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Talanta 60 (2003) 733–745
Talanta
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A surface plasmon resonance immunosensor for detecting a dioxin precursor using a gold binding polypeptide

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Received 9 October 2002; received in revised form 12 February 2003; accepted 12 February 2003

Abstract

A surface plasmon resonance (SPR) based biosensor was developed for monitoring 2,4-dichlorophenol, a known dioxin precursor, using an indirect competitive immunoassay. The SPR sensor was fabricated by immobilizing a gold-thin layer on the surface of an SPR sensor chip with an anti-(2,4-dichlorophenol) antibody using a gold binding polypeptide (GBP) and protein G. The SPR response based on the antigen–antibody reaction in a flow system was measured by injecting a 2,4-dichlorophenol sample solution into the flow system in which the SPR sensor was located. In a direct immunoassay system using the modified sensor chip, no significant SPR angle shift less than 0.001° was observed when a 25 ppm of 2,4-dichlorophenol solution was injected. In order to improve the sensitivity of the SPR sensor, an indirect competitive immunoassay method was used in conjunction with the SPR sensor system using 2,4-dichlorophenol conjugated with bovine serum albumin (BSA). In the competitive assay, a 350 ppm 2,4-dichlorophenol–BSA conjugate solution containing 2,4-dichlorophenol at various concentrations (10–250 ppb) were injected into the SPR sensor system. The sensitivity of this indirect immunoassay was found to be extremely sensitive, compared to the direct one, and a detection limit of 20 ppb was estimated. Verification that the use of GBP for immobilizing the antibody on the sensor chip enhanced the sensitivity to 2,4-dichlorophenol was obtained by comparing the procedure with another modification, in which BSA was used instead of GBP for immobilizing the antibody on the sensor chip. The affinity constant of 2,4-dichlorophenol and its conjugate to the antibody were estimated form the SPR response.

Keywords: SPR sensor; 2,4-Dichlorophenol; Dioxins; Endocrine disrupting chemicals; Gold binding polypeptide; Protein G

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0039-9140/03/$ - see front matter
doi:10.1016/S0039-9140(03)00139-5

1. Introduction

The hazardous effects of endocrine disrupting chemicals on humans and wildlife species have been well documented in recent years [1–5]. In particular, polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans, so-called dioxins, have a high toxicity, and their production and subsequent distribution from incinerators to the environment represent a serious health-related problem for society [6–9]. Therefore, sensitive and rapid analytical methods for the estimation of dioxins and dioxin-related compounds are urgently needed for the screening or monitoring of environmental pollution. A conventional method for the determination of dioxins and related compounds is based on gas chromatograph–mass spectrometry (GC–MS) [10–12], which is recognized as the one of the most reliable analytical methods currently available. Capability of the simultaneous determination of dioxins with different number of chlorine substituents is one of the advantages of this method. However, the method requires an expensive instrument is time-consuming and involves sample pre-treatment, and a skillful technique for operation. A biochemical method based on enzyme-linked immunosorbent assay (ELISA) has also been widely used for dioxin detection [13–18]. This method has some advantages such as high selectivity, high sensitivity, and low cost, but the procedure is somewhat complicated, and several hours are required for a single measurement.

An analytical method using a biosensor represents one of the most promising methods for overcoming such problems as those encountered for the GC–MS and ELISA methods. In an assay using a biosensor, a selective and sensitive detection can be achieved because the biomolecules used in the biosensor have a specific and strong affinity to the target molecule. Since an assay using a biosensor is extremely simple from the procedural point of view, rapid measurements would be expected. A surface plasmon resonance (SPR) system has been reported to be an effective transducer for biosensors [19–23] because the SPR system was originally developed for studying the interactions of biomolecules such as proteins and DNAs. This is due to the fact that the SPR system is highly sensitive and would be suitable for real-time and automated monitoring. Thus, an analytical method based on an SPR system might be useful for the sensitive detection of dioxins and related compounds. Karube et al. investigated the use of SPR for sensing 2,3,7,8-tetra-chlorodibenzo-*p*-dioxin, and reported the simple and rapid determination of the dioxin using such a system [23].

In this paper, we wish to report on an SPR sensing system for 2,4-dichlorophenol, a major dioxin precursor [24–27]. The method involves the immobilization of an anti-(2,4-dichlorophenol) antibody on a sensor chip. A method for detecting the dioxin precursor is also critical for anticipating the production of dioxin, since the production of dioxins from dichlorophenols has been verified and the presence of dichlorophenol therefore represents a marker dioxins, as for reported in several papers [24–27]. 2,4-Dichlorophenol is widely used in industrial materials such as dye intermediates, and moreover, the compound itself is also suspected to have an endocrine disrupting effect. Therefore, the development of a sensor for 2,4-dichlorophenol as a tool for the simple and rapid determination in environment analysis would be an important development. To achieve a highly sensitive detection of 2,4-dichlorophenol, we focused on two goals in this study: (1) to enhance the density of antibodies immobilized on the SPR sensor chip using a gold binding protein (GBP) [28,29], (2) to immobilize the antibodies in an orientation such that it would have a favorable binding site for the antigen using protein G.

2. Experimental

2.1. Materials

E. coli strain containing pSB3053 produced by one of the authors (S.B.) was used. The anti-(2,4-dichlorophenol) antibody was purchased form Shibayagi Co., Ltd (Gunma, Japan). 2,4-Dichlorophenol was obtained from Tokyo Chemical

Kogyo Co., Ltd (Tokyo, Japan). *N*-Hydroxysuccinimide and bovine serum albumin (BSA) were purchased from Wako Chemical Co. Ltd (Osaka, Japan). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was purchased from Dojindo Laboratories (Kumamoto, Japan). Protein G was obtained from Pharmacia (New Jersey, USA). 3-Bromopropylamine hydrobromide was purchased from Acros Organics (Janssens, Belgium). A sensor chip of a cover glass ($18 \times 18 \times 0.15$ mm^t), on which a thin gold layer, 45 nm in thickness, with the assistance of a 3 nm-thick of chrome layer between the cover glass and the gold-thin layer, was obtained from Eliotech (Tokyo, Japan). Other reagents and solvents were purchased commercially and were used without further purification.

