Flow Immunoassay for Nonionic Surfactants Based on Surface Plasmon Resonance Sensors

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A rapid, sensitive immunoassay based on a surface plasmon resonance sensor in a flow system for the determination of alkylphenol polyethoxylate (APEO) is described. The method is based on an indirect competitive reaction between an anti-APEO antibody in the sample solution and APEO immobilized on a sensor chip and APEO in the same sample solution. A sensor chip was prepared by immobilizing an APEO-horseradish peroxidase (APEO-HRP) conjugate on the thin gold film of the sensor chip. The adsorption constants for the APEO-HRP conjugate on the sensor chip and the surface density of the APEO-HRP adsorbed on the sensor chip at the saturated state were estimated to be $4.7 \times 10^5 \text{ M}^{-1}$ and $5.0 \times 10^{-14} \text{ mol/mm}^2$, respectively, using a Langmuir adsorption isotherm equation and results from the adsorption experiments. The affinity constants for the immunocomplexes of the anti-APEO antibody with the APEO conjugate on the sensor chip and for APEO in the sample solution were estimated to 2.0×10^6 and $5.1 \times 10^6 \text{ M}^{-1}$, respectively. A typical sigmoid calibration curve for APEO was obtained in the concentration range from 1 ppb to 1000 ppb. The detection limit, defined as the concentration of APEO, at which 85% of the sensor signal was observed, was *ca.* 10 ppb. The assay was applied to the determination of APEO in tap water in conjunction with a solid phase extraction pretreatment; APEO levels of approximately 50 ppt were successfully determined.

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Introduction

Because of their unique properties, nonionic surfactants as well as anionic surfactants have been extensively used in various fields as detergents, emulsifiers in foods, cosmetics and pharmaceuticals and additives in industrial concrete and carbonate boards.¹ Among the nonionic surfactants, alkylphenol polyethoxylates (APEOs) have been in widespread use, due to their superior surfactant activity. However, the contamination of environmental water by APEOs and their subsequent biological degradation to alkylphenol derivatives² makes them candidates for acting as endocrine-disrupting chemicals. Indeed the Japanese Water Supply Law has strictly lowered the levels of APEOs tolerated in tap water to 20 ppb.³ The official method for the determination of APEOs in tap water is a spectrophotometric solvent extraction after a solid phase extraction pretreatment. Since the sensitivity of the spectrophotometric solvent extraction is about the ppm level, APEOs in tap water must be enriched by 100 to 1000 times, if they are to be determined at the 1–10 ppb level. A further drawback is that toxic organic solvents are used in this method. One of the bioanalytical methods, an enzyme linked immunosorbent assay (ELISA), for the determination of

APEOs has been successfully developed as an environmentally friendly analytical method. This method is both sensitive and selective for APEOs and is capable for measurements of many samples by using a 96-microtiter plate, but it is laborious and time-consuming, involving procedures that include washing and the addition of additional reagents.

The surface plasmon resonance (SPR) sensor has recently been recognized as a sensitive analytical tool, especially for bioanalytical assays that are based on immunoreactions, *i.e.*, the so-called SPR immunosensors.^{4–8} The reason for its success is due to the fact that the SPR sensor is highly sensitive to chemical events, such as antibody-antigen binding, which occurs at the surface of the sensor chip in the vicinity of several hundred nanometers. The SPR sensor also has some advantages with respect to its ability to monitor chemical events in real time without the need for labeling with an enzyme or a dye, unlike the ELISA method. In this paper, we wish to report on a rapid and sensitive method for the determination of an APEO (a compound containing 10 oxyethylene units was used in this work) based on an SPR immunosensor. In the method, a sequential injection technique is utilized to automate the immunoassay, because the sequential injection technique can be employed to automate many procedures in the ELISA method, including washing and the addition of immune-reagents in the flow system *via* the use of a computer-controlled syringe pump and a switching valve. In this work, the effect of the solvent

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Fig. 1 Schematic flow diagram of the sequential injection system with the SPR sensor: (1) wash solution, (2) APEO-HRP conjugate solution, (3) blocking solution (BSA solution), (4) APEO sample solution containing an anti-APEO antibody, (5) dissociation solution (glycine/HCl solution at pH 2), (6) carrier solution (0.1 M phosphate buffer at pH 7.4).

matrix (methanol content) in the sample solution on the sensitivity of developed SPR immunosensor was also investigated. The reason for this is that the sensitivity of the method can be greatly increased, compared to the official method, when methanol is used for the preconcentration of APEO in tap water samples at the ppt level by the solid phase extraction procedure. In addition, the response of the SPR sensor to immunoreactions on the sensor surface was investigated on a theoretical basis using a Langmuir-type adsorption equation.

