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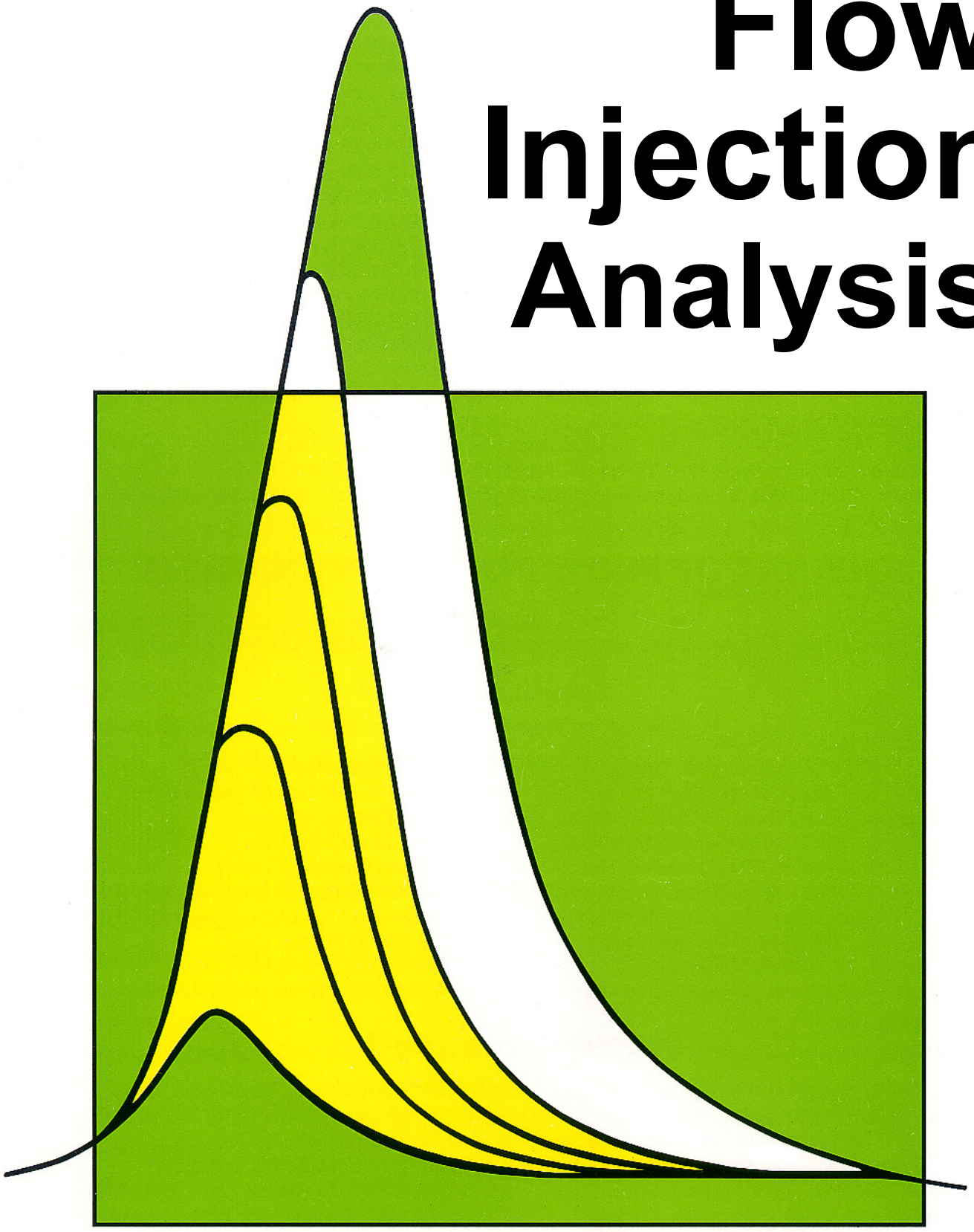

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<Minireview>

# Sequential Injection Flow Immunoassay Based on Surface Plasmon Resonance Sensors

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**Abstract**

Flow immunoassay methods for the determination of several environmental pollutants, such as 2,4-dichlorophenol, bisphenol A, trinitrophenol, pesticides and allergy related compounds, such as histamine and immunoglobulin E (IgE) based on a surface plasmon resonance (SPR) sensor are reviewed from the view point of methodology of immunoassay. In this review, two types of immobilization methods for an antibody or an antigen conjugate for the target molecule on the sensor chip are proposed. One is the immobilization method for the antibody of the target molecule, where the antibody is immobilized on the sensor chip through a gold binding polypeptide and Protein G by taking into account of high orientation of the antibody for an immunoreaction on the sensor chip. The other method is the immobilization of the antigen conjugate in two way, where an antigen conjugate with protein such as bovine serum albumin is immobilized by physical adsorption and an antigen conjugate is immobilized by amino-coupling with an alkane thiol compound, which is preliminary immobilized on the sensor chip as a self-assembled monolayer. In order to enhance the sensitivity of the SPR sensor by increasing the change in the mass on the sensor chip after the immunoreaction, a competitive immunoreactions is adopted for the both immobilization methods, where the antibody or the antigen conjugate is immobilized on the sensor chip. A sandwich immunoassay method for the allergy related compound, IgE, was demonstrated as a sensitive method for enhancement of the SPR sensor signal. In the present flow immunoassay, a sequential injection technique is utilized in order to automate the immunoassay protocol, which consists of many procedures.

**Keywords**: Flow immunoassay; sequential injection analysis; surface plasomon resonance sensor; immobilization technique.

## 1. Introduction

The hazardous effects of several environmental pollutants by so-called endocrine disrupting chemical on humans and wildlife species have created a serious environmental problem in recent years. In particular, dioxins have a high toxicity and their production and subsequent distribution from incinerators to the environment cause a serious health-related problem for society. A rapid and sensitive analytical method for monitoring such pollutants would be highly desirable. Therefore sensitive and rapid analytical methods for such environmental pollutants are urgently needed for screening or monitoring of environmental pollution. One of the reliable analytical methods is that based on gas chromatography or high performance liquid chromatography, coupled with mass spectroscopy, because these methods permit the identification of homologues and isomers. However, these methods typically involve time-consuming pretreatments of samples from the complicated matrices and expensive instrumentation. A biological method based on an enzyme-linked immunosorbent assay (ELISA) has been widely used for the determination of environmental pollutants as well as for the assay of diagnosis of human health. This is because this method has some advantages such as high selectivity and sensitivity, and low cost. However, in general, such types of ELISA method involve many laborious and time-consuming procedures such as the washing, addition of sample and reagents.

An analytical method based on a surface plasmon resonance (SPR) sensor has been recognized as a promising analytical method for environmental pollutants as an alternative of the ELISA method based on the interaction between an antibody and an antigen, because the SPR sensor was originally developed for studying the interactions of biomolecules sucn as proteins and DNAs. This is due to the fact that the SPR sensor is highly sensitive for the chemical events occurred at the surface of the sensor chip at the vicinity just in several hundred nanometers. A sequential injection analysis (SIA) is a suitable analytical technique for immunoassay, because many procedures such as the washing and additions of immune-reagents can be automated by using a computer-controlled syringe pump and a switching valve. In this review we wish to review flow immunoassay methods based on a surface plasmon resonance (SPR) sensor combined with the SIA technique for the determination of several environmental pollutants, such as 2,4-dichlorophenol, bisphenol A, trinitrophenol and pesticides as well as allergy related compounds, such as histamine and immunoglobulin E, from the view point of preparation of a sensor chip.

## 2. Principle of SPR sensor [1]

A conventional optical system of an SPR sensor based on a Kretchmann configuration is shown in Fig. 1. When an incident light with an angle width (e.g., 10 degree) under the total reflection condition through a prism to a sensor chip, on which a gold thin film (50-100 nm) is coated on a cover glass with an assistance of a chromium layer (e.g. 3 nm), an evanescent wave of a p-polarized incident light penetrated into the gold thin film, which contact to a dielectric medium, is coupled with a wave of free electron of the gold thin film, so-called surface plasmon wave. When the wave vectors of the both evanescent wave ($k_{ev}$) and surface plasmon wave ($k_{sp}$) are coincident, namely a resonance condition expressed by Eq. (2-1) is satisfied, a part of energy of the evanescent wave is used for excitation of the surface plasmon wave and the light intensity of the reflection light is decreased at a certain angle.

$$k_{ev} = k_p \sin\theta = (\omega/C)\,(\varepsilon\, n^2/(\varepsilon + n^2))^{1/2} = k_{sp} \qquad (2\text{-}1)$$

where $k_{ev}$ and $k_{sp}$ are the wave vectors of the evanescent wave and the surface plasmon wave, respectively. $\theta$ is the angle of the incident light and $\varepsilon$ and $n$ are the dielectric constant of a gold thin film and the refractive index of the medium contacted with the gold thin film, respectively. $\omega$ and C are the angular frequency and the speed of light, respectively.

The angle, at which the minimum in reflectivity is observed, is called as a resonance angle or an SPR angle ($\theta_1$ in Fig. 2). When the refractive index of the medium contacted to the gold thin film increases, the resonance angle is shifted towards the higher angle ($\theta_2$ in Fig. 2). Therefore, when the gold thin film is modified with a bio-recognition compound such as an antibody, molecular interaction with a target molecule to the bio-recognition compound such as an antigen induces the angle shift of the resonance angle ($\Delta\theta$ in Fig. 2).