2.2. Apparatus of SPR system

An SPR system was constructed from an SPR detector with a flow-cell (SPR-20, DKK) and a syringe pump (MODEL 22 221W, Harvard Apparatus, Inc.) and an injector (7125, Rheodyne) with a sample loop (300 μl). The flow-cell was assembled on the SPR detector by attaching a silicon packing with a thickness of 20 μm with a groove (3×10 mm) between the gold-layered-cover glass and a prism of the SPR detector. Matching oil with a refractive index of 1.516 was coated between the backside of the cover glass of the sensor chip and the prism. The cell volume of the SPR detector was approximately 6 μl. Though the SPR system is equipped with two flow channels, one flow channel was used for measurements because a data processing error occurs when two flow channel was used. Bulk refractive index change and non-specific binding were not compensated. All measurements were performed in an air-conditioned room at 24–26 °C.

2.3. Biosynthesis and purification of GBP

Biosynthesis and purification of GBP were performed according to the method reported by Brown [28]. *E. coli* strain containing pSB3053 was cultured, and cold osmotic flow shock fluid (OSF) containing the GBP, which contains alkaline phosphatase, was prepared. The GBP in the OSF was purified by an ultrafiltration and gel permeation column chromatography. The GBP was identified by the determination of the activity of alkaline phosphatase in the GBP and by SDS-polyacrylamide gel electrophoresis. The activity of the alkaline phosphatase was measured by monitoring the change in absorbance at 405 nm of the enzymatic product of *p*-nitrophenol in the presence of sufficient amounts of *p*-nitrophenyl phosphate.

2.4. Immobilization of anti-(2,4-dichlorophenol) antibody on the SPR sensor chip

A gold-thin-layered sensor chip was sonicated for 20 min in acetone, to clean the surface. The sensor chip was placed on a spin coater and 50 μl of an aqueous solution of GBP (2000 μg ml^{-1}) was dropped on it, followed by rotation at 1800 rpm. The resulting GBP-coated sensor chip was set into the flow-cell of an SPR detector, and 300 μl of a protein G solution (160 μg ml^{-1} in 10 mM sodium acetate/acetic acid buffer (pH 4.0)) containing the coupling reagents NHS (2000 μg ml^{-1}) and EDC (2000 μg ml^{-1}) was injected from the injector, where a 10 mM, pH 7.0 phosphate buffer solution (PBS) was continuously flowed by the syringe pump at the flow rate of 10 μl min^{-1}. The reaction time for the coupling of GBP and protein G was approximately 30 min, as estimated from the injection volume of the protein G solution and the flow rate of the PBS. A 300 μl aliquot of an anti-(2,4-dichlorophenol) antibody solution (50 ppm) was subsequently injected into the PBS stream to immobilize the antibody layer on the GBP layer/protein G layer of the sensor chip. Thus, a GBP layer/protein G layer/antibody layer was formed on the sensor chip, as shown in Fig. 1. The immobilization of GBP, protein G and the antibody were confirmed by monitoring the SPR response during the passage of the sample zone through the SPR detector. For comparison, a different sensor chip was also prepared in the same way by using BSA instead of GBP.

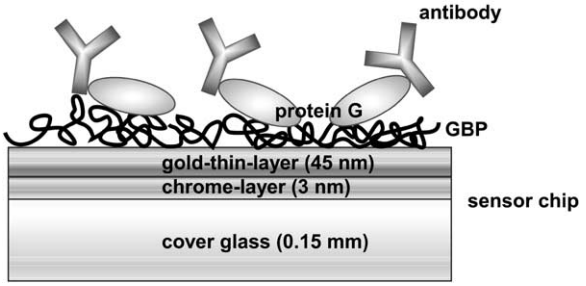


Fig. 1. Schematic diagram of an SPR sensor chip prepared in this study.

2.5. Direct detection of 2,4-dichlorophenol with the SPR sensor

Two milligrams of 2,4-dichlorophenol was dissolved in 20 ml of 10 mM PBS (pH 7.0) to a 100 ppm stock solution of 2,4-dichlorophenol, and 2–25 ppm of 2,4-dichlorophenol solutions were prepared from the stock solution and the PBS as a diluent. An SPR sensor chip modified with a GBP/protein G/anti-(2,4-dichlorophenol) antibody membrane or BSA/protein G/anti-(2,4-dichlorophenol) antibody membrane was placed in the flow-cell of the SPR detector. Three hundred microliter of a 2,4-dichlorophenol solution (2–25 ppm) was injected into a carrier stream (10 mM PBS at the flow rate of 30 μl min^{-1}), and the SPR response was monitored.

2.6. Preparation of 2,4-dichlorophenol–BSA conjugate

Twenty milliliters of a 4 M NaOH solution was added dropwise to 2,4-dichlorophenol (6.5 g, 40 mmol) in 250 ml of ethanol in a three-necked flask with stirring. 3-Bromopropylamine hydrobromide (8.8 g, 40 mmol) dissolved in 30 ml of ethanol, was then dropped into the reaction mixture, following by refluxing for 2 days at 85 °C under nitrogen atmosphere. The reaction mixture was cooled and acidified to pH 4.0 by adding an appropriate volume of 12 M HCl, and the resulting white precipitate by-product was removed by filtration. The filtrate was evaporated in vacuo, and the residue dissolved in diethylether. The diethylether phase was washed with a 1 M NaOH solution in a separatory funnel to remove starting materials. The diethylether phase was separated and evaporated to give 1.3 g (15%) as yellow oil (**1**, see Scheme 1).

