1st antibody layer on the concentration of TNP is the same as that of the concentrations of the 2nd antibody species in the incubation solution.

If we adopt a relationship [30] between the angle shift of the SPR sensor and the mass change on the sensor chip by binding the analyte in which the angle shift of 0.1° corresponds to a change in mass of 1 ng mm^{-2}, the following equation for the angle shift, $\Delta\theta$, of the present SPR sensor holds:

$$\Delta\theta = \alpha_{\mathrm{Ab2}}[\underline{\mathrm{Ab1\text{-}Ab2}}] + \alpha_{\mathrm{Ab2\text{-}Ag}}[\underline{\mathrm{Ab1\text{-}Ab2\text{-}Ag}}] + \alpha_{\mathrm{Ab2\text{-}AgC}}[\underline{\mathrm{Ab1\text{-}Ab2\text{-}AgC}}] \quad (14)$$

where α_{Ab2}, $\alpha_{\mathrm{Ab2\text{-}Ag}}$ and $\alpha_{\mathrm{Ab2\text{-}AgC}}$ are coefficients that are proportional to the molecular weight of the 2nd antibody, the 2nd antibody-TNP complex and the 2nd antibody-TNP-KLH conjugate complex, respectively. These values have dimensions of [$^\circ$ mm^2 nmol^{-1}]. The angle shift of the SPR sensor can be calculated from the surface concentrations of the 2nd antibody species from Eq. (14), using the surface concentrations of the 2nd antibody species on the 1st antibody layer calculated in the manner shown in Fig. 3 and by assuming $\alpha_{\mathrm{Ab2}} = 1.5 \times 10^4$, $\alpha_{\mathrm{Ab2\text{-}Ag}} = 1.5 \times 10^4$ and $\alpha_{\mathrm{Ab2\text{-}AgC}} = 7 \times 10^4$–$11 \times 10^4$ [$^\circ$ mm^2 nmol^{-1}]. The values for α are assumed, based on the fact that the molecular weight of the 2nd antibody and its complex with TNP is about 1.5×10^5 Da and that of KLH is about 7–11×10^5 Da [31]. The calculated angle shifts as a function of the concentration of TNP and as a parameter of $\alpha_{\mathrm{Ab2\text{-}AgC}}$, are shown in Fig. 4. This indicates that the angle shift is mainly dependent on the surface concentration of the 2nd antibody-TNP-KLH complex. In addition, the sensitivity to TNP is estimated to be enhanced by using the higher molecular weight TNP-KLH conjugate.

4. Results and discussion

4.1. Estimation of adsorption constant of the 1st antibody on the gold film of the sensor chip

In the first step, in the present immunoassay, the 1st antibody layer was formed on the gold film of the sensor chip by physical adsorption, as described in Section 2. In order to estimate the adsorption constant of the 1st antibody on the gold film and to evaluate the amount of the 1st antibody that was immobilized on the sensor chip, the angle shifts were measured when the 1st antibody at various concentrations were introduced into the sensor chip.

The adsorption of the 1st antibody on the gold film of the sensor chip is assumed to be in adsorption equilibrium. The adsorption constant, K_{ad}, can be expressed by the following equation:

$$K_{\mathrm{ad}} = \frac{[\underline{\mathrm{Ab1}}]}{C_{\mathrm{Ab1}}} \quad (15)$$

where $[\underline{\mathrm{Ab1}}]$ and C_{Ab1} are the surface concentration of the 1st antibody adsorbed on the sensor chip in nmol mm^{-2} units and the concentration of the 1st antibody in the solution adjacent to the sensor chip in mol dm^{-3} units, respectively. If the adsorption of the 1st antibody obeys a Langmuir-type adsorption equation, the following equation is derived from Eq. (15),

the same as Eqs. (11)–(13):

$$\frac{[\underline{\text{Ab1}}]}{[\underline{\text{Ab1}}]^{\text{T}}} = \frac{K_{\text{ad}}C_{\text{Ab1}}}{1 + K_{\text{ad}}C_{\text{Ab1}}} \tag{16}$$

