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Indirect Competitive Immunoassay for Bisphenol A, Based on a Surface Plasmon Resonance Sensor

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A method for the determination of bisphenol A (BPA), a representative endocrine-disrupting chemical, was developed using a surface plasmon resonance (SPR) sensor. The method is based on an indirect competitive immunoassay, where a BPA sample containing an anti-BPA antibody is introduced into the SPR sensor system. A sensor chip immobilized with a 2AET layer/ BPA layer membrane was prepared by depositing 2-aminoethanthiol (2AET) on a gold film on the sensor chip, followed by reacting the 2AET layer with esterified BPA. The resulting sensor chip was placed into the SPR sensor of a flow system, which consisted of a syringe pump and an injector. The sensor response, in the form of a resonance angle shift, was measured as a function of time before and after injecting different concentrations of BPA in a sample solution that contained the anti-BPA antibody at a constant concentration. In order to estimate the affinity constant of the anti-BPA antibody to BPA, which was immobilized on the sensor chip, the SPR angle shift was first measured by injecting an anti-BPA antibody solution at different concentrations into the SPR system. The affinity constant of the anti-BPA antibody to BPA immobilized on the sensor chip (K_1) was calculated to be 9.3×10^5 M^{-1} from the SPR angle shift data, assuming a Langmuir adsorption isotherm. BPA sample solutions (1–100 ppb) containing 40 ppm anti-BPA antibody were then injected into the SPR system, and the SPR angle shift was determined for each of the sample solutions. A conventional sigmoidal calibration curve, which was typically observed in a competition immunoassay, was obtained when the SPR angle shift was plotted against the BPA concentration. The affinity constant of the anti-BPA antibody to the free BPA (K_2) in the sample solution was estimated to be 1×10^7 M^{-1} by fitting the observed calibration curve to the theoretical one for a competitive inhibition assay. The BPA detection limit of the method was determined to be approximately 10 ppb.

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1. Introduction

Bisphenol A (BPA) represents a member of a class of endocrine-disrupting chemicals (EDCs), which are thought to mimic natural hormones and to cause abnormal sexual development and reduction in male fertility. BPA has been reported to have weak estrogenic properties[1,2] and, as a result, the release of BPA from many products made of polycarbonates is of some concern, since polycarbonate can contain BPA as a monomer residue. Because various plastics such as polycarbonates and epoxy resins are in widespread use, the potential for humans and wildlife species to be exposed to such harmful compounds is an important issue. In order to determine BPA levels in environments such as river water and seawater, a sensitive and rapid method is desirable. Although a chromatographic method can be used for the analysis of BPA, several time-consuming sample pretreatment steps are required.[3] Gas chromatography-mass spectrometry (GC-MS) is one of the reliable and currently used analytical methods for BPA.[4,5] However, the method requires expensive instrumentation, a complicated derivatization process, and expert skills. An enzyme-linked immunosorbent assay (ELISA) has been widely used for the determination of EDCs including BPA due to its advantages that include high selectivity, high sensitivity, high sample throughput, and low cost,[6,7] but a minimum of one hour is required to complete a single measurement.

Recently, a sensor-based surface plasmon resonance (SPR) technique has been applied to an effective transducer for several biosensors by immobilizing a specific receptor substance on the sensor chip.[8–17] The SPR sensor, in which an antibody for a target molecule is immobilized on the sensor chip, would be expected to be highly sensitive and selective due to a specific binding of the target molecule with the antibody. One of the advantages of the SPR sensor is that real-time and automated monitoring is possible without the need for labeling by a fluorophore or an enzyme. Shimomura *et al.* reported an SPR sensor for determining 2, 3, 7, 8-tetra-chlorodibenzo-*p*-dioxin, PCB, and atrazine using an SPR sensor chip, on which antibodies for these chemicals were immobilized by an amine coupling method.[14] We also reported an SPR sensor for 2, 4-dichlorophenol, as a dioxin precursor, using an SPR sensor chip, to which an anti-(2, 4-dichlorophenol) antibody was immobilized with the assistance of a gold-binding polypeptide (GBP) and protein G.[15] In our method as well as the method reported by Shimomura *et al.*, an antibody for the analyte was immobilized on the sensor chip. In a different approach, Sakai *et al.* fabricated a type of SPR immunosensor, in which the analyte (antigen) conjugated with bovine serum albumin (BSA), was immobilized on the sensor chip. They demonstrated the utility of this sensor for the highly sensitive and selective determination of methamphetamine[16] and benzopyrene[17] using an indirect competitive immunoassay.

In this paper, we report on an SPR immunosensor for BPA by adopting the method reported by Sakai *et al.* Namely, an SPR sensor chip, on the surface of which BPA was immobilized via a self-assemble alkane thiol monolayer. The rationale for this is that an SPR system using such an antigen-immobilized sensor chip would be suitable for the repeated measurement of several samples by one sensor chip, since the dissociation of the antigen-antibody complex can be achieved using a regeneration reagent solution after completion of the measurement.[16,17] An SPR system equipped with a BPA-immobilized

sensor chip was used for the determination of BPA. The affinity constants of the anti-BPA antibody to free BPA in a sample solution as well as to BPA immobilized on the SPR sensor chip were also evaluated from the concentration dependency of the SPR sensor response, assuming a Langmuir-type adsorption isotherm.