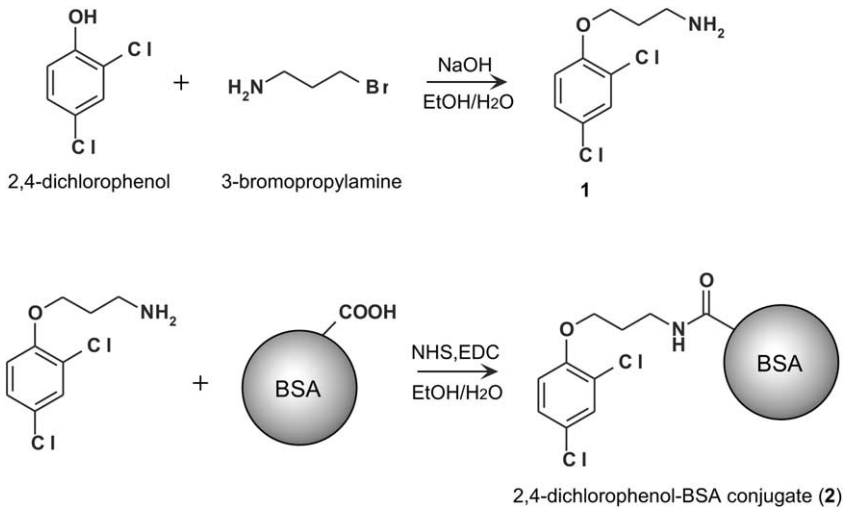
The product 1 (1.3 g) was dissolved in 65 ml of a mixture of ethanol and water (ethanol/water = 1/1, v/v), and acidified at pH 3.0 by adding an appropriate volume of 12 M HCl. The solvent was evaporated in vacuo, and the product was added to 5 ml of a 2 mg ml^{-1} BSA (10 mg, 0.14 μmol) solution at pH 4.0 containing EDC (100 mg, 0.5 mmol) to prepare product **2**. The reaction mixture was stirred for 2 days at room temperature, and then purified by dialysis (MWCO of a dialysis tube: 12 000–14 000 Da) in water. The dialyzed sample was lyophilized in vacuo to yield 7 mg of a white powder (**2**, see Scheme 1).

2.7. SPR response to 2,4-dichlorophenol–BSA conjugate

Two milligrams of 2,4-dichlorophenol–BSA conjugate was dissolved in 2 ml of 10 mM PBS (pH 7.0) to prepare 1000 ppm of the 2,4-dichlorophenol–BSA conjugate solution, and 17.5–350 ppm of 2,4-dichlorophenol–BSA conjugate solutions were prepared from the stock solution and the PBS as a diluent. An SPR sensor chip modified with a GBP/protein G/anti-(2,4-dichlorophenol) antibody membrane was placed in the flow-cell of the SPR detector. Three hundred microliter of the 2,4-dichlorophenol–BSA conjugate solution (17.5–350 ppm) was injected into a carrier stream (10 mM PBS at a flow rate of 30 μl min^{-1}), and the SPR response was monitored.

2.8. Competitive assay for 2,4-dichlorophenol

The SPR sensor chip modified with the antibody membrane (GBP/protein G/anti-(2,4-dichlorophenol) antibody membrane) was placed in the flow-cell of the SPR detector, and a sufficient amount of a 10 mg ml^{-1} BSA solution was then allowed to flow over it, to block any unmodified sites of the membrane in order to prevent any unspecific adsorption of the analyte on the antibody membrane. A sample solution of 2,4-dichlorophenol at



Scheme 1. Synthetic route for preparing the 2,4-dichlorophenol–BSA conjugate.

different concentrations (10, 50, 100, 500 ppb) was mixed with a 700 ppm 2,4-dichlorophenol–BSA conjugate solution at a 1:1 volume ratio, and 300 μl of the mixture of 2,4-dichlorophenol and its conjugate was then injected into the SPR system, where the PBS carrier (pH 7.0) was pumped at 30 μl min^{-1}. The SPR sensor chip was changed after each sample measurement.

3. Results and discussion

3.1. Design of modification of sensor chip

The sensitivity of an SPR immunosensor would be affected by the density of an antibody immobilized on the sensor chip. A higher sensitivity would be expected for the sensor chip immobilized with the antibody at higher density because the SPR sensor response is dependent on the amount of analyte adsorbed on the sensor chip. In the conventional immobilization of antibody on a gold surface, alkane thiol compounds are used, because of strong interaction between the thiol group and gold. In this case, the density of the antibody on the gold surface may not be as high, because the alkane thiol forms a monolayer membrane on the gold surface. Contrary to this immobilization method, Furlong et al. have reported a method using GBP for immobilizing the antibody on the SPR sensor chip [29]. GBP, originally reported by Brown [28] as a novel polypeptide having a specific affinity to gold, represents a promising material for use in enhancing the immobilization density of the antibody on the sensor chip. The GBP was extracted from selected bacteria that adhere to gold, and has a seven repeat amino acid sequence MHGKTQATSGTIQS. When the GBP solution is dropped on the surface of the gold layer of the sensor chip, it is readily immobilized, since the GBP has a strong affinity for the gold surface. Thus, the surface of the sensor chip would be coated with three-dimensional GBP, compared with the monolayer coating obtained using alkane thiol compounds.

Another factor should be noted in the design of the sensor chip. Namely, oriented immobilization of the antibody on the surface of the sensor chip is designed so as to effectively recognize the analyte. This is due to the fact that if the antibody is randomly immobilized on the sensor chip, the number of the effective active sites of the antibody may be reduced. The active site of the antibody on the sensor chip should be oriented toward the sample solution side. In order to satisfy the oriented immobilization of the antibody, we used protein G, which is known to bind the Fc moiety

of antibodies. Protein G was coupled with the GBP on the gold surface using a mixture of NHS and EDC, in the first step. In the next step, an anti-(2,4-dichlorophenol) antibody solution was flowed onto the resultant protein G-modified gold surface to give a sensor chip immobilized with the antibody. No denaturation of the variable region in the antibody would be expected during the immobilization step for the antibody on the protein G-modified gold surface, because the immobilization of the antibody was performed through the Fc moiety of the antibody. In addition, the antibody would be immobilized on the sensor chip at a high density because the effective surface area of the sensor chip was increased by the three-dimensional immobilization of protein G through GBP. In this study, another sensor surface, in which BSA was used instead of the GBP for the immobilization, was also prepared, in order to compare the performance of the SPR sensor modified with the antibody membrane prepared from GBP.

3.2. Evaluation of modified membrane

As described in Section 2, a containing of GBP was used to prepare a stable anchor membrane for the immobilization of the antibody. Fig. 2 shows resonance signals of the SPR responses obtained in air for the GBP-coated gold sensor chip and a non-modified bare gold sensor chip. As can be seen from Fig. 2, the resonance angle of the sensor signal obtained by the bare sensor chip is shifted to a higher angle by 0.3° when the sensor chip is coated by the GBP. This angle shift indicates that 0.3 ng mm^{-2} of GBP is immobilized on the sensor chip, according to the specification of the SPR instrument produced by BIACORE [30]. On the other hand, when the BSA was used instead of the GBP for the modification of the sensor chip, only about a 0.1° of angle shift was observed. This indicates that the mass of the BSA immobilized on the sensor chip is one-third that of the GBP.