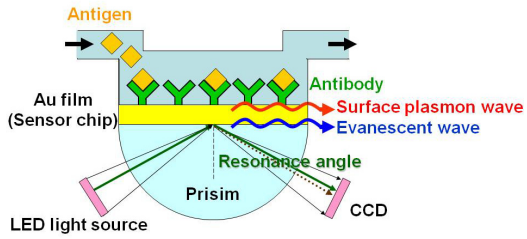


Fig. 1 Principle of SPR sensor.

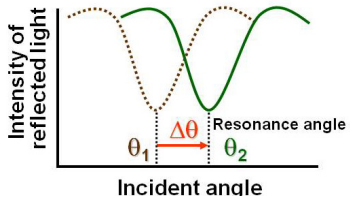


Fig. 2 SPR signal.

Since the sensor signal of the SPR sensor is governed by the change in the refractive index of a medium on the surface of the sensor chip when a chemical event such as binding of a target antigen to an antibody on the sensor chip occurs, the sensitivity of the SPR sensor can be enhanced by several techniques where the mass change on the sensor chip is induced for the chemical events. One of the effective methods for enhancement of mass change is utilization of a competitive immunoassay, where an antigen conjugate with a larger molecular weight protein and a target antigen are competitively bound to an antibody immobilized on the sensor chip. The binding of the antigen conjugate with a large molecular weight generates a larger angle shift of the SPR sensor. This situation is schematically shown in Fig. 3.

In this review, two types of the sensor chip immobilized with an antibody and with an antigen conjugate for the target molecule are described. Namely, one is the immobilization method for the antibody of the target molecule, where the antibody is immobilized on the sensor chip through a gold binding polypeptide and Protein G by taking into account of high orientation of the antibody for an immunoreaction on the sensor chip. The other method is the immobilization of the antigen conjugate in two ways, where an antigen conjugate with protein such as bovine serum albumin (BSA) is immobilized by physical adsorption and an antigen conjugate is immobilized by amino-coupling with an alkane-thiol compound, which is preliminary immobilized on the sensor chip via a gold-thiol interaction. In order to enhance the sensitivity of the SPR sensor by increasing the change in the mass on the sensor chip after the immunoreaction, a competitive immunoreactions is adopted for the both immobilization methods, where the antibody or the antigen conjugate is immobilized on the sensor chip. The present immunoassay was performed by using a sequential injection system combined with an SPR sensor as a detector shown in Fig. 4.

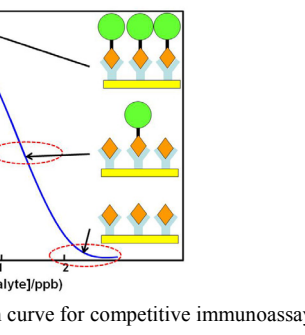


Fig. 3 Typical calibration curve for competitive immunoassay based on SPR sensor.

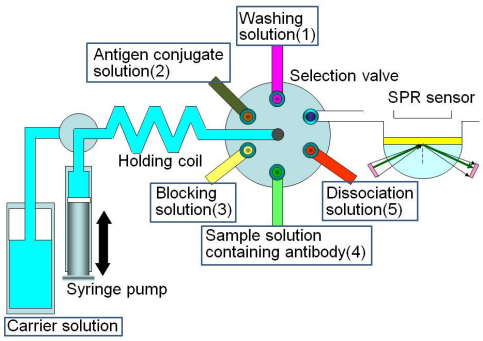


Fig. 4 Sequential injection system with surface plasmon resonance sensor for immunoassay.

## 3. Antibody immobilized sensor chip [2]

An SPR immunosensor method based on a competitive immunoassay using a sensor chip immobilized with an antibody for a target antigen is shown in this section as an example of the determination of 2,4-dichlorophenol. In the present system, an anti-2,4-dichlorohpneol antibody is immobilized on the sensor chip by using a gold binding polypeptide (GBP) with an assistance by protein G. The surface of the sensor chip is schematically shown in Fig. 5.

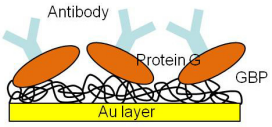


Fig. 5 Schematic diagram of an SPR sensor chip immobilized with antibody through protein G and gold binding polypeptide (GBP).

The target molecule of 2,4-dichlophenol and its conjugate of bovine serum albumin (BSA) are competitively bound to the anti-2,4-dichlorophenol antibody immobilized on the sensor chip. The competitive immunoreactions of the 2,4-dichlorophenol BSA conjugate, (expressed by $Ag_1$) and 2,4-dichlorophenol (express by $Ag_2$) with the antibody (expressed by Ab) immobilized on the sensor surface are assumed to be in the binding equilibrium and expressed by the following equations. The model is shown in Fig. 6.

$$\underline{Ab} + Ag_1 \rightarrow \underline{Ab\text{-}Ag_1} \qquad (3\text{-}1)$$

$$\underline{Ab} + Ag_2 \rightarrow \underline{Ab\text{-}Ag_2} \qquad (3\text{-}2)$$

where under bar indicates that the chemical species is on the sensor chip.

The affinity constants of the anti-2,4-dichlorophenol antibody with the 2,4-dichlorophenol BSA conjugate and 2,4-dichlorophenol are $K_1$ and $K_2$, respectively and can be expressed by the following equations.

$$K_1 = [\underline{\text{Ab-Ag}_1}]/[\underline{\text{Ab}}][\text{Ag}_1] \quad (3\text{-}3)$$

$$K_2 = [\underline{\text{Ab-Ag}_2}]/[\underline{\text{Ab}}][\text{Ag}_2] \quad (3\text{-}4)$$

where $[Ag_1]$ and $[Ag_2]$ stand for the molar concentration (mol $L^{-1}$) of the 2,4-dichlorophenol-BSA conjugate and 2,4-dichlorophenol, respectively. $[\underline{\text{Ab-Ag}_1}]$ and $[\underline{\text{Ab-Ag}_2}]$ stand for the surface concentration of the antibody complexes with 2,4-dichlorophenol-BSA conjugate and with 2,4-dichlorophenol on the sensor surface, respectively. $[\underline{\text{Ab}}]$ is the free anti-2,4-dichlorophenol antibody on the sensor surface. The under bar on $\underline{\text{Ab-Ag}_1}$, $\underline{\text{Ab-Ag}_2}$ and $\underline{\text{Ab}}$ means the surface concentration expressed in nmol $mm^{-2}$.

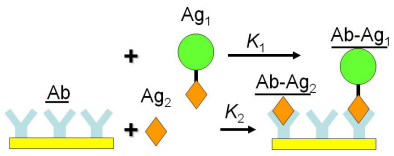


Fig. 6 Model of competitive immunoassay using a sensor chip immobilized with an antibody.

Firstly in order to evaluate $K_1$, a step, where a solution of the 2,4-dichlorophenol BSA conjugate is introduced on the sensor chip immobilized with the anti-2,4-dicholrophenol antibody, is considered In this case, the following equation holds for a mass balance of the antibody on the sensor surface.

$$[\underline{\text{Ab}}]^T = [\underline{\text{Ab-Ag}_1}] + [\underline{\text{Ab}}] \quad (3\text{-}5)$$

where $[\underline{\text{Ab}}]^T$ stands for the total concentration of the antibody on the sensor surface.
From Eqs. (3-3) and (3-5), a Langmuir-type adsorption equation is derived as follows, assuming that the concentration of $Ag_1$ is much larger than $[\underline{\text{Ab}}]^T$. Namely, the concentration of $Ag_1$ in the sample is assumed to be constant even after binding with the antibody on the sensor surface.

$$[\underline{\text{Ab-Ag}_1}]/[\underline{\text{Ab}}]^T = K_1[\text{Ag}_1]/(1 + K_1[\text{Ag}_1]) \quad (3\text{-}6)$$

If the angle shift of the SPR sensor, $\Delta\theta_1$, is proportional to the surface concentration of the conjugate adsorbed on the sensor surface ($[\underline{\text{Ab-Ag}_1}]$), the relative surface concentration of $[\underline{\text{Ab-Ag}_1}]$ to the total surface concentration of the antibody is expressed as follows.

$$[\underline{\text{Ab-Ag}_1}]/[\underline{\text{Ab}}]^T = \Delta\theta_1 / \Delta\theta_{1,\max} \quad (3\text{-}7)$$

where $\Delta\theta_{1,\max}$ means the angle shift of the SPR sensor when the antibody on the sensor surface is completely bound by the conjugate.
From Eqs. (3-6) and (3-7), the following equation is derived.

$$[\text{Ag}_1]/\Delta\theta_1 = [\text{Ag}_1]/\Delta\theta_{1,\max} + 1/\Delta\theta_{1,\max} K_1 \quad (3\text{-}8)$$

According to Eq. (3-8), $\Delta\theta_{1,\max}$ and $K_1$ are calculated from a slope and an intercept of the $[Ag_1]$ vs. $[Ag_1]/\Delta\theta_1$ plot, respectively.