Experimental

Chemicals and preparation of solutions

An anti-APEO antibody and a horseradish peroxidase-labeled APEO (APEO-HRP conjugate) were purchased from Japan Envirochemicals, Ltd. (Japan). A standard APEO solution, bovine serum albumin (BSA), HRP and pepsin were obtained from Wako Pure Chemicals Co. (Japan). All other chemicals were of reagent grade and were used without further purification. A solid phase extraction column (Sep-Pack C18) was purchased from Waters (USA). Deionized water treated with a MilliQ system (Millipore Co.) was used. A 0.1 M phosphate buffer solution (pH 7.4) was prepared by dissolving 0.20 g KH_2PO_4 , 1.14 g Na_2HPO_4 , 8.0 g NaCl and 0.20 g KCl into 1 L of deionized water. Both APEO-HRP conjugate solutions and APEO solutions at various concentrations were prepared using a 0.1 M phosphate buffer. A dissociation solution for an anti-APEO antibody complex with the APEO-HRP conjugate on the sensor chip was prepared by dissolving 0.75 g glycine in 50 mL of water containing 1 ppm pepsin and the resulting solution was adjusted to pH 2 with a 0.1 M HCl solution. Tap water samples containing various concentrations of APEO (1 ppt to 1 ppb) were prepared by spiking 1 L of Fukuoka city tap water with the standard APEO solution.

Flow system with an SPR sensor

The flow system with the SPR sensor was constructed from a portable type SPR sensor (DSPR-200, Mebius Advanced Technology Co., Japan)⁹ and a flow controller (PRO-6000,

Fig. 2 Protocol for the determination of APEO by indirect competitive immunoassay on the SPR sensor chip: (1) immobilization of an APEO-HRP conjugate, (2) blocking the unmodified sensor chip with BSA, (3) introduction of an APEO solution containing an anti-APEO antibody, (4) regeneration of the sensor chip by means of a dissociation solution.

Yabegawa Electric Industry Ltd., Japan). A schematic diagram of the flow system is shown in Fig. 1. The protocols for the immunoassay in the flow system were programmed using the flow controller and the sensor signals, as the shifts in the resonance angle were also stored in the memory of the flow controller. A bare sensor chip, comprised of a gold thin film (thickness 45 nm) deposited on a cover glass ($16 \times 16 \times 0.15$ mm) with an assistance of a thin chromium film (thickness 5 nm), was purchased from Eliotech Co. (Japan). The sensor chip was attached to the flow cell using a silicon sheet (0.5 mm thick) with two grooves (3×8 mm), one of which was used for a sample solution and the other for a reference solution, which was the same as the carrier solution. The flow cell, attached to the sensor chip, was placed on a prism of the SPR sensor after applying a coating of matching oil on the prism. The SPR sensor was placed in a thermostated incubator at $25 \pm 0.1^\circ\text{C}$.

Protocol of immunoassay

The sensor chip was soaked in piranha solution (conc. $\text{H}_2\text{SO}_4/30\% \text{H}_2\text{O}_2 = 3/1$, v/v) for 10 min. After washing with deionized water and ethanol three times and drying with nitrogen gas, the sensor chip was then assembled into the flow cell on the SPR sensor. The protocol used in the present immunoassay is schematically shown in Fig. 2. A 100 μL aliquot of a 100 ppm APEO-HRP conjugate solution was aspirated by the syringe pump into a holding coil; the selection valve was next turned to connect the holding coil to the inlet of the SPR sensor. The conjugate solution in the holding coil was then dispensed into the sample channel of the sensor chip by injecting a carrier solution (0.1 M phosphate buffer solution) at a flow rate of 20 $\mu\text{L}/\text{min}$ using the flow controller, as shown in Fig. 1. At this step ((1) in Fig. 2), the APEO-HRP conjugate was immobilized on the sensor chip by physical adsorption. In this work, HRP was used as a protein conjugate similar to BSA for immobilization of an antigen (APEO) on the sensor chip, not as a label enzyme for colorimetric detection. The reference channel of the SPR sensor was filled with the carrier solution. A 100 μL aliquot of a 1000 ppm BSA solution was then introduced onto the sensor chip using the syringe pump and the selection valve. At this step ((2) in Fig. 2) the bare part of the gold thin film on the sensor chip was blocked by BSA to prevent nonspecific adsorption of the anti-APEO antibody to be used in the subsequent protocol. This procedure was repeated twice more so as to achieve complete blockage by the BSA. A 100 μL