For immobilization of the antibody on the sensor chip with an orientation towards the sample solution side, protein G was coupled with the anchor membrane (the GBP layer or the BSA layer). The protein G solution containing NHS and EDC was injected over an interval of 30 min, as shown in Fig. 3. The resonance angle was monitored before and after passing the injected protein G solution through the detector and the resonance angle before injection of the protein G solution was plotted as angle shift 0° in Fig. 3. Since the volume of the protein G solution injected

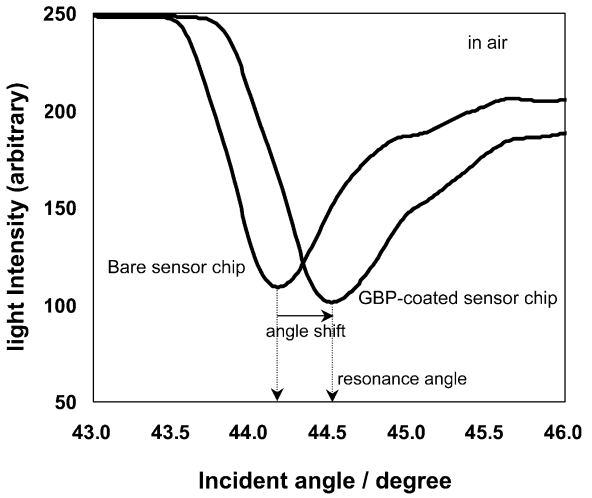


Fig. 2. Change of resonance signal of the SPR sensor by the immobilization of GBP on the sensor chip. The GBP solution (50 μl, 2000 μg ml^{-1}) was added to the sensor chip in a dropwise manner and centrifuged at 1800 rpm.

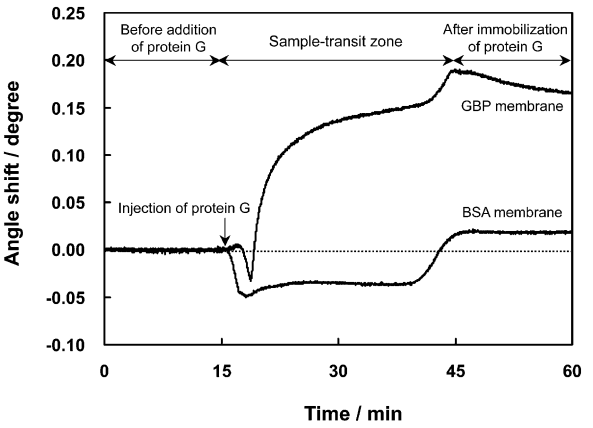


Fig. 3. SPR responses to the immobilization process of protein G on the sensor chip. The sensor chip: immobilized with the GBP or BSA, injected solution: protein G solution (160 μg ml^{-1}) containing NHS and EDC (2000 μg ml^{-1}, each), volume of injected solution: 300 μl, carrier solution and flow rate: 10 mM PBS (pH 7.0), 10 μl min^{-1}.

was 300 μl, the same zone was calculated to pass through the detector for 30 min. The protein G solution was prepared by dissolved in 10 mM sodium acetate/acetic acid buffer (pH 4.0), and the refractive index of the buffer is smaller than that of 10 mM PBS carrier (pH 7.0). This would be the reason why the SPR angle shift was decreased immediately just after the injection of the protein G solution in Fig. 3. The angle shift for the GBP membrane on the sensor chip gradually increased with time, reaching a plateau at 30 min. A further increase in angle shift was observed in the 43–45 min region. After passing the sample zone through the sensor, the angle shift reached a constant value. On the other hand, the angle shift for the BSA membrane on the sensor chip decreased during the passage of the sample zone through the detector and increased, eventually reaching a constant value. As shown in Fig. 3, 0.16° of angle shift was finally observed when the GBP membrane was used, on the other hand, only 0.02° of angle shift was reached when the BSA membrane was used. This result indicates that protein G was immobilized more efficiently on the GBP membrane than the BSA membrane. This difference in immobilization efficiency may be due to differences in the degree of affinity strength to the gold surface and the accumulated density of lysine residues between GBP and BSA.

For immobilization of the anti-(2,4-dichlorophenol) antibody, a solution of 50 ppm anti-(2,4-dichlorophenol) antibody (300 μl) was then injected into the carrier stream, where the PBS was allowed to flow at a rate of 30 μl min^{-1}. Fig. 4 shows the SPR response to the injected antibody solution for the GBP membrane immobilized with protein G and for the BSA membrane immobilized with protein G. In Fig. 4, the resonance angle of the SPR sensor before injection of the antibody solution is plotted as an angle shift of 0°. After the transient signal for the passage of the antibody solution through the detector, angle shifts of 0.80° and 0.31° were observed for the injection of the antibody to the GBP membrane and the BSA membrane, respectively. The plateau regions after 55 min in Fig. 4 indicate that the antibody was immobilized on the sensor surface as an adsorption equilibrium. The larger angle shift observed

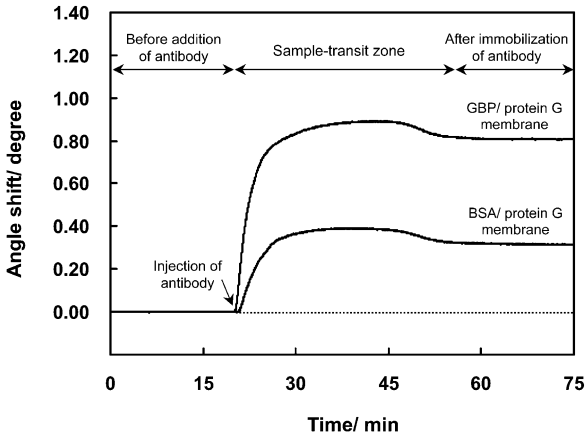


Fig. 4. SPR responses to the immobilization process of the antibody on the sensor chip. The concentration and volume of anti-(2,4-dichlorophenol) antibody: 50 μl ml^{-1} and 300 μl, carrier solution and flow rate: 10 mM PBS (pH 7.0), 10 μl min^{-1}.

for the GBP membrane indicates that much more antibody was immobilized on the GBP membrane. In general, an increase in an angle shift of 0.1° for the SPR response is approximately equivalent to 1 ng mm^{-2} of mass increase on the sensor surface [30]. Therefore, the amounts of immobilized GBP, protein G, and antibody on the sensor chip for the GBP membrane were estimated to be 3, 1.6 and 8 ng mm^{-2}, respectively, from Figs. 3 and 4. The GBP membrane (GBP/protein G/antibody) was used in subsequent experiments. The coefficient of variation of the angle shift for immobilization of the antibody was 7.4% ($n = 5$).