Next, a step, where a 2,4-dichlorophenol solution containing the 2,4-dichlorophenol BSA conjugate at a constant concentration is introduced on the sensor chip immobilized with the anti-2,4-dicholrophenol antibody, is considered 2,4-dichlorophenol and 2,4-dichlorophenol BSA conjugate are competitively bound to the anti-2,4-dichlorophenol antibody immobilized on the sensor chip. As the concentration of 2,4-dichlorophenol increased, amount of 2,4-dichlorophenol BSA conjugate bound to the sensor chip decrease and thus the resonance angle shift decreases. In this case, the following equation holds for a mass balance of the antibody on the sensor surface.

$$[\underline{\text{Ab}}]^T = [\underline{\text{Ab-Ag}_1}] + [\underline{\text{Ab-Ag}_2}] + [\underline{\text{Ab}}] \quad (3\text{-}9)$$

From Eqs. (3-3), (3-4) and (3-9), the following equations, which are also identical to the Langmuir-type adsorption equation in the case of Eq. (3-6), are obtained.

$$[\underline{\text{Ab-Ag}_1}]/[\underline{\text{Ab}}]^T = K_1[\text{Ag}_1]/(1 + K_1[\text{Ag}_1] + K_2[\text{Ag}_2]) \quad (3\text{-}10)$$

$$[\underline{\text{Ab-Ag}_2}]/[\underline{\text{Ab}}]^T = K_2[\text{Ag}_2]/(1 + K_1[\text{Ag}_1] + K_2[\text{Ag}_2]) \quad (3\text{-}11)$$

Since the concentrations of the 2,4-dichlorophenol BSA conjugate and 2,4-dichlorophenol are assumed to be constant after binding equilibrium, the initial values can be used for $[Ag_1]$ and $[Ag_2]$ in Eqs. (3-10) and (3-11). Therefore, the values of $[\underline{\text{Ab-Ag}_1}]$ and $[\underline{\text{Ab-Ag}_1}]$ can be calculated from Eqs. (3-9), (3-10) and (3-11) as a function of $[Ag_2]$ by assuming appropriate values of $[\underline{\text{Ab}}]^T$ and $K_2$. The angle shift in this case can be estimated by the following equation, taking into account of the molecular weight of the conjugate and the target molecule, according to the BIACOR's specification of the relationship between the resonance angle shift and the amount of protein adsorbed on the sensor chip.

$$\Delta\theta = \alpha_1[\underline{\text{Ab-Ag}_1}] + \alpha_2[\underline{\text{Ab-Ag}_1}] \quad (3\text{-}12)$$

where $\alpha_1$ and $\alpha_2$ are the coefficient that are proportional to the molecular weight of $Ag_1$ (2,4-dichlorophenol BSA conjugate) and $Ag_2$ (2,4-dichlorophenol), respectively. These values have a dimension of [deg $mm^2$ $nmol^{-1}$]. Therefore, the affinity constant of $K_2$ can be evaluated by curve fitting the observed SPR angle shift with the calculated lines from Eq. (3-12).

In order to evaluate the affinity constant of an anti-2,4-dichlorophenol antibody complex with the 2,4-dichlorophenol BSA conjugate on the sensor chip ($K_1$ in Eq. (3-3)), several 2,4-dichlorophenol BSA conjugate solutions were introduced on the sensor chip, where the anti-2,4-dichlorophenol antibody was immobilized with assistance of GBP and protein G, the angle shift of the SPR sensor was measured. The results are shown in Fig. 7. According to Eq. (3-8), $[Ag_1]/\Delta\theta_1$ was plotted against $[Ag_1]$ by using the observed angle shift shown in Fig. 7, $\Delta\theta_{1,\max} = 0.62^o$ and $K_1$=1.5x$10^6$ $M^{-1}$ were obtained.

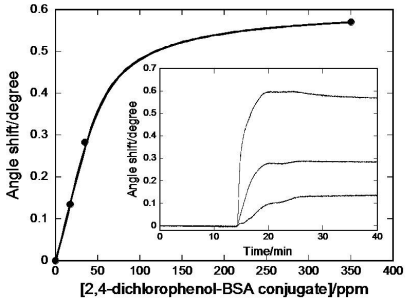


Fig. 7 Angle shift by adsorption of 2,4-dichlorophenol-BSA conjugate on the sensor chip.

In order to evaluate the affinity constant of an anti-2,4-dichlorophenol antibody complex with the 2,4-dichlorophenol on the sensor chip ($K_2$ in Eq. (3-4)), several 2,4-dichlorophenol solutions (0-250 ppb) containing the 2,4-dichlorophenol BSA conjugate at 350 ppm were introduced on the sensor chip, and the angle shift of the SPR sensor was measured. The observed angle shifts are plotted against the

concentration of 2,4-dichlorophenol, as shown in Fig. 8 (filled circles), together with the calculated lines of Eq. (3-12) by using $K_1$ (=1.5x10$^6$ M$^{-1}$) obtained and assuming $K_2$ and $\alpha_1$=6800 and $\alpha_2$=16, which are estimated from the molecular weight of 2,4-dichlorophenol BAS conjugate and 2,4-dichlorophenol. As can be seen from Fig. 8, the observed angle shift gradually approaches to the constant angle shift of 0.49$^o$ with increasing the concentration of 2,4-dichlorophenol, which may be due to the present antibody may bind with BSA conjugate somewhat. Therefore the additional term of 0.49$^o$ is added to Eq. (3-12) to simulate the observed angle shift and $K_2$=5x10$^7$ M$^{-1}$ was estimated from Fig. 8.

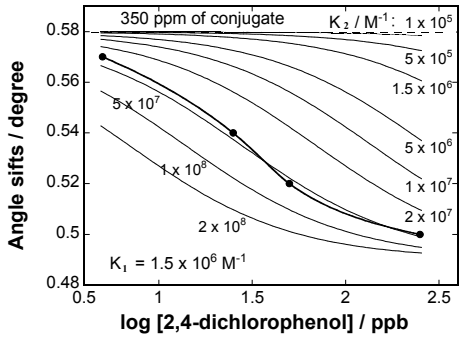


Fig. 8 Simulation of calibration curve. Angle shift is calculated against the concentration of 2,4-dichlorophenol as a parameter of the affinity constant of antibody-2,4-dichlorophenol, $K_2$ affinity constant of antibody-2,4-dichlorophenol-BSA conjugate complex, $K_1$ is assumed to be 1.5 x 10$^6$ M$^{-1}$. Filled circles are observed data.

## 4. Antigen immobilized sensor chip [3-7]

### 4.1 Utilization of self-assembled monolayer prepared by thiol compounds

An SPR immunosensor method based on a competitive immunoassay using a sensor chip immobilized with a conjugate of a target antigen is shown in this section, as an example of the determination of histamine [3]. In the present system, histamine is immobilized on the sensor chip by using a self-assembled monolayer of 11-mercaptoundecanoic acid as an anchor membrane, followed by an amino-coupling reaction with histamine. An anti-histamine antibody solution containing the target molecule of histamine is introduced on the sensor chip immobilized with the histamine conjugate. The anti-histamine antibody is competitively bound to histamine in the same sample solution and to the histamine conjugate immobilized on the sensor chip. The sensor chip is schematically shown in Fig. 9.

The competitive immunoreactions of the anti-histamine antibody, (expressed by Ab) with histamine (express by $Ag_2$) and the histamine conjugate (expressed by $Ag_1$) immobilized on the sensor surface are expressed by the following equation.

$$Ab + \underline{Ag_1} \rightarrow \underline{Ab\text{-}Ag_1} \qquad (4\text{-}1)$$

$$Ab + Ag_2 \rightarrow Ab\text{-}Ag_2 \qquad (4\text{-}2)$$

where $\underline{Ag_1}$ and $Ag_2$ denote the histamine conjugate

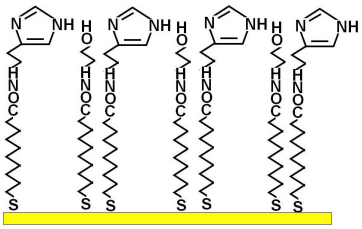

Fig. 9 Histamine conjugate immobilized sensor chip prepared by using a self-assembled monolayer.

immobilized on the sensor chip and histamine in the solution, respectively. Ab denotes the anti-histamine antibody in the solution. $\underline{Ab\text{-}Ag_1}$ and $Ab\text{-}Ag_2$ are immunocomplex of the anti-histamine antibody with the histamine conjugate on the sensor chip and that with histamine in the solution, respectively.

The affinity constants corresponding to immunoreactions Eqs (4-1) and (4-2) can be expressed by Eqs. (4-3) and (4-4), respectively.

$$K_1 = [\underline{Ab\text{-}Ag_1}]/[\underline{Ag_1}]\,[Ab] \qquad (4\text{-}3)$$

$$K_2 = [Ab\text{-}Ag_2]/[Ag_2]\,[Ab] \qquad (4\text{-}4)$$

where a bracket without under line denotes the concentration of chemical species in the solution expressed in mol dm$^{-3}$ and that with under line denotes the surface concentration of a chemical species immobilized on the sensor chip, expressed such as in nmol mm$^{-2}$.

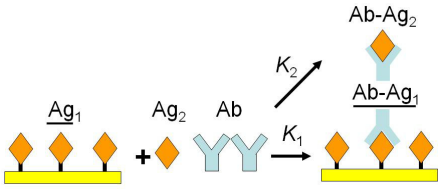


Fig. 10 Model of competitive immunoassay using the sensor chip immobilized with antigen conjugate.