aliquot of a preliminary incubated solution containing the anti-APEO antibody at a constant concentration of 50 ppm and an APEO sample solution at various concentrations (1 ppb – 1 ppm) was introduced onto the sensor chip at a flow rate of 20 $\mu\text{L}/\text{min}$ by the syringe pump. At this step ((3) in Fig. 2), the anti-APEO antibody was competitively bound to the APEO-HRP conjugate on the sensor chip and to the APEO in the sample solution. The angle shift of the SPR sensor between step (2) and step (3) is directly related to the concentration of APEO in the sample solution, because the angle shift is generated by the binding of the anti-APEO antibody to the APEO-HRP conjugate on the sensor chip. Finally, after measuring the angle shift of the SPR sensor, we introduced a 100 μL aliquot of a dissociation solution onto the sensor chip to dissociate the anti-APEO antibody from the immunocomplex of the anti-APEO antibody with the APEO-HRP conjugate on the sensor chip. At this step ((4) in Fig. 2), the sensor chip was regenerated and is in the same state as it was before the introduction of the sample solutions.

Application of present assay to tap water samples

A 1.0 L aliquot of tap water sample, spiked with various amounts of APEO (1 ppt, 50 ppt, 0.5 ppb and 1 ppb), was passed through a Sep-Pack C18 column at a flow rate of 20 mL/min using an extraction manifold (Waters Co.), after pretreatment of the column with 25 mL of methanol for conditioning. The APEO on the column was then eluted with 5.0 mL of methanol. To determine the APEO in the eluent, we diluted the eluent to one-tenth with deionized water, thus reducing the methanol content to 10%.

Theoretical Consideration of the Present Immunoassay Based on the SPR Sensor

Evaluation of the adsorption constant of the APEO-HRP conjugate on the sensor chip

In the first step of the immunoassay, the APEO-HRP conjugate is immobilized on the sensor chip by physical adsorption, as described in the previous section. In order to evaluate the adsorption constant of the APEO-HRP conjugate on the sensor chip and to evaluate the surface density of the APEO-HRP conjugate that was immobilized on the sensor chip, the angle shifts were measured using HRP instead of when the APEO-HRP conjugates at various concentrations were introduced onto the sensor chip. The adsorption constant, K_{ad} , can be expressed by Eq. (1):

$$K_{\text{ad}} = [\text{APEO-HRP}]/C_{\text{APEO-HRP}} \quad (1)$$

where $[\text{APEO-HRP}]$ and $C_{\text{APEO-HRP}}$ are the surface concentration of the APEO-HRP conjugate on the sensor chip in nmol/mm^2 units and the concentration of the APEO-HRP conjugate in the solution adjacent to the sensor chip in mol/dm^3 units, respectively.

If the adsorption of the APEO-HRP conjugate obeys a Langmuir-type adsorption isotherm, the following equation can be derived from Eq. (1):

$$[\text{APEO-HRP}]/[\text{APEO-HRP}]^{\text{T}} = \frac{K_{\text{ad}}C_{\text{APEO-HRP}}}{(1 + K_{\text{ad}}C_{\text{APEO-HRP}})} \quad (2)$$

where $[\text{APEO-HRP}]^{\text{T}}$ is the surface concentration of the APEO-HRP conjugate, when the sensor chip is completely covered with the APEO-HRP conjugate.

If the angle shift of the SPR sensor, $\Delta\theta$, is proportional to the surface concentration of the APEO-HRP conjugate adsorbed to the sensor chip, the ratio of the relative surface concentration to the total surface concentration of the APEO-HRP conjugate can be expressed as follows:

$$[\text{APEO-HRP}]/[\text{APEO-HRP}]^{\text{T}} = \Delta\theta/\Delta\theta_{\text{max}} \quad (3)$$

where $\Delta\theta_{\text{max}}$ denotes the angle shift of the SPR sensor when the APEO-HRP conjugate completely occupies the surface of the sensor chip.