3.3. Direct assay for 2,4-dichlorophenol

The response of the SPR sensor, the surface of which was modified with the GBP/protein G/anti-(2,4-dichlorophenol) antibody membrane, was examined for a 2,4-dichlorophenol sample by introducing 300 μl of 5–25 ppm sample solutions into the SPR system, without any regeneration procedure of the sensor surface. An SPR response was barely observed when a 5, 10 or 15 ppm of 2,4-dichlorophenol sample solutions were injected. The angle shift of the SPR sensor obtained by injecting a 25 ppm 2,4-dichlorophenol sample solution is shown in Fig. 5. As can be seen from

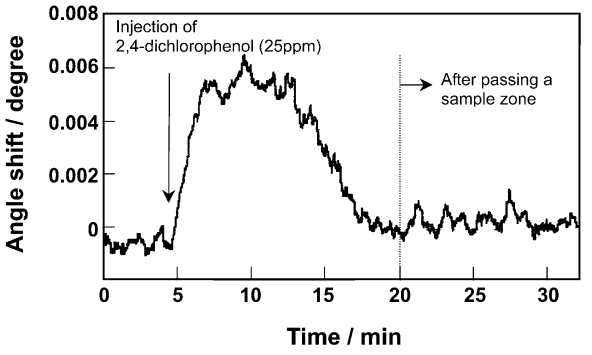


Fig. 5. SPR response to 2,4-dichlorophenol in a direct assay. Injected sample solution and volume: 25 ppm 2,4-dichlorophenol, 300 μl, sensor chip: immobilized with GBP membrane (GBP/protein G/antibody).

Fig. 5, the resonance angle is shifted to the positive side just after the injection of the sample solution. This may be due to the fact that the refractive index of the sample solution is higher than that of the sensor surface modified with the antibody membrane. After passing the sample zone through the SPR detector, the resonance angle returned to nearly the initial baseline. This angle shift from the baseline is as low as 0.001° although the SPR signal seems to be sluggish and noisy due to the angle resolution of the present SPR detection. This small angle shift cannot clearly verify the binding of 2,4-dichlorophenol to the antibody of the sensor membrane. The result is reasonable, considering the fact that the angle shift of the SPR sensor is dependent on a mass change on the sensor chip and that the molecular weight of 2,4-dichlorophenol is about 160.

3.4. Evaluation of affinity property of 2,4-dichlorophenol–BSA conjugate

As discussed in Section 3.3, the detection of 2,4-dichlorophenol at concentrations lower than 25 ppm could not be performed by the direct immunoassay. It is impossible to achieve a highly sensitive detection of 2,4-dichlorophenol in the ppb-order level using the direct assay, taking into account of the angle resolution of the present SPR sensor. In order to construct a more sensitive detection system for 2,4-dichlorophenol, we then investigated an indirect competitive detection method using a 2,4-dichlorophenol–BSA conjugate.

The 2,4-dichlorophenol–BSA conjugate was prepared by a coupling reaction between a 2,4-dichlorophenol derivative having an amino group and BSA, as described in Section 2.6. The response of the SPR sensor, the surface of which was modified with the GBP/protein G/anti-(2,4-dichlorophenol) antibody membrane, was investigated by injecting 300 μl of 17.5–350 ppm of the 2,4-dichlorophenol–BSA conjugate samples into the SPR system. As shown in the corner of Fig. 6, the plateau region of the angle shift observed after injection of the sample indicates that an expected antigen–antibody reaction occurred on the sensor surface. Although the molar concentration of the conjugate solutions was 1/100–1/1000 for the 2,4-dichlorophenol solution used for the direct assay, clear angle shifts were obtained for the conjugate samples. A little strange SPR response behavior observed for the 17.5 ppm sample in Fig. 6 may be due to the temperature variations during the measurement.

Fig. 6 shows the relationship between the concentration of 2,4-dichlorophenol–BSA conjugate and angle shift of the SPR sensor at equili-

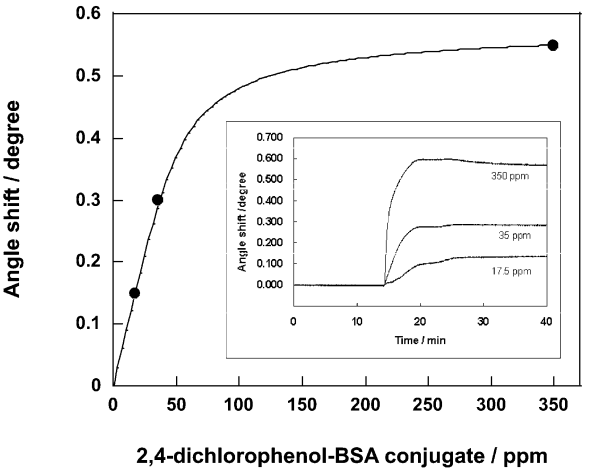


Fig. 6. Calibration curve for the 2,4-dichlorophenol–BSA conjugate. Conjugate solution: 17.5, 35, 350 ppm 2,4-dichlorophenol–BSA conjugate (300 μl), sensor chip: immobilized with the GBP membrane (GBP/protein G/antibody), carrier solution and flow rate: 10 mM PBS (pH 7.0), 10 μl min^{-1}.

brium. Although an insufficient number of data points were collected, the convex curve resembles a Langmuir-type adsorption isotherm. Namely, concentrations higher than 100 ppm, the angle shift approached a constant value. The site for the adsorption of the 2,4-dichlorophenol–BSA conjugate on the sensor surface immobilized with the antibody appears to be saturated with the BSA conjugate at higher concentration ranges. In preliminarily experiments, the regeneration of the sensor surface was attempted by injecting an HCl–glycine solution (pH 2) to remove the 2,4-dichlorophenol–BSA conjugate from the antibody. However, the plateau part of the angle shift did not return to the initial value. This indicates that the interaction between the antibody and 2,4-dichlorophenol is strong. In this work, a new sensor chip was used for each measurement.