Firstly a step, where a solution of the anti-histamine antibody is introduced on the sensor chip immobilized with the histamine conjugate, is considered. In this case, the following equation holds for a mass balance of the antibody on the sensor surface.

$$[\underline{Ag_1}]^T = [\underline{Ab\text{-}Ag_1}] + [Ab] \qquad (4\text{-}5)$$

where $[\underline{Ag_1}]^T$ is total surface concentration of the histamine conjugate on the sensor chip.

If a Langmuire-type adsorption is assumed to be hold for binding the anti-histamine antibody to the histamine conjugate on the sensor chip, the following equation can be derived.

$$[\underline{Ab\text{-}Ag_1}]/[\underline{Ag}]^T = [Ab]/(1 + K_1\,[Ab]) \qquad (4\text{-}6)$$

In this case, since the change in the SPR sensor signal, $\Delta\theta_1$, is proportional to the surface concentration of the anti-histamine antibody bound to the sensor chip, $\Delta\theta_1$ can be rewritten by the following equation.

$$\Delta\theta_1 / \Delta\theta_{1,max} = [Ab]/(1 + K_1\,[Ab]) \qquad (4\text{-}7)$$

where $\Delta\theta_{1,max}$ means the maximum of the angle shift of the SPR sensor, where the histamine conjugate was completely bound with the anti-histamine antibody.

From Eq. (4-7), the following equation can be derived.

$$1/\Delta\theta_1 = 1/([Ab]\,\Delta\theta_{1,max}) + K_1 / \Delta\theta_{1,max} \qquad (4\text{-}8)$$

When $1/\Delta\theta_1$ is plotted against 1/[Ab], a linear straight line would be obtained and $\Delta\theta_{1,max}$ and $K_1$ can be calculated from the values of slope and intercept of the straight line.

While, an affinity constant of the anti-histamine antibody with histamine in the solution can be estimated by the same manner as is assumed to be a Langmuir-type isotherm adsorption for the competitive immunoreaction of the anti-histamine antibody to histamine in the solution and the histamine conjugate immobilized on the sensor chip.

In order to evaluate the affinity constant of the immunocomplex of the anti-histamine antibody with histamine immobilized on the sensor chip (Eq. (4-3)), anti-histamine antibody solutions at several concentrations from 10 to 60 ppm were introduced on the sensor chip and the SPR angle shift $\Delta\theta_1$, was measured. From the obtained angle shifts for each anti-histamine antibody solutions, according to Eq. (4-8), $1/\Delta\theta_1$ is plotted against 1/[anti-histamine antibody] as shown in Fig. 11. As can be seen from Fig. 11, a good linear relationship between two parameters was obtained, indicating that the Langmuir-type adsorption holds. $\Delta\theta_1$ and $K_1$ were calculated to be 0.069° and 7.2 x $10^5$ $M^{-1}$, respectively, from the intercept and slope of the straight line in Fig. 11.

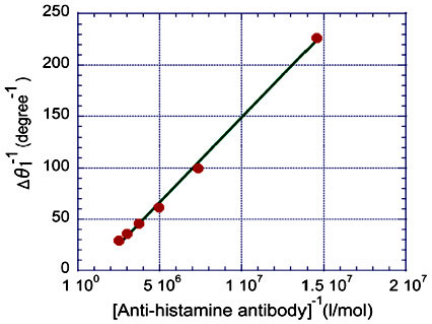


Fig. 11 Langmuir plot for calculation of the affinity constant of the immunocomplex of the anti-histamine antibody with histamine immobilized on the sensor chip

Next, histamine solutions at various concentrations containing the anti-histamine antibody at a constant concentration are introduced on the sensor chip. The competitive immunoreactions expressed by Eqs. (4-1) and (4-2) occur. As the concentration of histamine in the solution increases, the amount of the anti-histamine antibody bound to the histamine conjugate on the sensor chip decreases, thus the angle shift of the SPR sensor decreases.

From the mass balance in term of histamine in the incubation solution, the following equation holds.

$$[Ab]^T = [Ab] + [Ab\text{-}Ag_2] \qquad (4\text{-}9)$$

where $[Ab]^T$ is an initial concentration of the anti-histamine antibody in the incubation solution, and [Ab] and $[Ab\text{-}Ag_2]$ are a free anti-histamine antibody and a complex of the antibody with histamine, respectively, in the incubation solution.

When the incubation solution containing the anti-histamine antibody and histamine is introduced onto the sensor chip, the surface on which the histamine conjugate was immobilized, a free anti-histamine antibody is preferentially bound to the histamine conjugate immobilized on the sensor chip. The concentration of the free antibody is derived by using Eqs. (4-8) and (4-9) as follows.

$$[Ab] = [Ab]^T /(1 + K_2 [Ag_2]) \qquad (4\text{-}10)$$

By inserting Eq. (4-10) into Eq. (4-8) and rearranging, the following equation is obtained.

$$1/\Delta\theta_2 = 1/\Delta\theta_1 + K_2 [Ag_2] / (\Delta\theta_{1,max} K_1 [Ab]^T) \qquad (4\text{-}11)$$

where $\Delta\theta_2$ is the SPR angle shift observed when the incubation solution is introduced onto the sensor chip. When $1/\Delta\theta_2$ is plotted against $[Ag_2]$, a linear relationship would be expected between two parameters, and $K_2$ is obtained from the slope of the linear relationship.

To evaluate the affinity constant of the immunocomplex of the anti-histamine antibody with histamine in solution as well as to obtain a calibration curve for histamine, several incubation solution containing 50 ppm of the anti-histamine antibody and histamine at several concentrations from 3 to 10 ppb were introduced onto the sensor chip and then the angle shift, $\Delta\theta_2$, were measured. The sensorgram for the incubation solution obtained when the 10 mM HCl solution was used as a dissociation solution is shown in Fig. 12(a). The affinity constant $K_2$ can be evaluated from the sensorgram shown in Fig. 12(a), according to Eq. (4-11) by plotting $1/\Delta\theta_2$ against [Histamine]. As shown in Fig. 12(b), a good linear relationship between $1/\Delta\theta_2$ and [Histamine] was obtained, indicating that the Langmuir-type adsorption holds. $K_2$ was calculated to be 1.5 x $10^7$ $M^{-1}$, which was 20 times as large as $K_1$, indicating that the affinity of the anti-histamine antibody with histamine in the sample solution is larger than that with histamine immobilized on the sensor chip, which may be due to the effect of steric hindrance in the antibody binding to the somewhat hindered histamine immobilized on the sensor chip.

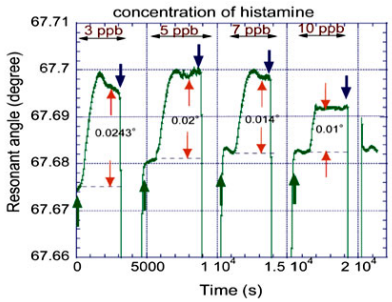


Fig. 12(a) Sensorgram for a histamine solution at various concentrations (3 -10 ppb) containing a 50 ppm anti-histamine antibody.

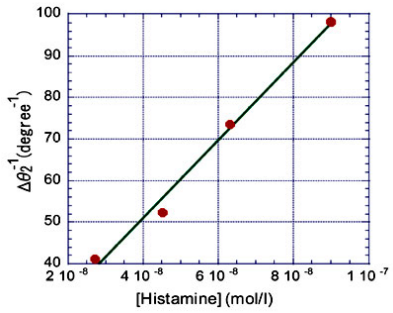


Fig. 12(b) Langmuir plot for calculation of the affinity constant of the immunocomplex of the anti-histamine antibody wit histamine in incubation solution. The data were taken from Fig. 12(a).

**4.2 Immobilization of antigen conjugate with protein by physical adsorption [7]**

In the present system for the determination of parathion methyl (PM), a parathion methyl conjugate with bovine serum albumin (PM-BSA) is immobilized on the sensor chip by physical adsorption.

An anti-PM antibody solution at a constant concentration containing the target molecule of PM at various concentrations is introduced onto the sensor chip immobilized with the PM conjugate. The anti-PM antibody is competitively bound to PM in the same sample solution and to the PM-BSA conjugate on the sensor chip. The adsorption constant defined as Eq. (4-12) was estimated by introducing the PM-BSA conjugate solution at various concentrations as well as the BSA solution as a reference onto a bear gold film sensor chip. The observed angle shifts are plotted against the concentration of PM-BSA conjugate solutions or BSA solutions. The results are shown in Fig. 13. In the same manner as for the evaluation of the affinity constant of the immunocomplex formed on the sensor chip, the adsorption constants were evaluated by plotting according to Eq. (4-13).

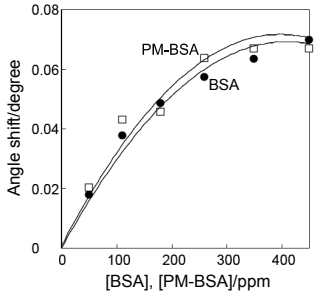


Fig. 13 Angle shift due to adsorption of BSA or PM-BSA conjugate.

$$K_{ad} = [\underline{\text{PM-BSA}}]/[\text{PM-BSA}] \quad (4\text{-}12)$$

$$[\text{PM-BSA}]/\Delta\theta = [\text{PM-BSA}]/\Delta\theta_{max} + 1/(\Delta\theta_{max} K_{ad}) \quad (4\text{-}13)$$

where $K_{ad}$ and $\Delta\theta_{max}$ are the adsorption constant and the angle shift at the situation that the sensor chip is completely bound by the PM-BSA or BSA.