If the angle shift of the SPR sensor, $\Delta\theta_1$, is proportional to the surface concentration of the 1st antibody adsorbed to the gold film, the relative surface concentration to the total surface concentration of the 1st antibody, $[\underline{\text{Ab1}}]^{\text{T}}$, can be expressed as follows:

$$\frac{[\underline{\text{Ab1}}]}{[\underline{\text{Ab1}}]^{\text{T}}} = \frac{\Delta\theta_1}{\Delta\theta_{1,\max}} \tag{17}$$

where $\Delta\theta_{1,\max}$ denotes the angle shift of the SPR sensor when the 1st antibody completely occupies the surface of the gold film.

The relationship between the concentration of the 1st antibody solution, which was introduced into the sensor chip, and the angle shift of the SPR sensor, $\Delta\theta_1$, is derived from Eqs. (16) and (17):

$$\frac{K_{\text{ad}}C_{\text{Ab1}}}{1 + K_{\text{ad}}C_{\text{Ab1}}} = \frac{\Delta\theta_1}{\Delta\theta_{1,\max}} \tag{18}$$

Rearranging Eq. (18), the following equation can be derived:

$$\frac{C_{\text{Ab1}}}{\Delta\theta_1} = \frac{C_{\text{Ab1}}}{\Delta\theta_{1,\max}} + \frac{1}{\Delta\theta_{1,\max}K_{\text{ad}}} \tag{19}$$

Fig. 5(a) shows the angle shift obtained when several concentrations of 1st antibody solutions were injected. As can be seen from Fig. 5(a), the angle shift gradually increased with increasing concentration of the 1st antibody. This indicates that the 1st antibody is adsorbed on the gold film of the sensor chip. From these data, $C_{\text{Ab1}}/\Delta\theta_1$ is plotted against C_{Ab1} as shown in Fig. 5(b). A linear relationship exists between $C_{\text{Ab1}}/\Delta\theta_1$ and C_{Ab1}, consistent with the estimation from Eq. (19). This indicates that the adsorption of the 1st antibody on the gold film holds for the Langmuir adsorption isotherm. The adsorption constant, $K_{\text{ad}} = 3.7 \times 10^9\ \text{M}^{-1}$ and the angle shift in the case that the gold film is completely adsorbed with the 1st antibody, $\Delta\theta_{1,\max} = 0.041^\circ$ are obtained from the intercept and the slope of the linear relationship in Fig. 5(b). The total surface concentration of the 1st antibody, $[\underline{\text{Ab1}}]^{\text{T}}$, is estimated to be $2.7 \times 10^{-6}\ \text{nmol mm}^{-2}$ from $\Delta\theta_{1,\max} = 0.041^\circ$, assuming the molecular weight of the 1st antibody to be 1.5×10^5 Da. Subsequent experiments were carried out using the gold film of the sensor chip after introducing a 100 ppm solution of 1st antibody. The surface concentration of the 1st antibody by this procedure is estimated to be $2.3 \times 10^{-6}\ \text{nmol mm}^{-2}$.

4.2. Estimation of binding constant of complex of 1st antibody with Fc moiety of 2nd antibody

The third step in the present immunoassay is the competitive binding of the 2nd antibody species in the incubation solution with the 1st antibody layer on the sensor chip. In order to estimate the binding constant for the complex of the 1st antibody on the gold film with an Fc moiety of the 2nd antibody, angle shifts were measured when various concentrations of the 2nd antibody solutions were introduced into the sensor chip, where the 1st antibody was immobilized using a 100 ppm solution of the 1st antibody. In this case, as described in Section 2, the blocking reagent solution was introduced onto the 1st antibody immobilized sensor chip prior to introduction of the 2nd antibody solution, in order to prevent a specific adsorption of the 2nd antibody. As can be seen from Fig. 6(a), since the 2nd antibody is bound to the 1st antibody on the sensor chip, an increase in the angle shift of the SPR sensor is observed, which is dependent on the concentration of the 2nd antibody solution injected into the sensor system. At concentrations of the 2nd antibody higher than 50 ppm, the angle shift reaches a constant angle shift.