The relationship between the concentration of the APEO-HRP conjugate solution, which was introduced onto the sensor chip, and the angle shift of the SPR sensor, $\Delta\theta$, can be derived from Eqs. (2) and (3):

$$K_{\text{ad}}C_{\text{APEO-HRP}}/(1 + K_{\text{ad}}C_{\text{APEO-HRP}}) = \Delta\theta/\Delta\theta_{\text{max}} \quad (4)$$

Rearranging Eq. (4), one can derive the following equation:

$$C_{\text{APEO-HRP}}/\Delta\theta = C_{\text{APEO-HRP}}/\Delta\theta_{\text{max}} + 1/(\Delta\theta_{\text{max}}K_{\text{ad}}) \quad (5)$$

When $C_{\text{APEO-HRP}}/\Delta\theta$ is plotted against $C_{\text{APEO-HRP}}$, a linear relationship is obtained if the Langmuir-type adsorption isotherm holds for the adsorption of the APEO-HRP conjugate on the sensor chip. The values for K_{ad} and $\Delta\theta_{\text{max}}$ can be calculated from the intercept and the slope of the linear relationship.

Evaluation of affinity constant of anti-APEO antibody immunocomplex with APEO-HRP conjugate on the sensor chip

In order to evaluate the affinity constant of an anti-APEO antibody immunocomplex with the APEO-HRP conjugate on the sensor chip, we introduced 100 μL aliquots of various concentrations of anti-APEO antibody solutions (20 – 400 ppm) onto the sensor chip, the surface of which was immobilized with the APEO-HRP conjugate by introducing a 100 ppm APEO-HRP conjugate solution.

The affinity constant (expressed as K_1) of the anti-APEO antibody (expressed as Ab) immunocomplex with the APEO-HRP conjugate (expressed as Ag_1) on the sensor chip are expressed by Eq. (6).

$$K_1 = [\text{Ag}_1\text{-Ab}]/[\text{Ag}_1]C_{\text{Ab}} \quad (6)$$

where $[\text{Ag}_1]$ and $[\text{Ag}_1\text{-Ab}]$ denote the surface concentrations of the APEO-HRP conjugate and the anti-APEO antibody immunocomplex with the APEO-HRP conjugate on the sensor chip, respectively. C_{Ab} is the concentration of the free anti-APEO antibody in the solution.

If a Langmuir-type adsorption is assumed to hold for the binding of the anti-APEO antibody to the APEO-HRP conjugate on the sensor chip, and if the total surface concentration of the APEO-HRP conjugate on the sensor chip is assumed to be $[\text{Ag}_1]^{\text{T}}$, the following equation can be derived:

$$[\text{Ag}_1\text{-Ab}]/[\text{Ag}_1]^{\text{T}} = K_1C_{\text{Ab}}/(1 + K_1C_{\text{Ab}}) \quad (7)$$

In this case, since the change in the angle shift of the SPR sensor $\Delta\theta_1$, is proportional to the surface concentration of the anti-APEO antibody bound to the APEO-HRP conjugate on the sensor chip, $\Delta\theta_1$ can be rewritten in the form of the following equation:

$$\Delta\theta_1/\Delta\theta_{1,\text{max}} = K_1C_{\text{Ab}}/(1 + K_1C_{\text{Ab}}) \quad (8)$$

where $\Delta\theta_{1,\max}$ denotes the maximum of the angle shift of the SPR sensor, where the anti-APEO antibody is completely bound to the APEO-HRP conjugate.

The following equation can be derived from Eq. (8):

$$C_{Ab}/\Delta\theta_1 = C_{Ab}/\Delta\theta_{1,\max} + 1/(K_1\Delta\theta_{1,\max}) \quad (9)$$

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

Evaluation of the affinity constant of the anti-APEO antibody immunocomplex with APEO in the solution

The affinity constant of the anti-APEO antibody immunocomplex with APEO in the solution can be evaluated in the same manner, assuming that the Langmuir-type adsorption isotherm would hold for the competitive immunoreactions of the anti-APEO antibody with APEO in the solution and the APEO-HRP conjugate on the sensor chip. The affinity constants of the anti-APEO antibody immunocomplex with APEO (expressed as Ag_2) in the solution are expressed by Eq. (10):

$$K_2 = C_{Ag_2-Ab}/C_{Ag_2}C_{Ab} \quad (10)$$

In the present competitive immunoassay, an incubation solution containing the anti-APEO antibody (Ab) and APEO (Ag_2) was introduced on the sensor chip, on which the APEO-HRP conjugate (Ag_1) is immobilized. Therefore the following mass balance equations should be considered for the anti-APEO antibody and APEO in the incubation solution and the APEO-HRP conjugate immobilized on the sensor chip.