As shown in Fig. 14, good linear relationships between [PM-BSA]/ $\Delta\theta$ and [PM-BSA] and [BSA]/ $\Delta\theta$ and [BSA] were obtained and $K_{ad}$ and $\Delta\theta_{max}$ were calculated to be 5.0 x $10^5$ $M^{-1}$ and 0.095° for the PM-BSA conjugate and 3.8 x $10^5$ $M^{-1}$ and 0.10° for BSA from the intercepts and the slopes of the linear relationships.

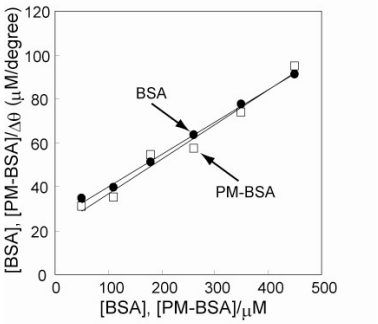


Fig. 14 Langmuir plot for estimation of adsorption constants of BSA and PM-BSA conjugate on sensor chip.

The sensorgram for the determination of PM is shown in Fig. 15. At the first step, aliquot of 100 μL of a 50 ppm PM-BSA conjugate solution is introduced on the sensor chip for immobilization of the PM-BSA conjugate. At the second step, the 10 mg $mL^{-1}$ BSA solution is introduced on the sensor chip twice for blocking the sensor surface to prevent nonspecific adsorption of antibody introduced in the subsequent procedures. Finally sample solutions of PM at various concentrations

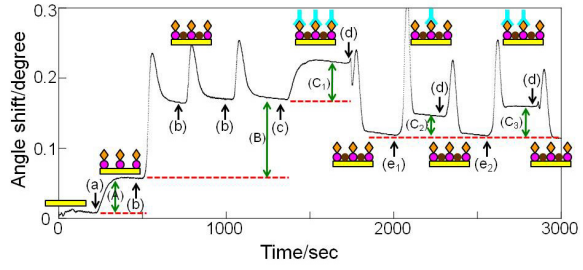


Fig. 15 Sensorgram for PM. Injected solution: (a) 50 ppm PM-BSA conjugate; (b) 10 mg $mL^{-1}$ BSA solution; (c) 60 ppm anti-PM antibody; (d) pH 2 HCl-glycine solution containing 1 ppm pepsin; (e1) 150 ppb PM solution containing 60 ppm anti-PM antibody; (e2) 100 ppb PM solution containing 60 ppm anti-PM antibody. Angle shift due to adsorption of PM-BSA conjugate on the sensor chip (A); blocking with BSA (B); binding anti-PM antibody to the PM-BSA conjugate on the sensor chip ($C_1$, $C_2$, $C_3$).

containing an anti-PM antibody at 60 ppm is introduced on the sensor chip and the change in the angle shift is measured. After measurement of each sample, the senor chip is regenerated to be the initial condition, where the PM-BSA conjugate is immobilized on the sensor chip, by introducing a dissociation solution of an HCl-glycine solution containing 1 ppm pepsin at pH 2. A calibration curve obtained by above procedures is shown in Fig. 16, indicating a typical sigmodial curve observed for a conventional immunoassay.

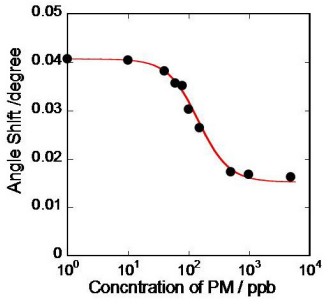


Fig. 16 Calibration curve for parathion methyl obtained by competitive immunoassay method.

## 5. Enhancement of sensitivity of SPR sensing by sandwich immunoassay [8]

As is described in section 2, since the sensitivity of the SPR sensor is governed by the change in the refractive index of a medium on the sensor chip, the competitive immunoassay was utilized in the SPR immunosensor method. For a larger molecular weight compounds such as several kinds of immunoglobulins, a sandwich immunoassay is an another approach for improvement of the sensitivity of the SPR immunosensor method for such kinds of target molecules. In the present section, the methodology of the sandwich immunoassay by the SPR sensor is demonstrated for the determination of IgE. We utilized the fact that IgE has two different domains, Cε2 and Cε3 and an anti-IgE(D) antibody and an anti-IgE(H) antibody selectively react with the domains of Cε2 and Cε3, respectively. Namely IgE can form a sandwich immunocomplex with the anti-IgE(D) antibody and the anti-IgE(H) antibody. Therefore, when the anti-IgE(D) antibody is immobilized on the sensor chip, the IgE immunocomplex with the anti-IgE(H) antibody is reacted with the anti-IgE(D) antibody on the sensor chip as a sandwich immunocomplex. The sensitivity of the sandwich immunoassay would be expected to be enhanced compared with the direct immunoassay, where IgE reacts with the anti-IgE(D) antibody immobilized on the sensor chip. The situations of the direct immunoassay and the sandwich immunoassay are shown in Fig. 17 (a) and (b), respectively.

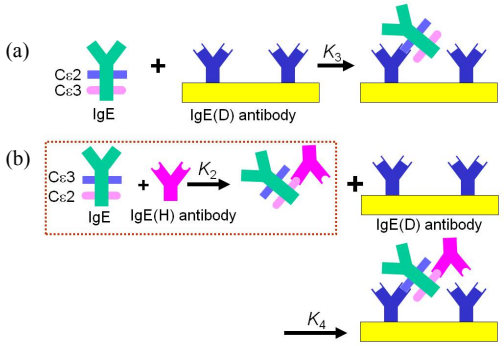


Fig. 17 Sandwich immunoassay for IgE by using two anti-IgE antibodies with different active recognition sites. (a) direct immunoassay; (b) sandwich immunoassay.

**5.1 Evaluation of the affinity constant for the anti-IgE(H) antibody complex with IgE on the sensor chip**

The affinity constant of the anti-IgE(H) antibody complex with IgE immobilized on the sensor chip can be evaluated by assuming a Langmuir-type adsorption as follows. When an anti-IgE(H) antibody solution is introduced on the sensor chip, the surface, on which IgE is immobilized by a biotin-streptavidin interaction, an immunoreaction via the Cε3 domain of the IgE that occurs on the sensor chip is expressed by Eq. (5-1).

$$\underline{\text{IgE}} + \text{anti-IgE(H)} \rightarrow \underline{\text{IgE-anti-IgE(H)}} \qquad (5\text{-}1)$$

where $\underline{\text{IgE}}$ denotes IgE immobilized on the sensor chip and anit-IgE(H) denotes the anti-IgE(H) antibody in the sample solution. $\underline{\text{IgE-anti-IgE(H)}}$ is an anti-IgE(H) antibody immunocomplex with IgE on the surface of the sensor chip.
The affinity constant, $K_1$, of the immunoreation Eq. (5-1) can be expressed by Eq. (5-2).

$$K_1 = \underline{[\text{IgE-anti-IgE(H)}]}/([\underline{\text{IgE}}] \times [\text{anti-IgE(H)}]) \qquad (5\text{-}2)$$

where a bracket without under line denotes the concentration of the chemical species in the solution expressed in mol $dm^{-3}$ and that with an under line denotes the surface concentration of the chemical species immobilized on the sensor chip expressed in nmol $mm^{-2}$. If a Langmuire-type adsorption is assumed to hold for the binding of the anti-IgE(H) antibody to IgE on the sensor chip, and the total surface concentration of the IgE antibody is assumed to be $[\underline{\text{IgE}}]^T$, the following equation can be derived.

$$[\underline{\text{anti-IgE(H)-IgE}}]/[\underline{\text{IgE}}]^T = [\text{anti-IgE(H)}]/(1 + K_1 \times [\text{anti-IgE(H)}]) \qquad (5\text{-}3)$$

In this case, since the change in the SPR sensor signal (angle shift), $\Delta\theta_1$, is proportional to the surface concentration of the anti-IgE(H) antibody bound to the sensor chip, $\Delta\theta_1$ can be rewritten in following form.

$$\Delta\theta_1 / \Delta\theta_{1,\max} = [\text{anti-IgE(H)}]/(1 + K_1 \times [\text{anti-IgE(H)}]) \qquad (5\text{-}4)$$

where $\Delta\theta_{1,\max}$ denotes the maximum of the angle shift of the SPR sensor, where all of IgE immobilized on the sensor chip is completely bound to the anti-IgE(H) antibody.
The following equation can be derived, from Eqs. (5-3) and (5-4).

$$1/\Delta\theta_1 = 1/\Delta\theta_{1,\max} + 1/([\text{anti-IgE(H)}] \times K_1 \times \Delta\theta_{1,\max}) \qquad (5\text{-}5)$$

When $1/\Delta\theta_1$ is plotted against 1/[anti-IgE(H)], a linear relationship would be obtained and $\Delta\theta_{1,\max}$ and $K_1$ can be evaluated from the values of slope and intercept of the straight line. Indeed, an anti-IgE(H) antibody solution at concentrations from 3 to 26 ppm was introduced on the sensor chip, on which IgE was immobilized, and the angle shift of the SPR sensor was measured. Since at a higher concentration range than 13 ppm, the angle shift was saturated at 0.035°, the angle shifts obtained at lower concentration range than 13 ppm were used for evaluation of $\Delta\theta_{1,\max}$ and $K_1$. In Fig. 18, the reciprocal of the angle shift is plotted against the reciprocal value of the concentration of the anti-IgE(H) antibody. A good linear relationship between the two parameters was obtained, indicating that the Langmuir-type adsorption holds. The affinity constant $K_1$ and $\Delta\theta_{1,\max}$ were estimated to be $5.8 \times 10^6$ $M^{-1}$ and 0.034°, respectively.