2. Materials and Methods

2.1 *Materials*

BPA, *N*-hydroxysuccinimide (NHS), and BSA were purchased from Wako Chemical Co. Ltd. (Osaka, Japan). Ethyl 6-bromohexanoate, *N, N'*-dicyclohexylcarbodiimide, and 2-aminoethanethiol were obtained from Kishida Chemical Co., Ltd. (Osaka, Japan). Anti-(bisphenol A) monoclonal antibody was donated by Takeda Chemical Industries, Ltd. (Osaka, Japan). A sensor chip comprised of a cover glass (18 mm×18 mm×0.15 mm^t), to which a thin gold layer, 45 nm in thickness, was deposited with the assistance of a 3-nm-thick chrome layer between the cover glass and the thin-gold layer, was obtained from Eliotech (Tokyo, Japan). All other reagents and solvents were purchased commercially and were used without further purification.

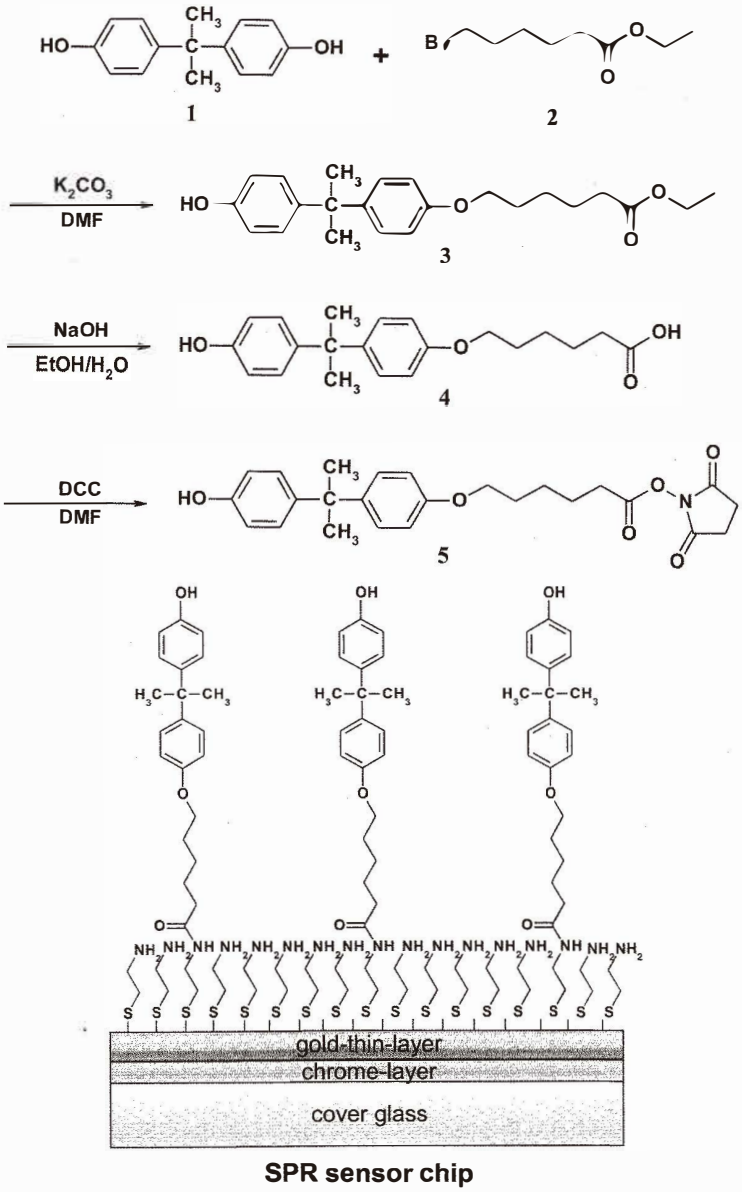
2.2 *Apparatus of SPR sensor system*

An SPR sensor 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. The flow cell was assembled on the SPR detector by attaching a 20-μm-thick silicon packing with a groove (3 mm×10 mm) on the gold-layered cover glass. A matching oil with a refractive index of 1.516 was coated between the back surface of the cover glass of the sensor chip and the prism of the SPR detector. The cell volume of the SPR detector was approximately 6 μL.

2.3 *Synthesis of bisphenol A succinimidyl ester*

The synthesis scheme for bisphenol A succinimidyl ethyl ester (**5**) is illustrated in Scheme 1 (a). BPA (**1**, 5.11 g, 22.4 mmol), ethyl 6-bromohexanoate (**2**, 5.00 g, 22.4 mmol), and potassium carbonate (4.60 g, 33.3 mmol) were added to 50 mL of DMF and the mixture was heated at 80–85°C for 2 h with stirring. The DMF was evaporated from the reaction mixture *in vacuo*, and the residue dissolved in 100 mL of ethyl acetate. The ethyl acetate phase was twice washed with 100 mL portions of pure water in a separatory funnel. The ethyl acetate phase was dried over $MgSO_4$ and filtered, and the filtrate concentrated under vacuum. The resulting residue was chromatographed on silica gel (ethyl acetate/hexane = 1/6, v/v) to yield 1.28 g of product **3** (yield : 15.5%) .

Product **3** (1.28 g, 3.45 mmol) was dissolved in 8 mL of a mixture of ethanol and water (ethanol/water = 7/3, v/v) containing NaOH (0.448 g, 7.99 mmol). The mixture was refluxed for 2 h at 75–80°C with stirring and was then concentrated under vacuum. The crude reaction mixture was dissolved in 10 mL of $CHCl_3$ and washed with a 0.1 M sodium citrate solution in a separatory funnel. The organic phase was evaporated and the resulting dark-orange residue was recrystallized from ethyl acetate and hexane to yield 0.633 g (53.2%) of a pale orange powder (**4**).