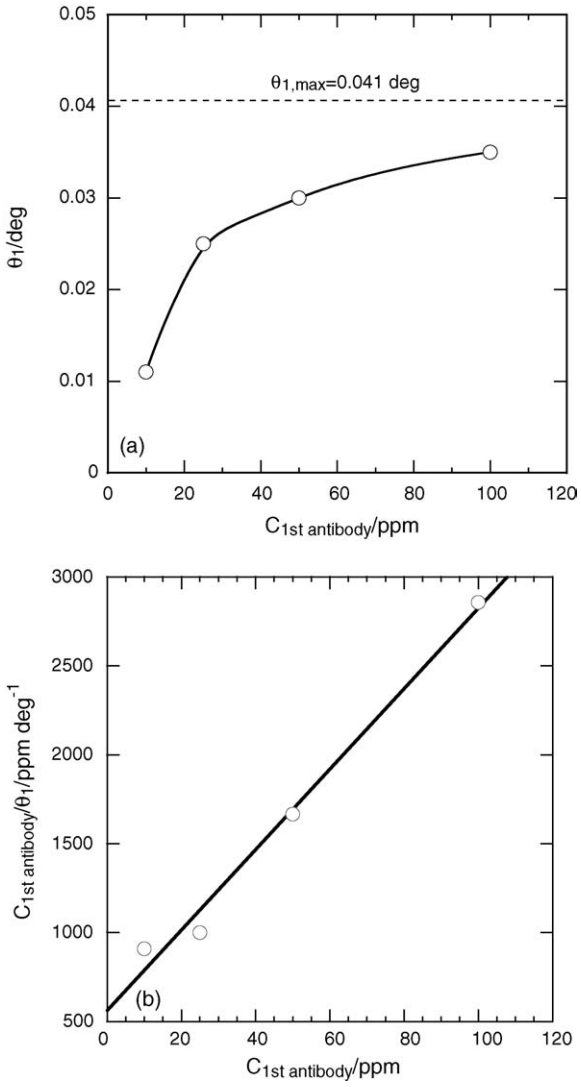

Fig. 5. (a) Relationship between angle shift and concentration of the 1st antibody injected into the SPR sensor system. Sample volume of the 1st antibody solution: 100 μL, carrier solution: phosphate buffer solution, pH 7.2, flow rate 20 μL/min. (b) Relationship between concentration of the 1st antibody and $C_{\text{1st antibody}}/\theta_1$. The experimental conditions are the same as in (a).