$$C_{Ab}^T = C_{Ab} + C_{Ag_2-Ab} \quad (11)$$

$$C_{Ag_2}^T = C_{Ag_2} + C_{Ag_2-Ab} \quad (12)$$

$$[Ag_1]^T = [Ag_1] + [Ag_1-Ab] \quad (13)$$

where C_{Ab}^T and $C_{Ag_2}^T$ are the initial (total) concentrations of the anti-APEO antibody and APEO in the incubation solution, respectively, and C_{Ab} and C_{Ag_2} are the corresponding free (uncomplexed) species in the incubation solution. C_{Ag_2-Ab} is the concentration of the anti-APEO antibody immunocomplex with APEO in the incubation solution.

When the incubation solution containing the anti-APEO antibody and APEO at initial concentrations C_{Ab}^T and $C_{Ag_2}^T$, respectively, is introduced onto the sensor chip, the angle shift of the SPR sensor, $\Delta\theta_2$, is due to the binding of the anti-APEO antibody free from APEO in the incubation solution to the APEO-HRP conjugate on the sensor chip. The concentration of the free antibody, C_{Ab} , can be derived using Eqs. (11) – (13) as follows:

$$C_{Ab} = C_{Ab}^T/(1 + K_2C_{Ag_2}^T) \quad (14)$$

By inserting Eq. (14) into Eq. (9) and rearranging, one can obtain the following equation:

$$1/\Delta\theta_2 = 1/\Delta\theta_{1,\max} + K_2C_{Ag_2}^T/(K_1C_{Ab}^T\Delta\theta_{1,\max}) \quad (15)$$

When $1/\Delta\theta_2$ is plotted against $C_{Ag_2}^T$, a linear relationship would be expected and K_2 is obtained from the slope using the parameters K_1 and $\Delta\theta_{1,\max}$, obtained in previous experiments and $C_{Ab}^T = 7.5 \times 10^{-5}$ M (50 ppb).

The theoretical calibration curve for the present immunoassay

Fig. 3 Typical sensorgram of the present immunoassay based on a surface plasmon resonance sensor: (a) 100 μ L aliquot of a 100 ppm APEO-HRP conjugate solution, (b) 100 μ L aliquot of a 1000 ppm BSA solution, (c) 100 μ L aliquot of a 100 ppm anti-APEO antibody solution, (d) 1 ppm pepsin solution (glycine/HCl solution at pH 2), (e) 100 μ L aliquot of an APEO solution containing 50 ppm anti-APEO antibody. Concentration of APEO: (e)0, 0 ppb; (e)1, 1 ppb; (e)2, 10 ppb; (e)3, 100 ppb.

by the SPR sensor, which involves plotting the angle shift obtained for the incubation solution against the concentration of APEO in the incubation solution, can be calculated from Eqs. (6), (10) and (11) – (13). Since the sensor signal is proportional to $[Ag_1-Ab]/[Ag_1]^T$, as described previously, the calibration curve can be simulated by inserting one of the solutions of the following the 2nd order Eq. (16) concerning the free antibody concentration, C_{Ab} , into Eq. (7).

$$K_2C_{Ab}^2 + (1 - K_2C_{Ab}^T + K_2C_{Ag_2}^T)C_{Ab} - C_{Ab}^T = 0 \quad (16)$$

Results and Discussion

Sensorgram obtained by SPR immunoassay

Figure 3 shows a typical sensorgram obtained using the protocol described in the experimental section. When a 100 μ L aliquot of a 100 ppm APEO-HRP conjugate solution was introduced onto a bare sensor chip ((a) in Fig. 3), the angle shift increased steeply, by about 0.076° , indicating the successful immobilization of the APEO-HRP conjugate on the sensor chip. In order to prevent nonspecific adsorption of the antibody used in a subsequent protocol, the un-immobilized portion of the sensor chip was blocked by introducing a 1000 ppm BSA solution onto the sensor chip three times. As indicated by the arrows (b) in Fig. 3, the angle shift was increased by about 0.082° for the first introduction of the BSA solution, but no further change was found for the third introduction. This indicates that the bare part of the sensor chip may be covered with BSA. The angle shift due to the immobilization of the APEO-HRP antibody is nearly equal to that due to blocking with BSA, indicating that about 50% of the sensor chip is covered with the conjugate. When a 100 μ L aliquot of a 100 ppm anti-APEO antibody solution was introduced onto the sensor chip ((c)1 in Fig. 3), the angle shift was increased by about 0.075° , indicating the binding of the anti-APEO antibody to the APEO-HRP conjugate on the sensor chip. As indicated by an arrow (d) in Fig. 3 for the introduction of a dissociation solution