Scheme 1. Synthesis of BPA succinimidyl ester (a) and a schematic diagram of the SPR sensor chip prepared in this study (b).

Product **4** (0.633 g, 1.84 mmol) was dissolved in 4 mL of DMF. Two milliliters of a DMF solution containing NHS (0.213 g, 1.84 mmol) and DCC (0.381 g, 1.84 mmol) was

then added dropwise to the DMF solution of product **4**, following by stirring for 5 h at room temperature. The resulting white precipitate, a by-product, was removed by filtration, and the yellow filtrate was evaporated *in vacuo*. The residue was dissolved in ethyl acetate and washed with a 0.05 M tartaric acid solution. The ethyl acetate phase was dried over $MgSO_4$ and filtered, and the filtrate was then evaporated *in vacuo* to yield 0.710g (87.9%) of product **5**. Products **3**–**5** were characterized by ^{1}H-NMR and elemental analysis.

2.4 *Immobilization of BPA on the SPR sensor chip*

A thin-gold-layered sensor chip was sonicated for 25 min in acetone and the chip was then soaked in a piranha solution (conc. H_2SO_4 / 30% H_2O_2 = 1/1, v/v) for 15 min. After washing with deionized water and drying, the sensor chip was then placed in the flow cell of the SPR detector, and 120 μL of a 0.01 M 2AET solution was injected from the injector, where pure water was allowed to continuously flow through the syringe pump at a flow rate of 4 μL/min. This procedure allowed the 2 AET layer to be immobilized on the sensor chip. Subsequently, 120 μL of an aqueous solution of 0.2 mg/mL of BPA succinimidyl ethyl ester (**5**) (containing 10% methanol) was injected into the stream in order to couple **5** with 2AET, which was immobilized on the thin-gold-layered sensor chip (Scheme 1 (b)). The immobilization of 2AET on the gold surface and the coupling of BPA succinimidyl ethyl ester with the 2AET layer were confirmed by monitoring the SPR response as the change in resonance angle during the passage of the sample zone through the SPR detector. Then, 100 μL of a 500 ppm BSA solution was injected into the carrier stream, to block any unmodified sites on the thin-gold-layered sensor chip in order to prevent any unspecific adsorption of the anti-BPA antibody, since a solution of BPA will be injected in the actual measurement of BPA. In this case, the carrier solution was changed from water to a 10 mM phosphate buffer containing 0.15 M NaCl (pH 7.2) and was allowed to flow at a rate of 5 μL/min.

2.5 *Measurements of the SPR response to the anti-BPA antibody to evaluate the affinity constant of the anti-BPA antibody to BPA on the sensor chip*

The SPR sensor chip immobilized with the 2AET/BPA layer was placed in the flow cell of the SPR detector. Solutions of anti-BPA antibody at different concentrations (10–40 ppm) containing 0.05% sodium azide were prepared. One hundred milliliters of the antibody solutions were injected into the SPR system, starting with the lower concentration sample, where the carrier solution, 10 mM PBS-0.15 M NaCl (pH 7.2), was pumped at a flow rate of 5 μL/min. The SPR responses to the antibody solutions were then monitored.

2.6 *Procedures of indirect competitive assay for BPA*

The SPR sensor chip immobilized with the 2AET/BPA layer was placed in the flow cell of the SPR detector. Solutions of BPA (2–200 ppb) at different concentrations were mixed with an 80 ppm anti-BPA antibody solution at a 1:1 volume ratio, and were incubated for 30 min at 25°C. A 140 μL aliquot of the incubated sample solutions was then injected into the SPR system, where the 10 mM PBS-0.15 M NaCl (pH 7.2) carrier solution was pumped at a flow rate of 5 μL/min. In order to repeat the assay, after the resonance angle of the SPR response reached a plateau due to immunoreaction of the antibody with the BPA, which

was immobilized on the sensor chip, 140 μL of a 0.01 M HCl solution was injected into the carrier stream to dissociate the antibody from the immunocomplex on the sensor chip.

3. Results and Discussion

3.1 *Detection strategy for BPA*

It is difficult, in general, to detect a chemical compound with a low molecular weight such as BPA, the target compound, directly with an SPR system with a high sensitivity due to the fact that the change in refractive index in the vicinity of the sensor chip is very small even when an immunoreaction is involved.[15] To overcome this difficulty, techniques based on a competitive immunoassay have been reported. Two methods are available for a competitive immunoassay in SPR determinations, as well as the conventional ELISA method. One involves an antibody for the target molecule, which is immobilized on the sensor chip of the SPR system. This method involves the introduction of a solution of the target molecule containing a high molecular weight conjugate of the target molecule to the sensor chip. In the other method, a target molecule is immobilized on the sensor chip, and a solution of the target molecule containing the antibody to the target molecule is introduced on the sensor chip. The latter method using a sensor chip immobilized with an antigen (target molecule for assay) is particularly suitable for repeated measurements using the same sensor chip, since the modified membrane on the sensor chip typically has a good stability. In the former case, the antibody immobilized on the sensor chip may be inactivated when the target molecule is dissociated from the immunocomplex by means of a dissociation solution after measurement. In the present work, a BPA-immobilized SPR sensor chip was prepared using 2AET as a component of the anchor layer. The immobilization would be expected to be stable due to the strong interaction of the thiol group of 2AET with gold and covalent bonding between 2AET and the esterified BPA. Sample solutions of BPA at different concentrations were mixed and incubated with the anti-BPA solution at a 1:1 volume ratio and the resulting solutions were injected into the SPR system. A portion of the antibody in the mixed solution would be complexed by an immunoreaction with the free BPA, and consequently, the SPR response would be reduced with increasing amounts of BPA in the sample solution, since the amount of antibody, that reacts with BPA immobilized on the sensor chip, would be reduced. In the present study, a 40 ppm anti-BPA antibody solution was used for the determination of BPA in the concentration range from 1 to 100 ppb. The affinity constants for the anti-BPA antibody to the free BPA in the sample solution as well as to the BPA immobilized on the SPR sensor chip were also evaluated by assuming a Langmuir-type adsorption isotherm and an equilibrium state for the immunoreaction.