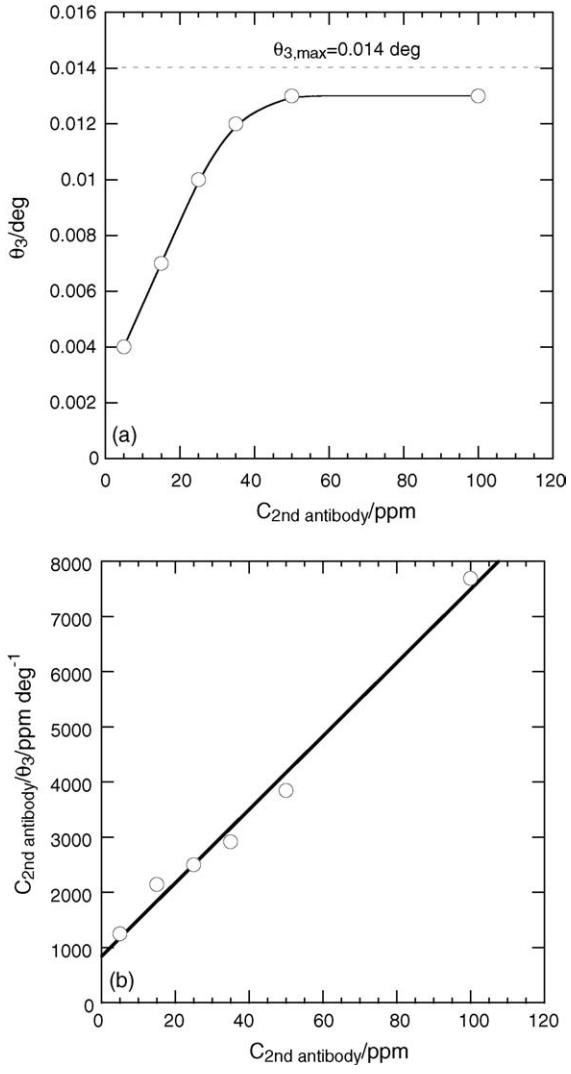

Fig. 6. (a) Relationship between angle shift and concentration of the 2nd antibody injected into the SPR sensor system. The 1st antibody was immobilized on the sensor chip by injecting 100 μL of 100 ppm solution, volume of the 2nd antibody solution: 100 μL, carrier solution: phosphate buffer solution, pH 7.2, flow rate 20 μL/min. (b) Relationship between concentration of the 2nd antibody and $C_{\text{2nd antibody}}/\theta_3$. The experimental conditions are the same as in (a).

In this experiment, the binding constant, K_3, is expressed by Eq. (7), as described in the theoretical section. If the angle shift of the SPR sensor, $\Delta\theta_3$, is proportional to the surface concentration of the 2nd antibody bound to the 1st antibody on the sensor chip, [Ab1-Ab2], the relative surface concentration to the total surface concentration of the 2nd antibody $[\underline{\text{Ab2}}]^{\text{T}}$ can be expressed as follows:

$$\frac{[\underline{\text{Ab1-Ab2}}]}{[\underline{\text{Ab2}}]^{\text{T}}} = \frac{\Delta\theta_3}{\Delta\theta_{3,\max}} \tag{20}$$

where $\Delta\theta_{3,\max}$ denotes the angle shift of the SPR sensor when the surface of the 1st antibody immobilized sensor chip is completely bound to the 2nd antibody.

The following Langmuir-type adsorption isotherm can be derived in a manner similar to Eq. (16):

$$\frac{[\underline{\text{Ab1-Ab2}}]}{[\underline{\text{Ab2}}]^{\text{T}}} = \frac{K_3 C_{\text{Ab2}}}{1 + K_3 C_{\text{Ab2}}} \tag{21}$$

where C_{Ab2} is the concentration of the 2nd antibody solution injected. From Eqs. (20) and (21), the following equation can be derived:

$$\frac{C_{\text{Ab2}}}{\Delta\theta_3} = \frac{C_{\text{Ab2}}}{\Delta\theta_{3,\max}} + \frac{1}{\Delta\theta_{3,\max} K_3} \tag{22}$$

According to Eq. (22), $C_{\text{Ab2}}/\Delta\theta_3$ was plotted against C_{Ab2} using the data shown in Fig. 6(a) in order to confirm that the Langmuir-type adsorption isotherm holds in the present system. As can be seen from Fig. 6(b), a good linear relationship between $C_{\text{Ab2}}/\Delta\theta_3$ and C_{Ab2} was found, indicating that the Langmuir-type isotherm adsorption isotherm holds. $\Delta\theta_{3,\max}$ and K_3 were calculated to be $\Delta\theta_{3,\max} = 0.014^\circ$ and $K_3 = 6.4 \times 10^8\ \text{M}^{-1}$, respectively, from the slope and intercept of the $C_{\text{Ab2}}/\Delta\theta_3$ versus C_{Ab2} plot.

The total surface concentration of the 2nd antibody, $[\underline{\text{Ab2}}]^{\text{T}}$, is estimated to be $9.3 \times 10^{-7}\ \text{nmol mm}^{-2}$ from $\Delta\theta_{3,\max} = 0.014^\circ$, assuming the molecular weight of the 2nd antibody to be 1.5×10^5 Da. This value is one-third of the total concentration of the 1st antibody on the sensor chip. Therefore, one-third of the 1st antibody on the sensor chip is effective in binding the 2nd antibody. In other words, two-third of the 1st antibody on the sensor chip is simply adsorbed to the gold film in a non-oriented state.