containing 1 ppm pepsin in a glycine/HCl solution at pH 2, a peak-shaped signal was observed just after the introduction of the dissociation solution. The appearance of the peak-shaped signal can be explained as follows. Since the refractive index of the dissociation solution is higher than that of the carrier solution, the SPR sensor responds to the change of the refractive index of the bulk solution when the dissociation solution passes through the sensor chip. After the dissociation solution passes, the angle shift returns to the initial value observed before the introduction of the anti-APEO antibody solution. This indicates that the sensor chip is successfully regenerated for additional immunoassays using a single sensor chip. In order to prepare a calibration graph for the determination of APEO, we successively introduced sample solutions of APEO at several concentrations (0, 1, 10 and 100 ppb) containing the anti-APEO antibody at a constant concentration of 50 ppm onto the sensor chip after the sensor chip was regenerated by the dissociation solution. The angle shift after introducing the sample solution decreased with increasing concentration of APEO in the sample solution, indicating that the amount of anti-APEO antibody bound to the APEO-HRP conjugate on the sensor chip was decreased by increasing the concentration of APEO due to competitive immunoreactions of the antibody in the sample solution. This angle shift observed for the sample solution without APEO can be used to calibrate the immunoassay, as described in a later section.

Evaluation of the adsorption constant of the APEO-HRP conjugate on the sensor chip

The APEO-HRP conjugate was immobilized on the sensor chip by physical adsorption in the first step of the immunoassay. The surface density of the immobilized APEO-HRP conjugate would be expected to affect the sensitivity of the method. In this work, the adsorption constant of HRP and the surface density of the immobilized HRP at saturated conditions were evaluated by using HRP instead of the APEO-HRP conjugate. Figure 4(a) shows the relationship between the concentration of an HRP solution and the observed angle shift of the SPR sensor when an HRP solution at several concentrations (20–1000 ppm) was introduced onto a bare sensor chip. In this experiment, the HRP solutions were successively introduced onto a single sensor chip and the observed angle shift was plotted against the sum of the concentration of the HRP solution for previous solutions. The data for the adsorption of HRP from a 10% aqueous methanol solution are also shown in Fig. 4(b). As can be seen from Fig. 4(a), the angle shift increased steeply with the concentration of HRP in a lower concentration range and gradually reached a constant value of 0.23° , which may be due to saturation of the adsorbed HRP on the sensor chip. While, in the case of the adsorption of HRP from the 10% methanol solution, the angle shift gradually increased with the concentration of HRP, compared with the adsorption from an aqueous solution. The angle shift at a concentration of 3000 ppm is about 0.2° , which is lower than the angle shift observed for adsorption from the aqueous solution. From the data shown in Fig. 4, $C_{\text{HRP}}/\Delta\theta$ is plotted against C_{HRP} according to Eq. (5), as shown inside of Fig. 4. A linear relationship exists between $C_{\text{HRP}}/\Delta\theta$ and C_{HRP} , consistent with the estimation based on Eq. (5). This indicates that the adsorption of HRP on the sensor chip holds for the Langmuir adsorption isotherm. In the case of the adsorption of HRP from the aqueous solution, $K_{\text{ad}} = 4.7 \times 10^5 \text{ M}^{-1}$ and $\Delta\theta_{\text{max}} = 0.23^\circ$ were obtained from the intercept and slope of the linear relationship shown inside of Fig. 4. In the case of adsorption from the 10% methanol solution, moreover, $K_{\text{ad}} = 3.1 \times 10^5 \text{ M}^{-1}$ and $\Delta\theta_{\text{max}} = 0.20^\circ$ were also obtained, which

Fig. 4 The relationship between the angle shift of the SPR sensor due to the adsorption of HRP to the sensor chip and the concentration of HRP solution introduced onto the sensor chip and the relationship between C_{HRP} and $C_{\text{HRP}}/\Delta\theta$ for evaluating the adsorption constant and the surface density of HRP on the sensor chip (inside figure). (a) HRP solution prepared using a 0.1 M phosphate buffer solution, (b) HRP solution containing 10% methanol.