3.2 *Immobilization of BPA on the sensor chip*

For the immobilization of BPA on the sensor chip, 2AET was used to prepare a stable anchor membrane, by utilizing the strong affinity of the thiol group of 2AET to gold.[18] Figure 1 shows the angle shift of the resonance signals of the SPR sensor after the injection of 120 μL of a 0.01 M 2AET solution and subsequent injection of 120 μL of a 0.2 mg/mL esterified BPA (**5**) solution. As can be seen from Fig. 1, the angle shift increased steeply

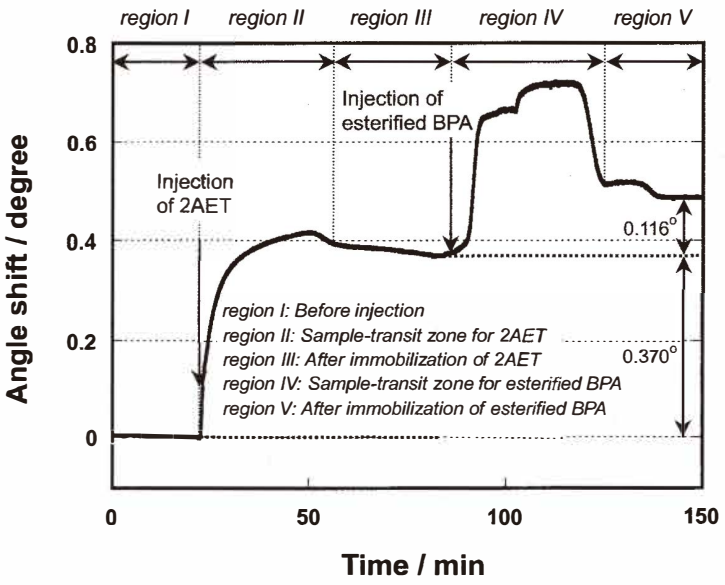


Fig. 1. SPR responses in the immobilization process of BPA on the sensor chip. Injected solution and volume: 0.01 M 2AET (120 μL), 0.2 mg/mL **5** (120 μL), carrier solution and flow rate: pure water, 4 μL/min.

during the sample zone corresponding to 2AET passing through the SPR detector and an angle shift of 0.370° was observed after the sample zone had passed through. This suggests that 2AET was immobilized on the surface of the SPR sensor chip. According to the specifications of the SPR instrument published by BIACORE, an angle shift of 0.1° corresponds to a mass change of 1 ng/mm^2 on the surface of the sensor chip.[19] Therefore, the angle shift of 0.370° indicates that 2AET was immobilized on the sensor chip at a surface concentration of 3.70 ng/mm^2 (4.80×10^{-2} nmol/mm^2). When 120 μL of 0.2 mg/mL **5** was injected into the SPR system after the angle shift reached a plateau, an angle shift of 0.116° was observed. This indicates that **5** was coupled to 2AET on the sensor chip thus verifying that a sensor chip immobilized with BPA had been prepared. According to the BIACORE specifications, the BPA derivative was calculated to be immobilized on the sensor chip at a surface concentration of 1.16 ng/mm^2 (2.64×10^{-3} nmol/mm^2). This value indicates that approximately 1/18 of the amino groups in the 2AET membrane were coupled with **5**. Thus, the sensor chip immobilized with the BPA derivative was evaluated for its binding affinity with the anti-BSA antibody. When 100 μL of a 500 ppm BSA solution was injected into the SPR system equipped with the 2AET layer/ BPA layer sensor chip, no significant change in SPR angle shift was observed before and after the injection. This finding indicates the absence of any unmodified sites available to be adsorbed by the bulky BSA, because 2AET and 2AET-BPA were immobilized at a density as high as 3.70 ng/mm^2 on the sensor chip.

3.3 *Evaluation of affinity property of anti-BPA antibody with BPA on the sensor chip*

The affinity properties of the anti-BPA antibody used in this work to BPA were investigated. Figure 2 shows the SPR response when 100 μL of antibody solutions (10 ppm, 20 ppm, 30 ppm and 40 ppm) were successively injected into the SPR system equipped with the sensor chip. Resonance angle shifts, 0.035°, 0.054°, 0.069° and 0.067°, respectively, were observed for the injection of 10 ppm, 20 ppm, 30 ppm and 40 ppm of the antibody solutions, when the sample zone passed through the SPR detector. The angle shifts clearly indicate that the antibody was bound to the immobilized BPA. In this case, since the dissociation procedure between each injection was not carried out, the antibody may have accumulated on the sensor chip via the formation of an immunocomplex with the BPA. The resonance angle shift at equilibrium was plotted against the sum of the concentration of the antibody for the previous samples. The results are shown in Fig. 3. After the successive injection of four different concentrations of antibody solution, a 0.225° resonance angle shift was observed. This indicates that 1.5×10^{-5} nmol/mm^2 (molecular weight of antibody: *ca.* 150,000) of the antibody was immobilized on the sensor chip.