4.3. Effect of incubation time

Incubation time would be important in the second step of the immunoassay, because immunoreaction between the 2nd antibody and TNP and the TNP-KLH conjugate in the incubation solution is approaching equilibrium. A short incubation is desirable for a rapid immunoassay. The effect of incubation time on the sensitivity of the SPR detection was investigated. A 25 ppm solution of the 2nd antibody and a 100 ppm solution of the TNP-KLH conjugate were incubated in the phosphate buffer solution for times in the range of 5–120 min and the resulting solution was introduced into the sensor chip, where the 1st antibody was immobilized on the gold film of the sensor chip by injecting 100 μL of a 100 ppm solution of the 1st antibody first, followed by the blocking solution. The angle shift of the SPR sensor was nearly constant for incubation times was longer than 30 min. Even for an incubation time of 5 min, the angle shift was 83% of the angle shift observed at 30 min. This indicates that the present immunoreaction is relatively fast in a homogeneous solution. In subsequent experiments, an incubation time of 30 min was adopted.

4.4. Effect of composition of incubation solution

As can be estimated from theoretical considerations, the composition of the 2nd antibody and the TNP-KLH conjugate

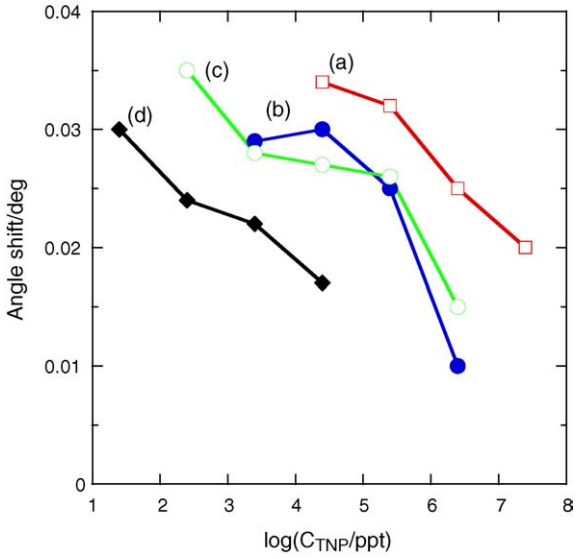

Fig. 7. Relationship between angle shift and concentration of TNP in the incubation solution. Concentrations of TNP-KLH conjugate and 2nd antibody in the incubation solution: (a) 100/25 ppm, (b) 150/25 ppm, (c) 150/35 ppm and (d) 200/35 ppm, respectively. The 1st antibody was immobilized on the sensor chip by injecting 100 μL of a 100 ppm solution, volume of the incubation solution: 100 μL, carrier solution: phosphate buffer solution, pH 7.2, flow rate 20 μL/min.

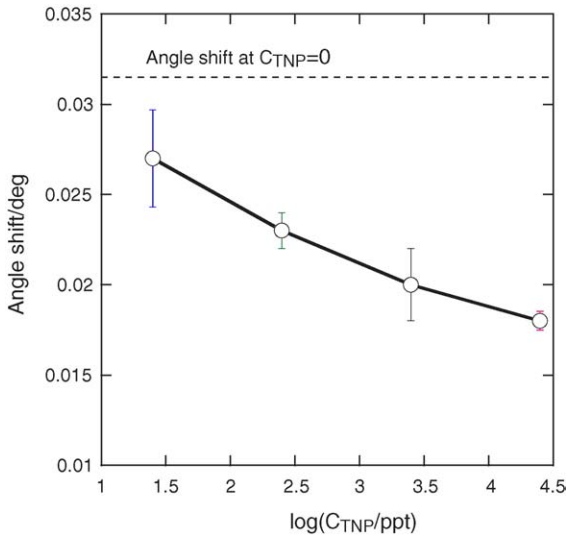

Fig. 8. The calibration curve for TNP. Incubation solution: mixed solution containing 200 ppm TNP-KLH conjugate, 35 ppm 2nd antibody and TNP. Volume injected: 100 μL, carrier solution: phosphate buffer solution, pH 7.2, flow rate 20 μL/min. The error bar was obtained from triplicate measurements.