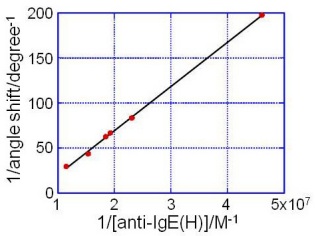


Fig. 18 Langmuir plot for evaluation of affinity constant of anti-IgE(H) antibody complex with IgE immobilized on sensor chip

**5.2 Evaluation of affinity constant of anti-IgE(H) antibody complex with IgE in solution**

An affinity constant of the anti-IgE(H) antibody complex with IgE in solution can be determined in the same manner as is assumed to be a Langmuir-type adsorption for the competitive immunoreaction of the anti-IgE(H) antibody to IgE in the solution and to IgE immobilized on the sensor chip. The immunoreaction of the anti-IgE(H) antibody with IgE via the Cε3 domain in IgE in the solution is expressed by Eq. (5-6) (*c.f.* $K_2$ in Fig. 17).

$$\text{IgE} + \text{anti-IgE(H)} \rightarrow \text{IgE-anti-IgE(H)} \qquad (5\text{-}6)$$

where IgE and IgE-anti-IgE(H) are IgE and an immunocomplex of the anti-IgE(H) antibody with IgE in the incubation solution. The affinity constant of the immunocomplex, $K_2$, of Eq. (5-6) is expressed by Eq. (5-7).

$$K_2 = [\text{IgE-anti-IgE(H)}] / ([\text{IgE}] \times [\text{anti-IgE(H)}]) \qquad (5\text{-}7)$$

From the mass balance in terms of the anti-IgE(H) antibody in the incubation solution, the following equation holds.

$$[\text{anti-IgE(H)}]^T = [\text{anti-IgE(H)}] + [\text{IgE-anti-IgE(H)}] \qquad (5\text{-}8)$$

where $[\text{anti-IgE(H)}]^T$ is the initial total concentration of the anti-IgE(H) antibody in the incubation solution, in this study, $[\text{anti-IgE(H)}]^T$ was 7.8 ppm, and [anti-IgE(H)] and [IgE-anti-IgE(H)] are the free anti-IgE(H) antibody and an immunocomplex of the anti-IgE(H) antibody with IgE, respectively, in the incubation solution.

When the incubation solution containing the free anti-IgE(H) antibody and the immunocomplex of the anti-IgE(H) with IgE, is introduced on the sensor chip, on which IgE is immobilized on the surface, only the free anti-IgE(H) antibody is bound to IgE immobilized on the sensor chip. The immunocomplex of the IgE-anti-IgE(H) flows out from the sensor chip because the active site for the Cε3 domain in IgE is already occupied with the anti-IgE(H) antibody. The concentration of the free anti-IgE(H) antibody in the incubation solution is derived using Eqs. (5-7) and (5-8) as follows.

$$[\text{anti-IgE(H)}] = [\text{anti-IgE(H)}]^T / (1 + K_2 \times [\text{IgE}]) \qquad (5\text{-}9)$$

By inserting Eq. (5-9) into Eq. (5-5) and rearranging the terms, the following equation is obtained.

$$1/\Delta\theta_2 = 1/\Delta\theta_1 + K_2 \times [\text{IgE}] / (\Delta\theta_{1,\max} \times K_1 \times [\text{anti-IgE(H)}]^T) \qquad (5\text{-}10)$$

where $\Delta\theta_2$ is the SPR angle shift observed when the incubation solution is introduced on the sensor chip. When $1/\Delta\theta_2$ is plotted against [IgE], a linear plot would be expected between the two parameters, and $K_2$ can be obtained from the slope of the line. Indeed, an incubation solution containing the anti-IgE(H) antibody at a concentration of 7.8 ppm and IgE in the concentration range from 0.65 to 9.3 ppm was introduced on the sensor chip, the angle shifts from 0.012° to 0.003° were

observed. A good linear relationship between the reciprocal angle shift and the concentration of IgE is obtained, as shown in Fig. 19. According to Eq. (5-10), the affinity constant of the anti-IgE(H) antibody complex with IgE in incubation solution, $K_2$=4.1x10$^7$ M$^{-1}$, was obtained from the slope of the linear relationship. This value is about 8 times larger than the affinity constant of the anti-IgE(H) antibody complex with IgE on the sensor chip. This is due to the fact that the reactivity of IgE immobilized on the sensor chip to the anti-IgE(H) antibody may be reduced after immobilization on the sensor chip.

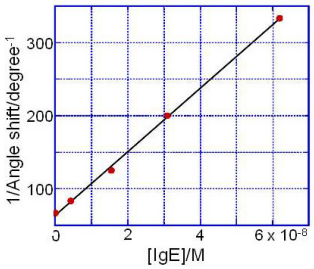


Fig. 19 Langmuir plot for evaluation of affinity constant of anti-IgE(H) antibody complex with IgE

### 5.3 Evaluation of affinity constants of the complex of IgE and an immunocomplex of anti-IgE(H)-IgE with an anti-IgE(D) antibody immobilized on sensor chip

The affinity constant for the IgE complex with the anti-IgE(D) antibody immobilized on the sensor chip (see in Fig. 17), $K_3$, can be evaluated by Eq. (5-11), in the same manner as Eq. (5-5) was derived.

$$1/\Delta\theta_3 = 1/\Delta\theta_{3,\max} + 1/([\text{IgE}] \times K_3 \times \Delta\theta_{3,\max}) \qquad (5\text{-}11)$$

where the value of $\Delta\theta_3$ is the SPR angle shift produced when an IgE solution is introduced onto the sensor chip, $\Delta\theta_{3,\max}$ is the maximum of the SPR angle shift, where all of the anti-IgE(D) antibody immobilized on the sensor chip is completely bound to IgE.

When the value for $1/\Delta\theta_3$ is plotted against 1/[IgE], a linear relationship between the two parameters can be obtained. $K_3$ and $\Delta\theta_{3,\max}$ can be evaluated from the value of the slope and the intercept of the straight line.

The affinity constant for the immunocomplex of IgE-anti-IgE(H) with the anti-IgE(D) antibody on the sensor chip, $K_4$ (see in Fig. 17), can be evaluated from Eq. (5-12), which is derived from the same manner as Eq. (5-11)

$$1/\Delta\theta_4 = 1/\Delta\theta_{4,\max} + 1/([\text{IgE-anti-IgE(H)}] \times K_4 \times \Delta\theta_{4,\max}) \qquad (5\text{-}12)$$

where $\Delta\theta_4$ is the SPR angle shift derived from an IgE solution containing an excess of the anti-IgE(H) antibody is introduced onto the sensor chip, and $\Delta\theta_{4,\max}$ is the maximum SPR angle shift, where all of the anti-IgE(D) on the sensor chip is completely bound to the IgE-anti-IgE(H) immunocomplex. In this case, [IgE] term in Eq. (5-11) is replaced with the term [IgE-anti-IgE(H)] in Eq. (5-12), because IgE in the incubation solution can be considered to have been converted to the form of the immunocomplex of IgE-anti-IgE(H) under conditions of an excess of the anti-IgE(H) antibody in the incubation solution.

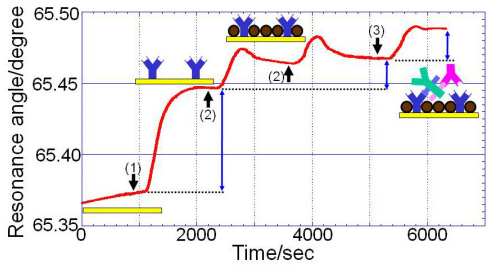


Fig. 20 Sensorgram for IgE by sandwich immunoassay. Injection of (1) 100 ppm anti-IgE(D) antibody, (2) 1000 ppm BSA for blocking, (3) incubation solution of 650 ppb IgE containing 50 ppm anti-IgE(H) antibody.

When the value of $1/\Delta\theta_4$ is plotted against the value of 1/[IgE-anti-IgE(H)], a linear relationship between two parameters is obtained. $K_4$ and $\Delta\theta_{4,\max}$ can be estimated from the value of the slope and intercept of the straight line. The concentration of the immunocomplex of IgE-anti-IgE(H) in the incubation solution can be regarded as the same concentration as that of IgE.