From the results shown in Fig. 3, the affinity constant of the anti-BPA antibody to BPA immobilized on the sensor chip can be evaluated assuming a Langmuir-type adsorption model. In the present system, the anti-BPA antibody is assumed to be in binding

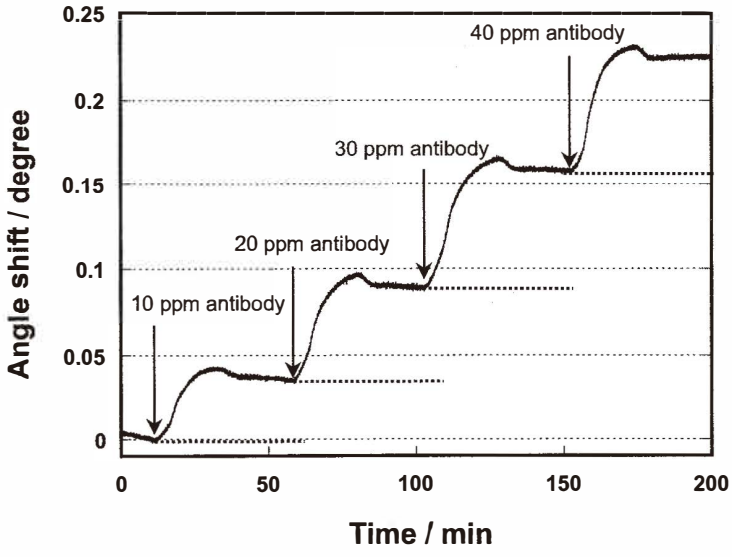


Fig. 2. SPR responses to the anti-BPA antibody. Injected sample: 10, 20, 30, or 40 ppm anti-BPA antibody, injected volume: 100 μL, carrier solution and flow rate: 10 mM PBS-0.15 M NaCl (pH 7.2), 5 μL/min.

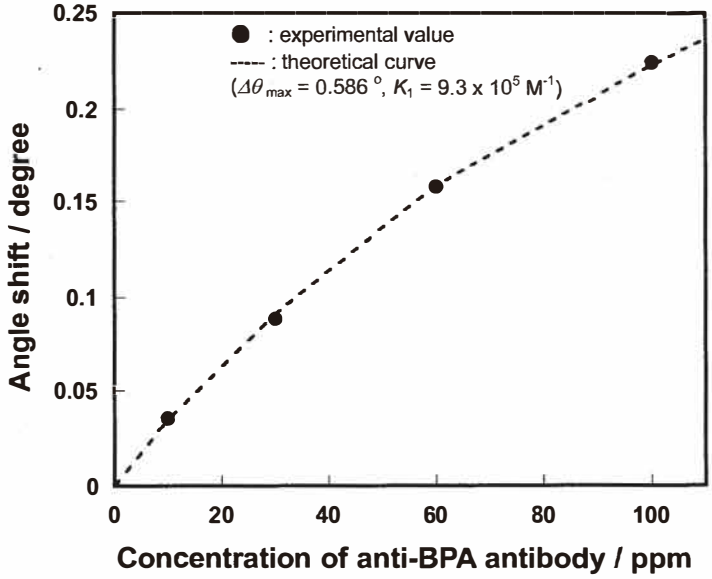


Fig. 3. Relationship between angle shift and concentration of anti-BPA antibody. The closed circles and dotted lines denote the experimental data and a theoretical curve, respectively. The theoretical curve was calculated assuming a Langmuir isotherm ($\Delta\theta_{max}$ = 0.586° K_1 = 9.3 × 10^5 M^{-1}). Injected sample: 10, 20, 30 or 40 ppm anti-BPA antibody, injected volume: 100 μL, carrier solution and flow rate: 10 mM PBS-0.15 M NaCl (pH 7.2), 5 μL/min.

equilibrium with the immobilized BPA. If the binding equilibrium is assumed to obey a Langmuir-type adsorption model, the affinity constant between the anti-BPA antibody and BPA immobilized on the sensor chip (K_1) can be expressed by the following equation.

$$K_1 = \underline{[BPA\text{-}Ab]}/[BPA][Ab] \quad (1)$$

Where [Ab] denotes the molar concentration (mol/L) of the anti-BPA antibody, and $\underline{[BPA\text{-}Ab]}$ and $\underline{[BPA]}$ denote the surface concentration (nmol/mm^2) of the BPA complex with the antibody and that of the uncomplexed BPA on the sensor surface, respectively. The underlines indicate the surface concentration of the chemical species expressed in nmol/mm^2.

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

$$\underline{[BPA]}^T = \underline{[BPA\text{-}Ab]} + \underline{[BPA]} \quad (2)$$

Where $\underline{[BPA]}^T$ represents the total surface concentration of BPA on the sensor surface.

A Langmuir-type adsorption isotherm can be derived from eqs. (1) and (2), assuming that the concentration of [Ab] in the sample is constant, even after binding with the immobilized BPA.

$$[\underline{\text{BPA–Ab}}]/[\text{BPA}]^{\text{T}} = K_1[\text{Ab}]/(1 + K_1[\text{Ab}]) \quad (3)$$

If the angle shift of the SPR sensor, $\Delta\theta$, is proportional to the surface concentration of the antibody bound to the BPA on the sensor chip ([BPA-Ab]), the relative surface concentration of [BPA-Ab] to the total surface concentration of the BPA immobilized on the sensor chip ($[\text{BPA}]^{\text{T}}$) can be expressed as follows.

$$[\underline{\text{BPA–Ab}}]/[\text{BPA}]^{\text{T}} = \Delta\theta/\Delta\theta_{\text{max}} \quad (4)$$

where $\Delta\theta_{\text{max}}$ denotes the angle shift of the SPR sensor when the BPA on the sensor surface is completely bound to the antibody.