in the incubation solution would greatly affect the sensitivity of the detection of TNP, because TNP and the TNP-KLH conjugate bind competitively to the 2nd antibody depending on the composition of the incubation solution and the resulting 2nd antibody species bind to the 1st antibody layer on the sensor chip, which is dependent on the fraction of the 2nd species in the incubation solution. In the same protocol described in Section 2, effect of the composition of the incubation solution was investigated. Fig. 7 shows the relationship between angle shift and the concentration of TNP in the incubation solution, where the concentration of the 2nd antibody and the TNP-KLH conjugate are kept constant. As can be seen from Fig. 7, by comparing (a) and (b) or (c) and (d), it is clear that a lower detection of TNP is better for an incubation solution containing the TNP-KLH conjugate at a higher concentration when the concentration of the 2nd antibody is the same. By comparing (b) and (c), it is clear that a lower detection of TNP is better for an incubation solution containing the 2nd antibody at higher concentrations when the concentration of the TNP-KLH conjugate is the same. This result indicates that detection of lower levels of TNP is improved by increasing the concentrations of the 2nd antibody and the TNP-KLH conjugate. This may be due to the fact that the fraction of the complex of the 2nd antibody-TNP-KLH conjugate in the incubation solution increased and the resulting complex was preferentially bound to the 1st antibody layer on the sensor chip. The repeatability of the calibration curve for TNP was examined under optimum conditions, in which the concentrations of the 2nd antibody and the TNP-KLH conjugate were 35 and 200 ppm, respectively, in the incubation solution.

4.5. Calibration curve for TNP and repeatability of measurements

Under optimum conditions, the calibration curve for TNP was obtained, as shown in Fig. 8. The error for the determination of 25 ppt is as large as 40%, but in the concentration region higher than 250 ppt, a good relationship between the concentration of TNP and the angle sift of the SPR sensor exists, with a relatively smaller error. The repeatability of the determination of 2.5 ppb for four measurements showed a coefficient of variation of 12.8%.

5. Conclusion

A one-pot immunoreaction method was applied to the present SPR sensor system for the determination of TNP as a model of TNT, in an attempt to develop a landmine detector. Although the immobilization of the 1st antibody occurred by physical adsorption, about 30% of the adsorbed 1st antibody was found to be effective for binding to the 2nd antibody. This may be due to the fact that the active site of the 1st antibody for recognition of the Fc moiety of the 2nd antibody is not hindered by adsorption to the gold film of the sensor chip, even though a special oriented immobilization technique for the 1st antibody was not utilized. The more effective immobilization of the 1st antibody is currently under investigation using a fragment of the 1st antibody by splitting or cutting the Fab moiety.

One of the drawbacks of the present method is the fact that the free 2nd antibody is bound to the 1st antibody layer as well as the complex of the 2nd antibody with the TNP-KLH conjugate, as described in the introduction section. However,

utilization of a much higher molecular weight TNP conjugate would be expected to lead to an increased sensitivity to TNP. KLP was used as the conjugate protein in the present work. However, if a protein conjugate with much higher molecular weight by polymerization of the protein could be used, the detection limit would be improved and this scenario is also under investigation.