are somewhat smaller than the values for adsorption from the aqueous solution. This may be due to the fact that the adsorptivity of HRP on the gold thin film from 10% methanol media is weaker than that from the aqueous media. In our previous study dealing with immunoassay for parathion methyl (PM) using an SPR sensor, the adsorption constants for bovine serum albumin (BSA) and a PM-BSA conjugate were found to be 3.8×10^{-5} and $5.0 \times 10^{-5} \text{ M}^{-1}$, respectively.¹⁰ The order of magnitude of the adsorption constant of HRP obtained in this work is similar to that of BSA or the PM-BSA conjugate reported in the previous work. This similarity in the extent of adsorption of the two proteins on the sensor chip is reasonable, judging from the molecular weights of the two proteins ($M_{\text{BSA}} = 67 \text{ kDa}$, $M_{\text{HRP}} = 44 \text{ kDa}$).

The surface density of HRP immobilized on the sensor chip can be estimated from the angle shift, taking into account the fact that an angle shift of 0.1° is known to correspond to the adsorption of protein at a surface density of 1.0 ng/mm^2 , according to BIACOR's specification.¹¹ The maximum angle shift calculated for a hypothetical state in which the sensor surface is completely covered with HRP is $\Delta\theta_{\text{max}} = 0.23^\circ$. This value corresponds a surface density of HRP of 2.3 ng/mm^2 *i.e.* $5.0 \times 10^{-14} \text{ mol/mm}^2$, when the molecular weight of HRP is taken into account. In preparing the sensor chip for the present immunoassay, we controlled the surface density of the APEO-HRP conjugate immobilized on the sensor chip by changing the concentration of the APEO-HRP conjugate solution, in order to prevent steric hindrance to the binding of the anti-APEO antibody to the APEO-HRP conjugate on the sensor chip. In this case, the concentration of the APEO-HRP conjugate solution was selected as 100 ppm and about a half the maximum value of the angle shift, $\Delta\theta_{\text{max}}$ was observed. This indicates that the surface density of the APEO-HRP conjugate on the sensor chip is about $2.5 \times 10^{-14} \text{ mol/mm}^2$.

Evaluation of the affinity constant of the anti-APEO antibody immunocomplex with the APEO-HRP conjugate on the sensor chip

In order to evaluate the affinity constant of the anti-APEO antibody complex with the APEO-HRP conjugate on the sensor chip, we introduced various concentrations of anti-APEO antibody solution (0–400 ppm, C_{Ab}) onto the sensor chip, where the APEO-HRP conjugate was immobilized and we then measured the angle shift of the SPR sensor ($\Delta\theta_1$). When the value of $C_{Ab}/\Delta\theta_1$ was plotted against C_{Ab} using the observed angle shifts, a linear relationship between two parameters was obtained, indicating that the Langmuir-type adsorption isotherm holds for the binding of the antibody to the antigen on the sensor chip. The values $\theta_{1,max} = 0.097^\circ$ and $K_1 = 2.0 \times 10^6 \text{ M}^{-1}$ were calculated from the values of the slope and the intercept of the straight line. The surface concentration of the anti-APEO antibody is estimated to be $6.5 \times 10^{-15} \text{ mol/mm}^2$ from the calculated $\theta_{1,max}$ value. The value of the affinity constant of the immunocomplex, $K_1 = 2.0 \times 10^6 \text{ M}^{-1}$, and is of a similar order of magnitude to that of other immunocomplexes, such as an anti-histamine antibody, an anti-parathion methyl antibody with corresponding antigens, reported in our previous papers.^{10,12}

Evaluation of the affinity constant of the anti-APEO antibody immunocomplex with APEO in aqueous solution

To evaluate the anti-APEO antibody immunocomplex with APEO in aqueous solution, we measure the angle shift of the SPR sensor ($\Delta\theta_2$), for incubation solutions containing various concentrations of APEO (0–40 ppb, C_{APEO}), while the anti-APEO antibody was introduced, at a constant concentration of 50 ppm onto the sensor chip. According to Eq. (15), in a plot of $1/\Delta\theta_2$ against C_{APEO} , a linear relationship was obtained and $K_2 = 5.1 \times 10^6$ was calculated from the slope of the linear relationship. The affinity constant for the anti-APEO antibody immunocomplex with APEO in aqueous solution is nearly the same as that for the APEO-HRP conjugate on the sensor surface.