In order to estimate the affinity constant of the the IgE-anti-IgE(H) immunocomplex with the anti-IgE(D) antibody immobilized on the sensor chip, $K_4$, incubation solutions containing the anti-IgE (H) antibody at a constant concentration of 50 ppm and IgE at different concentrations from 13 ppb to 1 ppm were introduced on the sensor chip. A calibration curve for IgE by the sandwich immunoassay is also obtained by this procedure. A typical sensorgram obtained for the incubation solution containing 650 ppb IgE is shown in Fig. 20, together with the sensorgram for immobilization of the anti-IgE(D) on the sensor chip and for blocking with BSA.

The angle shifts obtained for the incubation solutions containing IgE at different concentrations are plotted against the concentration of IgE, as shown in Fig. 21, together with the angle shifts obtained by the direct immunoassay, where the IgE solutions at different concentrations from 2.5 ppm to 20 ppm were introduced on the sensor chip immobilized with the anti-IgE(D) antibody. As can be seen from Fig. 21, the sensitivity of the sandwich immunoassay calculated from the slope of the strait line is about 4.5 times higher than that of the direct immunoassay.

The affinity constants, $K_3$ and $K_4$, are estimated to be 2.3x10$^6$ M$^{-1}$ and 4.9x10$^7$ M$^{-1}$, respectively from the intercepts and slopes of the curves (1) and (2) in Fig. 22, using Eqs. (5-11) and (5-12) and the data in Fig. 21. The higher sensitivity of the sandwich immunoassay than that of the direct immunoassay may be due to the fact that the affinity constant for the IgE-anti IgE(H) complex with the anti-IgE(D) antibody on the sensor chip is about 20-times larger than that for the IgE complex with

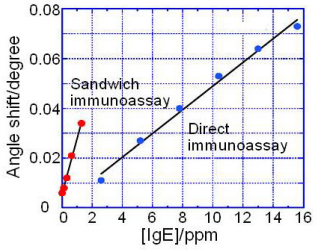


Fig. 21 Comparison of sensitivity of the sandwich immunoassay with that of the direct immunoassay for the determination of IgE.

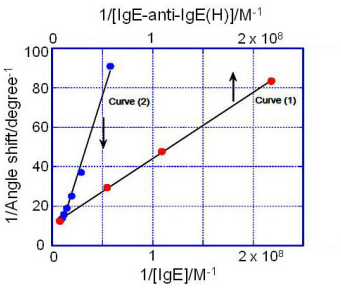


Fig. 22 Langmuir plot for estimation of affinity constants for IgE complex of IgE-anti-IgE(H) immunocomplexed with anti-IgE(D) immobilized on sensor chip. Curves (1) and (2) were obtained from data of the sandwich and direct immunoassay, respectively.

the anti-IgE(D) antibody on the sensor chip, as well as the molecular weight of the former complex is twice as that of the latte complex.

**6. Enhancement of sensitivity of SPR sensor by using an antigen-protein conjugate with large molecular weight [9]**

A competitive immunoassay based on one-pot immunoreactions can be applied to the SPR sensor based method by using an antigen-protein conjugate with large molecular weight (keyhole limpet hemocyanine, KLH) for enhancement of the sensitivity. This method is demonstrated by the determination of trinitrophenol (TNP).

The principle of this method is shown in Fig. 23. A sensor chip immobilized with the first antibody (Ab1 in Fig. 23), which recognizes the Fc moiety of the second antibody (Ab2 in Fig. 23) for a target molecule is used. An incubation solution containing the target antigen (Ag in Fig. 23), an antigen conjugate with a large molecular weight (AgC in Fig. 23) and the 2nd antibody is introduced on the sensor chip. In the incubation solution, the competitive immunoreactions occur between Ab2 and Ag or Ab2 and AgC. When this incubation is introduced onto the sensor chip, where the 1st antibody is immobilized on the sensor chip, the free 2nd antibody and the 2nd antibody complex with the antigen or the antigen conjugate are competitively bound to the 1st antibody. In this case, since an antigen conjugate with a large molecular weight protein is used, the SPR sensor signal is expected to mainly depend on the amount of the antigen conjugate reacted with the 1st antibody on the sensor chip. The SPR sensor signal can be simulated by assuming the chemical equilibria of the immunoreactions in the incubation solution as well as on the sensor chip.

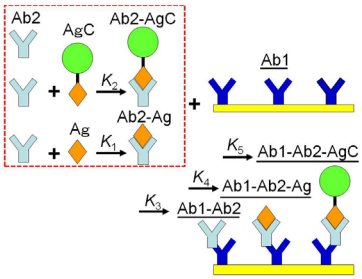


Fig. 23 Enhancement of sensitivity of SPR immunoassay by using a large molecular weight conjugate.

Fig. 24 shows a typical sensorgram obtained when the following three solutions are sequentially introduced to the bare sensor chip; 100 ppm of the 1st antibody solution, a blocking solution, and an inculation solution containing 25 ppm of the 2nd antibody, 250 ppb of TNP and 100 ppm of TNP-KLH conjugate.

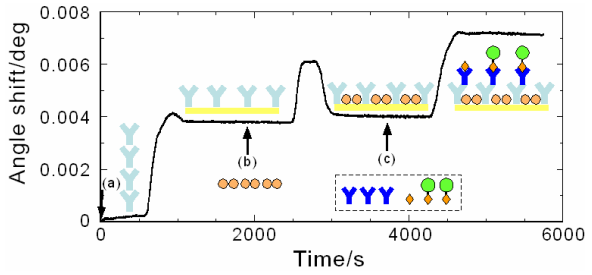


Fig. 24 Sensorgram for the determination of trinitrophenol. Injection of (a) the 1st antibody, (b) blocking reagent, (c) incubation solution containing 25 ppm 2nd antibody, 250 ppb TNP and 100 ppm TNP-KLH conjugate.

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 to the 1st antibody immobilized on the sensor chip. The chemical equilibrium in the incubation solution and the competitive binding are considered as follows.

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 affinity constants and the mass balance concerning with the 2nd antibody, TNP and the TNP-KLH conjugate.

$$Ab2 + Ag \rightarrow Ab2\text{-}Ag$$
$$K_1 = C_{Ab2\text{-}Ag}/(C_{Ab2} \cdot C_{Ag}) \quad (6\text{-}1)$$

$$Ab2 + AgC \rightarrow Ab2\text{-}AgC$$
$$K_2 = C_{Ab2\text{-}AgC}/(C_{Ab2} \cdot C_{AgC}) \quad (6\text{-}2)$$

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

$$C_{Ab2}{}^T = C_{Ab2} + C_{Ab2\text{-}Ag} + C_{Ab2\text{-}AgC} \quad (6\text{-}3)$$

$$C_{AgC}{}^T = C_{AgC} + C_{Ab2\text{-}AgC} \quad (6\text{-}4)$$

$$C_{Ag}{}^T = C_{Ag} + C_{Ab2\text{-}Ag} \quad (6\text{-}5)$$

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

From Eqs. (6-1)-(6-5), an equation concerning with the concentration of the free 2nd antibody, $C_{Ab2}$, is derived.

$$K_1K_2\,C_{Ab2}{}^3 + (C_{Ag}{}^T K_1K_2 + C_{AgC}{}^T K_1K_2 - K_1K_2C_{Ab2}{}^T + (K_1+K_2) - K_1K_2\,(C_{Ag}{}^T + C_{AgC}{}^T - C_{Ab2}{}^T) + K_1 + K_2)\,C_{Ab2}{}^2 + (K_1C_{Ag}{}^T + K_2C_{AgC}{}^T - (K_1 + K_2)C_{Ab2}{}^T + 1)\,C_{Ab2} - C_{Ab2}{}^T = 0 \quad (6\text{-}6)$$

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

The concentrations of the 2nd antibody-TNP complex, $C_{Ab2\text{-}Ag}$, and the 2nd antibody-TNP-KLH conjugate complex, $C_{Ab2\text{-}AgC}$, were calculated from $C_{Ab2}$ and Eqs. (6-4) and (6-5). In Fig. 25, the calculated $C_{Ab2}$, $C_{Ab2\text{-}Ag}$ and $C_{Ab2\text{-}AbC}$ are plotted as a function of the total concentration of TNP when the following values are assumed: $K_1$ = 1.0 x $10^7$ $M^{-1}$, $K_2$ = 0.8 x $10^7$ $M^{-1}$, and $C_{Ab2}{}^T$ = 2.0 x $10^{-6}$ M, $C_{AgC}{}^T$ = 4.0 x $10^{-6}$ M. As can be seen from Fig. 25, the concentration of the 2nd antibody-TNP-KLH conjugate complex gradually decreases with increasing concentrations of TNP, while the concentration

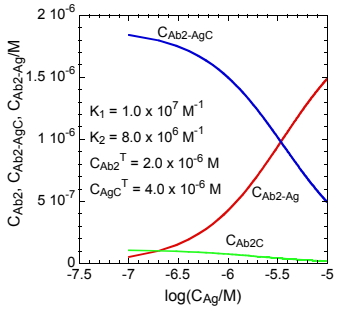


Fig. 25 Concentration of 2nd antibody, 2nd antibody complex with antigen and antigen conjugate in the incubation solution calculated from Eqs. (6-4) to (6-6), assuming $K_1$=1.0x$10^7$ $M^{-1}$, $K_2$=0.8x$10^7$ $M^{-1}$, $C^T_{Ab2}$=2.0x$10^{-6}$ M, $C^T_{AgC}$=4.0x$10^{-6}$ M.