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References

[1] I.B. Bakaltcheva, F.S. Ligler, C.H. Patterson, L.C. Shriver-Lake, Anal. Chim. Acta 399 (1999) 13–20.
[2] M. Bader, T. Goen, J. Muller, J. Angerer, J. Chromatogr. B. 710 (1998) 91–99.
[3] R. Batlle, H. Carlsson, E. Holmgren, A. Colmsjo, C. Crescenzi, J. Chromatogr. A. 963 (2002) 73–82.
[4] R. Batlle, H. Carlsson, P. Tollback, A. Colmsjo, C. Crescenzi, Anal. Chem. 75 (2003) 3137–3144.
[5] J.V. Goodpaster, V.L. Mcguffin, Anal. Chem. 73 (2001) 2004–2011.
[6] A.M. Jimenez, M.J. Navas, J. Hazard. Mater. 106A (2004) 1–8.
[7] T. Khayamian, M. Tabrizchi, M.T. Jafari, Talanta 59 (2003) 327–333.
[8] Z. Naal, J.H. Park, S. Bernhard, J.P. Shapleigh, C.A. Batt, H.D. Abruna, Anal. Chem. 74 (2002) 140–148.
[9] M.E. Walsh, Talanta 54 (2001) 427–438.
[10] J. Wang, G. Chen, M.P. Chatrathi, A. Fujishima, D.A. Tryk, D. Shin, Anal. Chem. 75 (2003) 935–939.
[11] J. Yinon, Trends Anal. Chem. 21 (2002) 292–301.
[12] C.A. Groom, A. Halasz, L. Paquet, S. Thiboutot, G. Ampleman, J. Hawari, J. Chromatogr. A 1072 (2005) 73–82.
[13] E.R. Goldman, I.L. Medintz, J.L. Whitley, A. Hayhurst, A.R. Clapp, H.T. Uyeda, J.R. Deschamps, M.E. Lassman, H. Mattoussi, J. Am. Chem. Soc. 127 (2005) 6744–6751.
[14] A. Üzer, E. Erçağ, R. Apak, Anal. Chim. Acta 534 (2005) 307–317.
[15] L.M. Dorozhkin, V.A. Nefedov, A.G. Sabelnikov, V.G. Sevastjanov, Sens. Actuators B 99 (2004) 568–570.
[16] L.A. Pinnaduwage, A. Wig, D.L. Hedden, A. Gehl, D. Yi, T. Thundat, R.T. Lareau, J. Appl. Phys. 95 (2004) 5871–5875.
[17] A.V. Kuznetsov, A.V. Evsenin, I.Yu. Gorshkov, O.I. Osetrov, D.N. Vakhtin, Appl. Radiat. Isot. 61 (2004) 51–57.
[18] P.T. Charles, J.G. Rangasammy, G.P. Anderson, T.C. Romanoski, A.W. Kusterbeck, Anal. Chim. Acta 525 (2004) 199–204.
[19] X. Fu, R.F. Benson, J. Wang, D. Fries, Sens. Actuators B 106 (2005) 296–301.
[20] M. Altstein, A. Bronshtein, B. Glattstein, A. Zeichner, T. Tamiri, J. Almog, Anal. Chem. 73 (2001) 2461–2467.
[21] D.R. Shankaran, K.V. Gobi, T. Sakai, K. Matsumoto, K. Toko, N. Miura, Biosens. Bioelectron. 20 (2005) 1750–1756.
[22] D.R. Shankaran, K.V. Gobi, T. Sakai, K. Matsumoto, T. Imato, K. Toko, N. Miura, Sens. Actuators B 100 (2004) 450–454.
[23] T. Sakai, A. Torimaru, K. Shinahara, N. Miura, T. Imato, K. Toko, K. Matsumoto, Sens. Mater. 15 (2003) 439–452.
[24] N. Soh, T. Tokuda, T. Watanabe, K. Mishima, T. Imato, T. Masadome, Y. Asano, S. Okutani, O. Niwa, S. Brown, Talanta 60 (2003) 733–745.
[25] N. Miura, M. Sasaki, G. Sakai, K.V. Gobi, Chem. Lett. (2002) 342–343.
[26] N. Miura, M. Sasaki, K.V. Gobi, C. Kataoka, Y. Shoyama, Biosens. Bioelectron. 18 (2003) 953–959.
[27] K.V. Gobi, N. Miura, Sens. Actuators B 103 (2004) 265–271.
[28] N. Soh, T. Watanebe, Y. Asano, T. Imato, Sens. Mater. 15 (2003) 423–438.
[29] A. Hemmi, T. Imato, Y. Aoki, M. Sato, N. Soh, Y. Asano, C. Akasaka, S. Ohkubo, N. Kaneki, K. Shimada, Sens. Actuators B 108 (2005) 893–898.
[30] Available from: http://www.biacore.com/home.lasso.
[31] R.D. Swerdlow, R.F. Ebert, P. Lee, C. Bonaventura, K.I. Miller, Comp. Biochem. Physiol. 113B (1996) 537–548.