From the result shown in Fig. 6, the affinity constant of the immobilized antibody to the 2,4-dichlorophenol–BSA conjugate can be evaluated by assuming a Langmuir-type adsorption model [31].

In the present system, the 2,4-dichlorophenol–BSA conjugate (Ag_1) is assumed to be in binding equilibrium with the antibody on the sensor surface. An affinity constant (K_1) can be expressed by the following equation.

$$K_1 = [\overline{Ab - Ag_1}]/[\overline{Ab}][Ag_1] \quad (1)$$

where $[Ag_1]$ denotes the molar concentration (mol/l) of the 2,4-dichlorophenol–BSA conjugate. $[\overline{Ab - Ag_1}]$ and $[\overline{Ab}]$ denote the surface concentration of the 2,4-dichlorophenol–BSA conjugate complex with the antibody and that of the free antibody on the sensor surface, respectively. The bar over the $Ab-Ag_1$ and Ab indicates that the surface concentration expressed in nmol mm^{-2}.

In addition, the following equation holds for a mass balance of the antibody on the sensor surface.

$$[\overline{Ab}]^T = [\overline{Ab - Ag_1}] + [\overline{Ab}] \quad (2)$$

where $[\overline{Ab}]^T$ represents the total concentration of the antibody on the sensor surface.

From Eqs. (1) and (2), a Langmuir-type adsorption equation is derived as follows, assuming that the concentration of Ag_1 is much larger than $[\overline{Ab}]^T$. Namely, the concentration of Ag_1 in the sample is assumed to be constant even after binding with the antibody on the sensor membrane.

$$[\overline{Ab\text{-}Ag_1}]/[\overline{Ab}]^T = K_1[Ag_1]/(1 + K_1[Ag_1]) \quad (3)$$

If the angle shift of the SPR sensor, $\Delta\theta_1$, is proportional to the surface concentration of the conjugate adsorbed to the sensor membrane ($[\overline{Ab - Ag_1}]$), the relative surface concentration of $[\overline{Ab - Ag_1}]$ to the total surface concentration of the antibody can be expressed as follows.

$$[\overline{Ab - Ag_1}]/[\overline{Ab}]^T = \Delta\theta_1/\Delta\theta_{1,max} \quad (4)$$

where $\Delta\theta_{1,max}$ denotes the angle shift of the SPR sensor when the antibody on the sensor surface is completely bound by the conjugate.

From Eqs. (3) and (4), the following equation is derived.

$$[Ag_1]/\Delta\theta_1 = [Ag_1]/\Delta\theta_{1,max} + 1/\Delta\theta_{1,max}K_1 \quad (5)$$

According to Eq. (5), $\Delta\theta_{1,max}$ and K_1 are calculated from the slope and intercept of the $[Ag_1]$ vs. $[Ag_1]/\Delta\theta_1$ plot, respectively. As evaluated from the results in Fig. 6, $\Delta\theta_{1,max} = 0.62^\circ$ and $K_1 = 1.5 \times 10^6\ M^{-1}$ were obtained. The values obtained for the present immunoreaction are reasonable, compared to the values reported for the immunoreaction between methamphetamine–BSA conjugate and its antibody [31].

3.5. Competitive assay for 2,4-dichlorophenol

Since the 2,4-dichlorophenol–BSA conjugate was found to be amenable to a competitive assay, the sensitivity of the detection for 2,4-dichlorophenol using a competitive assay was examined using the BSA conjugate. The sensitivity of the SPR response is known to be largely dependent on the refractive index or dielectric constant in the vicinity of the SPR chip surface. Therefore, the SPR response (angle shift) for the adsorption of a high molecular weight compound would be larger than that for the adsorption of a low molecular weight compound, provided the number of molecules is the same for each compound. The molecular weight of 2,4-dichlorophenol is about 160, while the molecular weight of 2,4-dichlorophenol–

BSA conjugate is about 68 000. The SPR response would be expected to be enhanced if the 2,4-dichlorophenol–BSA conjugate is bound to the SPR sensor chip. In fact, when the 2,4-dichlorophenol–BSA conjugate was introduced to the SPR system with the GBP/protein G/anti-(2,4-dichlorophenol) antibody membrane, a large SPR angle shift was observed, as shown in Fig. 6. In order to examine how the extent of 2,4-dichlorophenol inhibits the binding of the BSA conjugate with the antibody on the sensor membrane, a 350 ppm solution of the conjugate solution containing 250 ppb of 2,4-dichlorophenol was injected to the SPR system. As shown in Fig. 7, the angle shift for the mixed solution is smaller than that obtained by injecting the 350 ppm conjugate solution. This indicates that the binding of 2,4-dichlorophenol to the antibody on the sensor chip competes with that of the conjugate. Namely, the addition of 2,4-dichlorophenol to the conjugate solution reduces the amount of conjugate bound to the antibody on the sensor chip. Fig. 8 shows a calibration curve obtained from measurements using mixed sample solutions containing various concentration of 2,4-dichlorophenol and 350 ppm of the conjugate. The calibration curve is concave in shape. In the lower concentration range, the angle shift steeply decreased with increasing concentrations of 2,4-dichlorophenol,

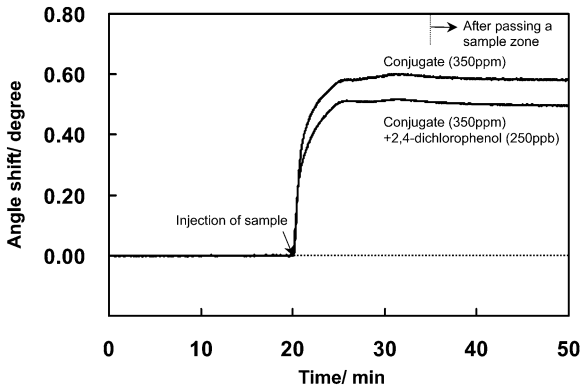


Fig. 7. SPR response for the conjugate solution with and without 2,4-dichlorophenol. Injected solution: 350 ppm 2,4-dichlorophenol–BSA conjugate or 350 ppm 2,4-dichlorophenol–BSA conjugate containing 250 ppb 2,4-dichlorophenol, carrier solution and flow rate: 10 mM PBS (pH 7.0), 10 μl min^{-1}.