From eqs. (3) and (4), the following equation can be derived.

$$[\text{Ab}]/\Delta\theta = [\text{Ab}]/\Delta\theta_{\text{max}} + 1/\Delta\theta_{\text{max}}K_1 \quad (5)$$

According to eq. (5), $[\text{Ab}]/\Delta\theta$ was plotted against [Ab] using the data shown in Fig. 3 in order to confirm that the Langmuir-type adsorption isotherm holds in the present system. As can be seen from Fig. 4, a good linear relationship between $[\text{Ab}]/\Delta\theta$ and [Ab] was found, indicating that the Langmuir-type adsorption isotherm holds. $\Delta\theta_{\text{max}}$ and K_1 were calculated to be $\Delta\theta_{\text{max}} = 0.586°$ and $K_1 = 9.3\times10^5$ M^{-1}, respectively, from the slope and intercept of the [Ab] vs. $[\text{Ab}]/\Delta\theta$ plot. Using the values, $\Delta\theta_{\text{max}} = 0.586°$ and $K_1 = 9.3\times10^5$ M^{-1} obtained from Fig. 4, the experimental data shown in Fig. 3 is in good agreement with the theoretical curve shown by the dotted line.

3.4 *Indirect competitive assay for BPA*

In Section 3.3, it was confirmed that the modified sensor chip with a 2AET layer/ BPA layer membrane had affinity for the anti-BPA antibody. Therefore, determination of BPA based on an indirect competitive assay was performed by using the system. The modified sensor chip was placed in the flow cell of the SPR detector and the SPR response change in resonance angle shift for a BPA sample solution containing the anti-BPA antibody was monitored for a competitive assay.

When 50 μL of a blank solution (containing 0 ppb BPA and 40 ppm anti-BPA antibody) was injected into the SPR system, a resonance angle shift of 0.086° was observed. The angle shift of 0.086° was smaller than the value of 0.106°, expected from the results shown in Fig. 3. This is probably because the volume of sample solution injected here (50 μL) was half that injected in Fig. 3 (100 μL). In order to dissociate the antibody from the immunocomplex formed on the sensor surface, several dissociation solutions were examined. When 0.01 M HCl was injected into the SPR system, the SPR angle shift returned to the initial value within 0.01°, as shown in Fig. 5. This indicates that HCl solution is suitable for dissociating the antibody from the immunocomplex and that the sensor chip can be

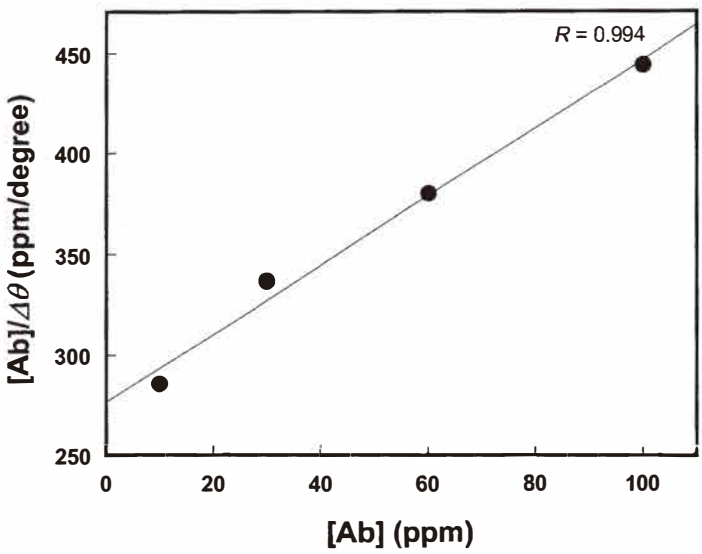


Fig. 4. Relationship between [Ab] and [Ab]/$\Delta\theta$.

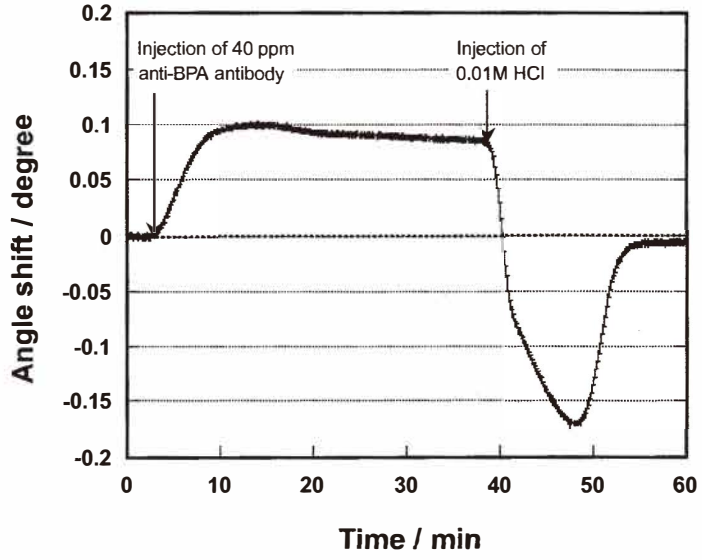


Fig. 5. Regeneration of the sensor chip by injecting a dissociation solution. Injection sample: 40 ppm anti-BPA antibody (50 μL), dissociation solution: 0.01 M HCl (140 μL), carrier solution and flow rate: 10 mM PBS-0.15 M NaCl (pH 7.2), 5 μL/min.