Application of the present assay to an analysis of tap water samples

A calibration curve for APEO obtained by the present immunoassay using the SPR sensor is shown in Fig. 5(a). The angle shifts shown in a typical sensorgram in Fig. 3 ((e)0)–((e)3), were plotted against the concentration of APEO in the incubation solution. In this case, the angle shifts obtained as the result of introducing a 100 μL aliquot of the incubation solution containing various levels of APEO (1 ppb–100 ppm) and the anti-APEO antibody at a constant concentration (50 ppm) were normalized by the angle shift obtained by introducing a 50 ppm anti-APEO antibody solution without APEO. Therefore, the angle shift resulting from the introduction of the antibody solution is regarded as 100% and the angle shift observed after blocking by a BSA solution is regarded as 0% for the calibration. This normalization of the sensor signal may be effective even in the case where the surface density of the APEO-HRP conjugate is varied to some extent in the immobilization conditions, since the absolute angle shift observed for the sample solution is dependent on the surface density of the conjugate. Indeed, for the determination of APEO using an aqueous matrix with a 10% methanol content, almost the same calibration curve was obtained using the angle shift normalized by that observed for the anti-APEO antibody solution with the 10% methanol matrix, as shown in Fig. 5(b). The simulated calibration curve is overlaid as a plot of $[Ag_1-Ab]/[Ag_1]^T$ vs. C_{Ab}^T in Fig. 5 by a dotted line, which was calculated from Eqs. (7) and (16) using

Fig. 5 Calibration curve for APEO by the present immunoassay based on an SPR sensor. Sample solution matrix: (a) ● 0.1 M phosphate buffer solution containing NaCl and KCl, (b) □ 10% methanol aqueous solution. Analytical conditions: 100 ppm APEO-HRP solution for immobilization, 1000 ppm BSA solution for blocking, APEO sample solution containing 50 ppb anti-APEO antibody for competitive immunoassay, 1 ppm pepsin solution prepared by a glycine/HCl solution at pH 2. The dotted line is calculated from Eqs. (7) and (16) by using $K_1 = 2.0 \times 10^6 \text{ M}^{-1}$, $K_2 = 5.1 \times 10^6 \text{ M}^{-1}$ and $C_{Ab}^T = 7.5 \times 10^{-5} \text{ M}$ (50 ppm).

the estimated affinity constants, K_1 and K_2 , and the initial concentration of the anti-APEO antibody, $C_{Ag_2}^T$, in the incubation solution. The limit of detection of the present immunoassay is about 10 ppb, as judged from the calibration curve shown in Fig. 5 at an APEO concentration at which 85% of the sensor signal is observed. The limit of detection obtained in the present immunoassay is sufficient for the Japanese Water Supply Law. The coefficient of variation (CV) for repeated assays of a sample solution containing 20 ppb APEO was about 40%.

In order to apply the present method to the determination of APEO in tap water samples at much lower levels as ppt order, we utilized a solid phase extraction procedure to enrich APEO. Although the solid phase extraction was not able to recover APEO from a sample solution at the 1 ppt level, for sample solutions containing at 50 ppt–1 ppb APEO, the recoveries of 98–150% were obtained by the present method combined with the solid phase extraction pretreatment.

Conclusions

A flow immunoassay method for APEO based on an SPR sensor was developed and was applied to the determination of APEO in tap water samples. The detection limit of the present method was *ca.* 10 ppb. A sensor chip can be used repeatedly for about 20 times by regeneration using a dissociation solution. A methanol content of 10% in the sample solution was found to have no significant effect on the immunoassay and 50 ppt APEO in a tap water sample could be detected, when preconcentration by solid phase extraction using methanol as an eluent was used. The affinity constants for the anti-APEO antibody immunocomplex with the APEO-HRP conjugate on the sensor chip and with APOE in aqueous solution were estimated to be 2.0×10^6 and $5.1 \times 10^6 \text{ M}^{-1}$, respectively. By use of the affinity constants obtained, the calibration curve can be simulated by an equation derived by assuming a Langmuir adsorption isotherm

for the anti-APEO antibody bound to the APEO-HRP conjugate on the sensor chip. The analytical throughput of the present method using a dual-channel SPR sensor is lower than that of an ELISA method using a 96 microtiter plate at this stage. A multichannel type SPR would be expected to be available for high-throughput immunoassay.

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