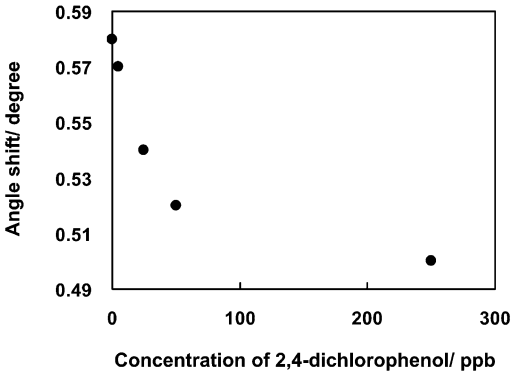


Fig. 8. Calibration curve for 2,4-dichlorophenol in the competitive assay. Injected sample: 350 ppm 2,4-dichlorophenol–BSA conjugate containing 5, 25, 50 or 250 ppb 2,4-dichlorophenol, injected volume: 300 μl, carrier solution and flow rate: 10 mM PBS (pH 7.0), 10 μl min^{-1}, sensor membrane: GBP membrane (GBP/protein G/antibody).

caused by competitive binding the conjugate to the antibody on the sensor chip. This type of calibration curve is typical for a competitive assay. The detection limit of this assay for 2,4-dichlorophenol cannot be estimated from the reproducibility of each data point in Fig. 8 since the calibration curve were obtained by a single experiment. As described in Section 3.2, since the reproducibility of the angle shift for immobilization of the antibody was 7.4%, each data point in Fig. 8 may include an 7.4% error. It may not be a defined detection limit, however, if the detection limit is defined as the concentration of 2,4-dichlorophenol where the angle shift was deviated from 0.04° from the angle shift for 0 ppb 2,4-dichlorophenol in Fig. 8, the detection limit is estimated to be 20 ppb.

In order to evaluate the validity of the calibration curve, a calibration curve was simulated based on a Langmuir-type adsorption model. In the competitive assay, 2,4-dichlorophenol and its BSA conjugate are bound competitively with the antibody to the sensor membrane. The affinity constant of 2,4-dichlorophenol to the antibody immobilized on the sensor chip (K_2) is expressed by the following equation as well as that of the 2,4-dichlorophenol–BSA conjugate discussed in Section 3.4.

$$K_2 = [\overline{Ab - Ag_2}]/[\overline{Ab}][Ag_2] \quad (6)$$

where $[Ag_2]$ is the molar concentration of 2,4-dichlorophenol, and $[\overline{Ab-Ag_2}]$ the surface concentration of 2,4-dichlorophenol bound to the antibody on the sensor chip. In the competitive assay, the total surface concentration of antibody ($[\overline{Ab}]^T$) is expressed as follows.

$$[\overline{Ab}]^T = [\overline{Ab-Ag_1}] + [\overline{Ab}] \quad (7)$$

From Eqs. (1), (6) and (7), the following equations, which are also identical to the Langmuir-type adsorption equation in the case of Eq. (3), are obtained.

$$[\overline{Ab-Ag_1}]/[\overline{Ab}]^T = K_1[Ag_1]/(1 + K_1[Ag_1] + K_2[Ag_2]) \quad (8)$$

$$[\overline{Ab-Ag_2}]/[\overline{Ab}]^T = K_2[Ag_2]/(1 + K_1[Ag_1] + K_2[Ag_2]) \quad (9)$$

The affinity constant of the 2,4-dichlorophenol–BSA conjugate (K_1) was calculated to be 1.5×10^6 M^{-1} in the previous discussion. The 350 ppm conjugate solution corresponds to $[Ag_1] = 5 \times 10^{-6}$ M, $[\overline{Ab-Ag_1}]$ and $[\overline{Ab-Ag_2}]$ can be calculated from Eqs. (7)–(9) as a function of $[Ag_2]$ by assuming appropriate values for $[\overline{Ab}]^T$ and K_2.

The value of $[\overline{Ab}]^T$ can be estimated from the degree of angle shift in the SPR measurement, because this value corresponds to a situation where all the binding sites on the sensor chip are completely covered with the conjugate. In general, an angle shift of 0.1° is approximately equivalent to 1 ng mm^{-2} of mass increase on the sensor surface [30]. The molecular weights of 2,4-dichlorophenol–BSA conjugate and 2,4-dichlorophenol are about 68 000 and 160, respectively. Thus, according to the above conversion, 6800° and 16° of angle shifts would be observed when the conjugate and 2,4-dichlorophenol are adsorbed on the sensor chip at concentrations of 1 nmol mm^{-2}, respectively. As can be seen from Fig. 8, the angle shift in the SPR response is 0.58° when the concentration of 2,4-dichlorophenol is zero and gradually approaches 0.49° with increasing concentrations of 2,4-dichlorophenol added to the 350 ppm 2,4-dichlorophenol–BSA conjugate solution. If 2,4-dichlorophenol inhibits the binding of the conjugate, the angle shift would be expected to approach 0.001°, judging from its molecular weight and the result of the direct assay. This discrepancy between the experimentally observed shift angle and that expected may be due to the fact that the anti-(2,4-dichlorophenol) polyclonal antibody used in this study contains another type of antibody, which only recognizes the 2,4-dichlorophenol–BSA conjugate and not 2,4-dichlorophenol. The (2,4-dichlorophenol) polyclonal antibody as an impurity may induce the binding of a different kind of BSA conjugate, and this may cause the background angle shift (0.49°) in the SPR response. Although this speculation may not be correct, the angle shift actually changed from 0.58° to 0.50° due to the competitive binding of 2,4-dichlorophenol at an added concentration of 250 ppb. If this change in the angle shift (0.08°) is assumed to be due to the fact that the 2,4-dichlorophenol–BSA conjugate occupying all the binding sites is replaced with 2,4-dichlorophenol, the total surface concentration of the antibody can be estimated from the conversion between the angle shift and the mass of the conjugate bound to the sensor surface. The total surface-concentration of the anti-(2,4-dichlorophenol) antibody ($[\overline{Ab}]^T$) was estimated to be 1.5×10^{-5} nmol mm^{-2} by assuming that a 0.1° of angle shift increase is equivalent to a mass increase of 1 ng mm^{-2} on the sensor surface. In addition, if the angle shift caused by the binding of the hypothesized anti-(2,4-dichlorophenol–BSA conjugate) antibody is assumed to be a constant value of 0.49°, judging from the calibration curve in Fig. 8. The angle shift in the present competitive assay ($\Delta\theta$) can be estimated by the following equation.