regenerated for the next measurement. After the regeneration of the sensor chip, sample solutions of BPA (1–100 ppb), containing 40 ppm of the anti-BPA antibody, which were incubated at 25°C for 30 min beforehand, were injected into the SPR system. The surface of the sensor chip was regenerated after each measurement by injecting a 0.01 M HCl solution. Figure 6 shows the SPR responses to the blank and BPA sample solutions used in the competitive assay. Since the angle shift observed for a sample solution containing BPA at 1 ppb was almost the same as that observed for the blank solution without BPA, these data are omitted from Fig. 6. The angle shifts observed for the 10–100 ppb samples were clearly dependent on the concentration of BPA in the sample solution. A calibration curve, in which the resonance angle shift at the plateau is plotted against the BPA concentration observed in the SPR system, is shown in Fig. 7. The angle shift decreases with increasing concentration of free BPA in the sample solution containing the anti-BPA antibody at a constant concentration. This is due to the fact that the amount of anti-BPA antibody, available for binding with the immobilized BPA, decreased with increasing BPA concentration in the sample. The reason for this is that the anti-BPA antibody in the sample solution competitively binds with the free BPA in the sample solution. From Fig. 7, it can be concluded that BPA can be quantitatively determined in the range of 10–100 ppb using the present SPR system.

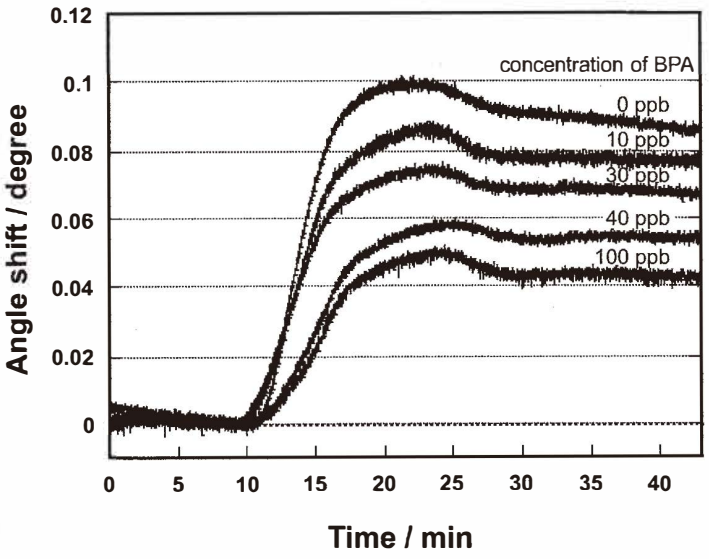


Fig. 6. SPR responses to anti-BPA antibody in the competitive assay. Injected sample: 40 ppm anti-BPA antibody containing BPA (0, 10, 30, 40 or 100 ppb), injected volume: 50 μL, carrier solution and flow rate: 10 mM PBS-0.15 M NaCl (pH 7.2), 5 μL/min.

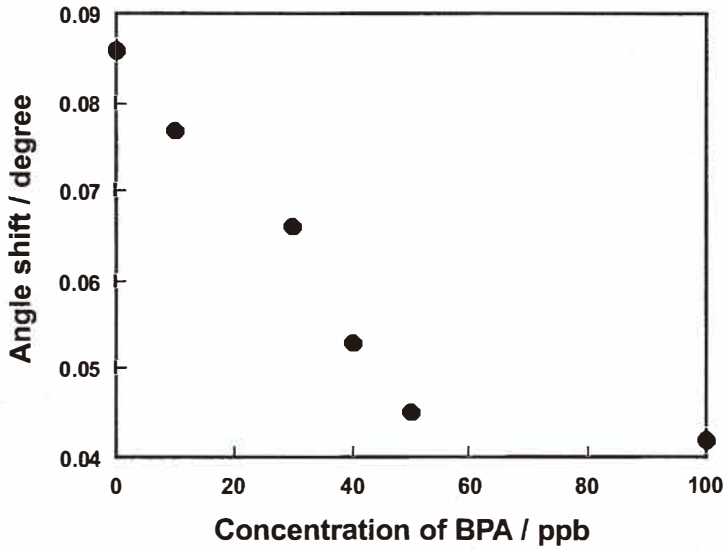


Fig. 7. Calibration curve for BPA in the competitive assay. Injected sample: BPA (0, 10, 30, 40, 50 or 100 ppb) containing 40 ppm anti-BPA antibody, injected volume: 50 μL, carrier solution and flow rate: 10 mM PBS-0.15 M NaCl (pH 7.2), 5 μL/min.

3.5 *Evaluation of affinity constant of the antibody to BPA in solution from the competitive assay*

For the purpose of evaluating the validity of the calibration curve shown in Fig. 7, the observed calibration curve was simulated based on a Langmuir-type adsorption model taking into account the chemical equilibrium of the immunoreactions between the antibody and the free BPA in the sample solution and the BPA immobilized on the sensor chip. In the competitive assay, the binding of some amount of anti-BPA antibody in the sample solution to the BPA immobilized on the sensor chip is inhibited by an immunoreaction between the anti-BPA antibody and the free BPA in the sample solution. The affinity constant for the anti-BPA antibody to the free BPA in the sample solution (K_2) can be expressed by the following equation.

$$K_2 = [\text{BPA-Ab}]/[\text{BPA}][\text{Ab}] \tag{6}$$

Where [BPA] and [Ab] are the molar concentrations of the free BPA and the free antibody in the sample solution, respectively, and [BPA-Ab] is the molar concentration of the BPA-antibody complex in the sample solution.