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 onto 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.

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

$$K_3 = [\underline{\text{Ab1-Ab2}}]/[\underline{\text{Ab1}}] \cdot C_{\text{Ab2}} \quad (6\text{-}7)$$

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

$$K_4 = [\underline{\text{Ab1-Ab2-AgC}}]/[\underline{\text{Ab1}}] \cdot C_{\text{Ab2-AgC}} \quad (6\text{-}8)$$

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

$$K_5 = [\underline{\text{Ab1-Ab2-Ag}}]/[\underline{\text{Ab1}}] \cdot C_{\text{Ab2-Ag}} \quad (6\text{-}9)$$

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.

$$[\underline{\text{Ab1}}]^{\text{T}} = [\underline{\text{Ab1-Ab2}}] + [\underline{\text{Ab1-Ab2-AgC}}] + [\underline{\text{Ab1-Ab2-Ag}}] \quad (6\text{-}10)$$

where $[\underline{\text{Ab1}}]^{\text{T}}$ 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 antibody binds 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.

$$[\underline{\text{Ab1-Ab2}}]/[\underline{\text{Ab1}}]^{\text{T}} = K_3 C_{\text{Ab2}}/(1 + K_3 C_{\text{Ab2}} + K_4 C_{\text{Ab2-AbC}} + K_5 C_{\text{Ab2-Ag}}) \quad (6\text{-}11)$$

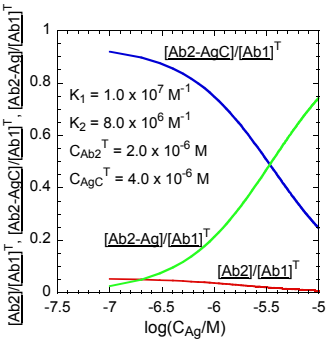


Fig. 26 Surface concentration of 2nd antibody, 2nd antibody complex with antigen and 2nd antibody complex with conjugate on the 1st antibody layers as function of the concentration of antigen calculated from Eqs. (6-11) to (6-13), assuming $K_3 = K_4 = K_5 = 6.8 \times 10^8$ M$^{-1}$.

$$[\underline{\text{Ab1-Ab2-AgC}}]/[\underline{\text{Ab1}}]^{\text{T}} = K_4 C_{\text{Ab2-AgC}}/(1 + K_3 C_{\text{Ab2}} + K_4 C_{\text{Ab2-AbC}} + K_5 C_{\text{Ab2-Ag}}) \quad (6\text{-}12)$$

$$[\underline{\text{Ab1-Ab2-Ag}}]/[\underline{\text{Ab1}}]^{\text{T}} = K_5 C_{\text{Ab2-Ag}}/(1 + K_3 C_{\text{Ab2}} + K_4 C_{\text{Ab2-AbC}} + K_5 C_{\text{Ab2-Ag}}) \quad (6\text{-}13)$$

where $C_{\text{Ab2}}$, $C_{\text{Ab2-Ab}}$ and $C_{\text{Ab2-AbC}}$ 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. (6-10)-(6-13) by using the concentrations of the 2nd species shown in Fig. 25 and assuming the binding constant, $K_3 = 6.8 \times 10^8$ M$^{-1}$. This value was obtained experimentally by the same procedures as described previously. Fig. 26 shows the surface concentrations of the 2nd antibody species normalized by the total surface concentration of the 1st antibody. As can be easily estimated from the concentration of the 2nd antibody species in the incubation 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 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 degree 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.

$$\Delta\theta = \alpha_{\text{Ab2}} [\underline{\text{Ab1-Ab2}}] + \alpha_{\text{Ab2-Ag}} [\underline{\text{Ab1-Ab2-Ag}}] + \alpha_{\text{Ab2-AgC}} [\underline{\text{Ab1-Ab2-AgC}}] \quad (6\text{-}14)$$

where $\alpha_{\text{Ab2}}$, $\alpha_{\text{Ab2-Ag}}$ and $\alpha_{\text{Ab2-AgC}}$ 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 [deg 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. (6-14), using the surface concentrations of the 2nd antibody species on the 1st antibody layer calculated in the manner shown in Fig. 27 and by assuming $[\text{Ab1}]^{\text{T}} = 2.7 \times 10^6$ nmol mm$^{-2}$, $\alpha_{\text{Ab2}} = 1.5 \times 10^4$, $\alpha_{\text{Ab2-Ag}} = 1.5 \times 10^4$ and $\alpha_{\text{Ab2-AgC}} = 7 \times 10^4 - 11 \times 10^4$ [deg mm$^{-2}$ nmol$^{-1}$]. The values for $\alpha$ are assumed, based on the fact that the molecular weight of the 2nd antibody and its complex with TNP is about $1.5 \times 10^5$ Da and that of KLH is about $7 - 11 \times 10^5$ Da. The calculated angle shifts as a function of the concentration of TNP and as a parameter of $\alpha_{\text{Ab2-AgC}}$, are shown

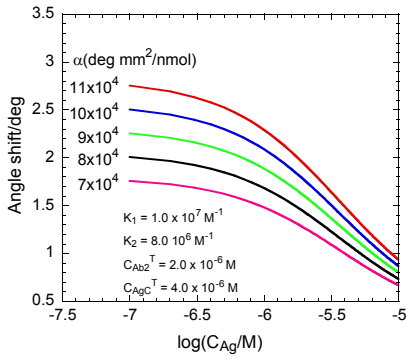


Fig. 27 Theoretical calibration curve for TNP based on Eq. (6-14), assuming $\alpha_{\text{Ab2}} = 1.5 \times 10^4$, $\alpha_{\text{Ab2-Ag}} = 1.5 \times 10^4$ and $\alpha_{\text{Ab2-AgC}} = 7 \times 10^4$-$11 \times 10^4$ [deg. mm$^2$ nmol$^{-1}$].

in Fig. 27. 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.

The effects of the concentrations of TNP-KLH and the 2nd antibody in the incubation solution on the sensitivity of the present method were examined and the relationships between the angle shift of the SPR sensor and the concentration of TNP in the incubation solution were obtained. The results are shown in Fig. 28. Although the angle shift observed is much smaller than that calculated from Eq. (6-14), shown in Fig. 27, by two orders of magnitude due to over estimation of the affinity constants of the 2nd antibody complexes with TNP and the TNP-KLH conjugate as well as affinity constants of the 1st antibody complexes formed on the sensor chip, the tendency of the angle shift on the concentration of TNP is similar to that shown in Fig. 28. 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 also 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 the detection of lower levels of TNP is improved by increasing the concentration 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 is preferentially bound to the 1st antibody layer on the sensor chip.

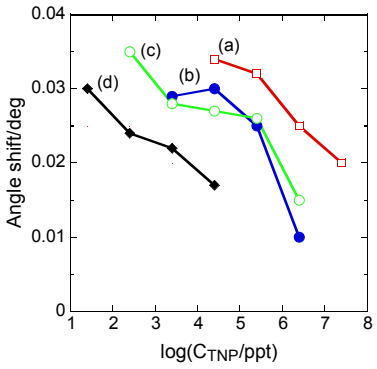


Fig. 28 Relationship between angle shift and concentration of TNP in the incubation solution. Concentration of TNP-KLH conjugate and 2nd antibody in the incubation solution: (a) 100/25 ppm, (b) 150/25 ppm, (c) 150/35 ppm and (d) 200/35 ppm, respectively. The 1st antibody was immobilized on the sensor chip by injecting 100 μL of a 100 ppm solution, volume of the incubation solution: 100 μL, carrier solution: phosphate buffer, pH 7.2, flow rate 20 μL $min^{-1}$.

## 5. Conclusion

A flow immunoassay based on an SPR sensor combined with the sequential injection technique was reviewed from the view point of the methodology of immunoassay. Taking into account of the sensitivity of the SPR sensor, a competitive immunoassay is expected to be preferable because a large angle shift can be induced by binding an antibody in an incubation solution containing a target antigen to an antigen conjugate immobilized on the sensor chip. When an antibody for a target molecule is immobilized on the sensor chip, a competitive immunoreaction of the target antigen and an antigen conjugate with a large molecular weight protein is also expected to be preferable to induce a large angle shift of the SPR sensor. These expectations were realized by demonstrating several experimental data for competitive immunoassays of environmental pollutants and allergy related compounds. A sandwich immunoassay was also a preferable method for enhancement of the sensitivity of the SPR sensor because the mass change on the sensor chip is expected to be more than double than the mass change obtained by the direct immunoassay, which experimental results were demonstrated by the immunoassay for one of allergy related compounds, IgE. In this review, the affinity constants of several immunoractions of an antigen with an antibody on the sensor chip as well as in an incubate solution were evaluated by assuming a Langmuir adsorption isotherm. The SPR sensor has an advantage for evaluation of affinity constant of the immunocomplex formed on the sensor chip.

The SPR sensor was also useful for monitoring a binding phenomenon of several saccharides with a polymer with boronic acid moiety immobilized on the sensor chip [10]. The development of a portable SPR sensor is desired for determination of environmental pollutants on site and we have developed a portable SPR sensor with a microchip flow cell [11-13] as well as an optical interface membrane alternative to matching oil [14]. A review of such kind of instrumentation and devices will be described elsewhere.

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