$$\Delta\theta = 6800[\overline{Ab-Ag_1}] + 16[\overline{Ab-Ag_2}] + 0.49 \quad (10)$$

where 6800 and 16 are the coefficients that are proportional to the molecular weight of Ag_1 (BSA conjugate) and Ag_2 (2,4-dichlorophenol), respectively. These values have a dimension of [deg mm^2 $nmol^{-1}$].

By using Eqs. (8)–(10), the relationship between $\Delta\theta$ and the concentration of added 2,4-dichlorophenol, $[Ag_2]$, can be theoretically calculated, as a parameter K_2. Fig. 9 shows the theoretical calibration curves, in which K_2 is varied in the range of 0.1×10^6 M^{-1} to 200×10^6 M^{-1}. In this case, the

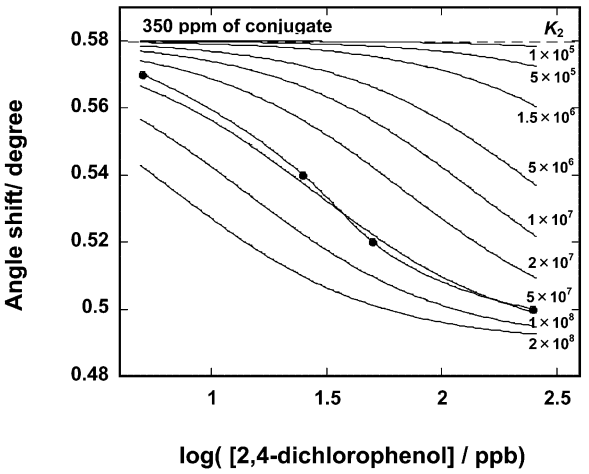


Fig. 9. Theoretical calibration curves in the competitive assay. Values of K_2 are indicated in the figure. Filled circles are experimental data. $[Ag_2]$ is converted to ppb units. Calculated from Eqs. (8)–(10) assuming $K_1 = 1.5\times10^6\ M^{-1}$, $[\overline{Ab}]^T = 1.5\times10^{-5}$ nmol mm^2, $[Ag_2] = 5\times10^{-6}$ M (350 ppm BSA conjugate).

concentration of Ag_2 is converted to ppb units. In the case where K_2 is smaller than K_1, which was obtained experimentally for the affinity constant between the BSA conjugate and the antibody, the slope of the calibration curve slowly declines, while the slope becomes steeper with an increase in K_2 values. The dependence of K_2 on the slope of the calibration curve can be easily understood for the competitive binding of the conjugate and 2,4-dichlorophenol. Namely, a small change in angle shift would be expected for a small value of K_2 because of the preferential binding of the conjugate with the antibody on the sensor chip. On the contrary, when the value of K_2 is large, the angle shift should decrease dramatically because 2,4-dichlorophenol, which has a small molecular weight is, preferentially bound to the sensor chip. The angle shift obtained experimentally in the present study are consistent with the theoretical curve calculated by using $K_2 = 5\times10^7\ M^{-1}$. This indicates that the affinity constant of 2,4-dichlorophenol for the antibody is estimated to be 33-fold larger than that of the 2,4-dichlorophenol–BSA conjugate. The difference in the affinity constants between 2,4-dichlorophenol and its conjugate may be explained by the fact that the ability of the 2,4-dichlorophenol–BSA conjugate to recognize the anti-(2,4-dichlorophenol) antibody is smaller than that of the 2,4-dichlorophenol because of the modification of the phenol moiety with the BSA.

4. Conclusion

An SPR sensor for 2,4-dichlorophenol, a major dioxin precursor, was fabricated and its sensitivity evaluated by direct and competitive assays. In the case of the SPR sensor, the surface of the sensor chip was modified with an anti-(2,4-dichlorophenol) antibody using GBP and protein G in order to enhance the density of the immobilized antibody and to orientate the antibody efficiently so as to increase the number of sites available for binding to the analyte. Although the angle shift of the SPR sensor to 25 ppm of 2,4-dichlorophenol was as small as 0.001° by a direct assay, the sensitivity of the sensor was greatly enhanced by a competitive assay using a conjugate prepared by coupling 2,4-dichlorophenol to BSA. A lower detection limit of the present method was estimated to be 20 ppb from the coefficient of variation of the angle shift for immobilization of the antibody on the sensor chip. The affinity constant of the anti-(2,4-dichlorophenol) antibody to the 2,4-dichlorophenol–BSA conjugate was calculated to be $1.5\times10^6\ M^{-1}$ from the SPR response obtained by injecting conjugate samples into the SPR system. The affinity constant of the anti-(2,4-dichlorophenol) antibody to the 2,4-dichlorophenol was estimated to be $5\times10^7\ M^{-1}$ by fitting the experimental data to the theoretical calibration curve in the competitive assay. These affinity constants estimated with present work may have 7–8% error, taking into account of the coefficient of variation of immobilization of the antibody.

Acknowledgements

The authors are grateful for Prof. Clement E. Furlong (University of Washington, USA) for his helpful assistance of purification of GBP. We thank Prof. Mizuo Maeda and Dr Masaharu Murata (Kyushu University, Japan) for helpful

discussion about the preparation of the modified sensor chip. This work was supported by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan.

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