In addition, the mass balances of the antibody and BPA in the sample solution are expressed by eqs. (7) and (8), respectively.

$$[\mathrm{Ab}]^{\mathrm{T}} = [\mathrm{BPA\text{-}Ab}] + [\mathrm{Ab}] + 1/d[\underline{\mathrm{BPA\text{-}Ab}]} \tag{7}$$

$$[\mathrm{BPA}]^{\mathrm{T}} = [\mathrm{BPA\text{-}Ab}] + [\mathrm{BPA}] \tag{8}$$

where $[\mathrm{Ab}]^{\mathrm{T}}$ and $[\mathrm{BPA}]^{\mathrm{T}}$ are the total concentrations of antibody and BPA in the sample solution, respectively, $\underline{[\mathrm{BPA\text{-}Ab}]}$ denotes the surface concentration of the BPA complex with the antibody, and d denotes the thickness of a layer of the BPA complex with the antibody on the sensor chip. The third term of right-hand side in eq. (7) means the concentration of the immunocomplex of the antibody-BPA immobilized on the sensor chip. However, it seemed reasonable to ignore the term because the amount of BPA immobilized on the sensor chip is very small compared with that of the antibody complexed with BPA in the sample solution. Therefore, eq. (7) can be approximated to eq. (9).

$$[\mathrm{Ab}]^{\mathrm{T}} \sim [\mathrm{BPA\text{–}Ab}] + [\mathrm{Ab}] \tag{9}$$

From eqs. (6), (8) and (9), the concentration of the free BPA in the sample solution was calculated by assuming several K_2 values and a theoretical curve for the competitive assay was constructed by substituting the calculated value of the concentration of free BPA into

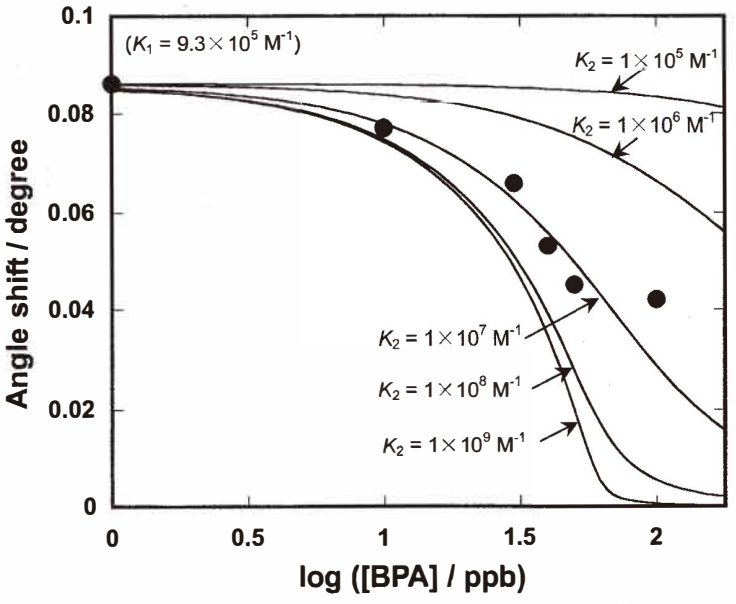


Fig. 8. Theoretical calibration curves in the competitive assay. Values of K_2 are indicated in the figure. Closed circles indicate experimental data. Theoretical calibration curves are calculated from eqs. (5), (6), (8), and (9) assuming $\Delta\theta_{max} = 0.586°$, $K_1 = 9.3 \times 10^5$ M^{-1}, $[\mathrm{Ab}]^{\mathrm{T}} = 2.67 \times 10^{-7}$ M (40 ppm), $K_2 = 1 \times 10^5$ M^{-1} – 1×10^9 M^{-1}.

eq. (5). The theoretical calibration curves, in which K_2 values are varied in the range of 1×10^5 M^{-1} to 1×10^{-9} M^{-1}, are shown in Fig. 8. As can be seen from Fig. 8, the experimental data are in good agreement with the theoretical calibration curve calculated using a value of $K_2 = 1 \times 10^7$ M^{-1}. The efficiency of the immunoreaction of the antibody to BPA immobilized on the sensor chip is reduced compared with that of an immunoreaction of the antibody with free BPA in the sample solution since the immobilized BPA was chemically modified by coupling with 2AET and its configuration may have been altered as a result at the immobilization. This represents a possible reason the affinity constant of anti-BPA antibody to BPA immobilized on the sensor chip ($K_1 = 9.3 \times 10^5$ M^{-1}) is 1/10-fold lower than that of the anti-BPA antibody to free BPA ($K_2 = 1 \times 10^7$ M^{-1}).

4. Conclusion

An SPR-based immunosensor equipped with a sensor chip modified with a 2AET layer/ BPA layer membrane was used in a competitive assay for BPA. In this case, we adopted the competitive method where a BPA immobilized sensor chip and a BPA sample solution containing the anti-BPA antibody at 40 ppm were used. Depending on the concentration of BPA in the solution containing the anti-BPA antibody, the SPR angle shift decreased due to an inhibition in the immunoreaction of the antibody with BPA immobilized on the sensor chip by the free BPA in the solution. A good correlation was obtained between SPR angle shifts and BPA concentration. The affinity constant of the anti-BPA antibody to the BPA immobilized on the sensor chip and that of the anti-BPA antibody to the free BPA were calculated to be 9.3×10^5 M^{-1} and 1×10^7 M^{-1}, respectively, assuming a Langmuir-type adsorption isotherm on the sensor chip and that the immunoreaction in the solution was at equilibrium.

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