Regenerable Surface Plasmon Resonance (SPR)-based Immunosensor for Highly Sensitive Measurement of Sub-ppb Levels of Benzo(a)pyrene

Norio Miura,* Makoto Sasaki, Go Sakai,† and K. Vengatajalabathy Gobi
Advanced Science and Technology Center for Cooperative Research, Kyushu University, Kasuga, Fukuoka 816-8580
†*Faculty of Engineering Sciences, Kyushu University, Kasuga, Fukuoka 816-8580*

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A regenerable surface plasmon resonance (SPR)-based immunosensor functioning with the indirect competitive immunoreaction of monoclonal antibody between the analyte (antigen) in testing solution and antigen-bovine serum albumin conjugate immobilized on immunoprobe provided a rapid in situ estimation (response time: about 15 min) of benzo(a)pyrene in the concentration range of 0.1–300 ppb.

Polycyclic aromatic hydrocarbons (PAHs) are known to exhibit strong carcinogenic properties and to be one of the endocrine disrupting chemicals. Among the metabolites of PAHs, benzo(a)pyrene (BaP) is extensively studied and it is often monitored as an indication of the potential carcinogenicity of PAHs. Though high pressure liquid chromatography, gas chromatography–mass spectrometry, enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay-based methods developed for the analysis of BaP are accurate and sensitive at sub-ppb levels, these methods involve time-consuming purification steps and/or tedious radio-labeling procedures.[1–3] Thus there is a strong demand for instrumentation that will allow the rapid in situ analysis of PAHs and their metabolites, specifically BaP, in various liquid samples at low cost. When ELISA method is coupled with an optical transducer, low-cost, regenerable and simple immunosensors can be developed. Among the various optical sensing methodologies, a SPR-based sensor has been widely regarded as a useful tool for detecting chemicals especially in liquid media.[4–6] SPR phenomenon is highly sensitive to little changes in refractive index adjacent to the sensor surface. A change in refractive index can be obtained when an analyte binds to the sensor surface. We have recently reported SPR-based immunosensors for sensitive detection of drugs, such as morphine and methamphetamine.[7–9] In this work, we report a SPR-based immunosensor for the highly sensitive and rapid in situ estimation of BaP, a potential endocrine disrupter.

BaP has no immunogenicity due to its small molecular weight and thus it should be derived into a conjugate with protein. BaP-bovine serum albumin conjugate (BaP–BSA) was newly synthesized by covalently binding BaP with BSA using a short aliphatic chain spacer (-$COCH_2CH_2CO$-). Benzo(a)pyrene was acylated to ethyl 4-oxo-4-(benzo(a)pyrene)butyrate, which was then reacted with dicyclohexylcarbodiimide and N-hydroxysuccinimide to obtain succinimidyl 4-oxo-4-(benzo(a)pyrene)butyrate; the succinimidyl ester was coupled to BSA by amide bond (-CO-NH-) formation. The molecular weight (MW) of BSA and BaP-BSA was determined using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF MS). An anti-BaP–BSA monoclonal antibody (MW: ca. 150000) was produced from a hybrid cell fused with myeloma cell line and spleen cell of mice immunized with BaP–BSA. BaP–BSA and anti-BaP–BSA antibody are gifts from Kyoto Electronics Manufacturing Co., Ltd. The sensor assembly used here was composed of a SPR angle measuring instrument (SPR-20, DKK, Japan), a flow cell and a microtube pump. Gold plate prepared by sputtering gold on a thin glass plate with chromium as an adhesion layer was used as base for a sensor chip (immunoprobe) and was attached to the cylindrical prism of SPR instrument by using a refractive index matching liquid. The flow cell was placed on the gold plate and test solutions were circulated at a flow rate of 0.45 cm^3/min. Each of BaP–BSA, BSA, the monoclonal antibody and BaP was dissolved in phosphate buffer saline (PBS; contains 1 vol% ethanol; pH 7.2), and a fixed volume (1 cm^3) of these solutions was circulated through the flow cell. Through out the investigation, room temperature was controlled at $25 \pm 0.3\,^{\circ}C$.

The principle of the present BaP sensor we developed is based on an indirect inhibition method. Incident angle of the sensor chip having immobilized BaP–BSA increases when contacted with the monoclonal antibody solution. At a properly selected concentration of the monoclonal antibody, the change in incident angle will depend on the concentration of BaP which coexists, because of the immunoreaction between BaP and the monoclonal antibody. From the dependence of the incident angle shift, the concentration of BaP would be determined.

The MWs of BSA and BaP–BSA were determined to be 66431 and 75881, respectively, and the difference in MW between BSA and BaP–BSA was used to calculate the average number of BaP units appended to BaP–BSA to be ca. 28. BaP–BSA was immobilized on the sensor chip by a simple physical adsorption. While the sensor chip was exposed to the flow of BaP–BSA solution (70 ppm), the incident angle of the sensor increased rapidly and reached a steady state in about 10 min. A very small incident angle shift was observed on followed exposure to the flow of a carrier solution (PBS), confirming that the BaP–BSA immobilization is strong enough. The increment in incident angle due to the adsorption of BaP–BSA was about 0.30 degree. Then the chip was exposed to the flow of BSA solution (1000 ppm) in order to block non-specific adsorption sites of the chip; the incident angle was increased by ca. 0.05 degree by the exposure of BSA. After this treatment, the incident angle was no longer changed by the flow of 20 ppm BSA solution or PBS.

The BaP–BSA immobilized chip thus prepared was exposed to the flow of the antibody solutions of various concentrations. On the exposure to the antibody, the incident angle increased and attained a steady state in about 15 min. Then PBS was flowed to remove any non-specifically bound antibody from the chip; a very small change in the incident angle was observed. The incident angle shift increased rapidly with increasing concentration of anti-BaP–BSA antibody solution up to ca. 22 ppm and then

increased meagerly above this concentration (Figure 1). From this figure, the use of 22 ppm antibody solution was considered to be the best for the BaP sensing experiments based on the inhibition method. The antibody anchored to the chip by antigen–antibody binding was not removed on exposure to the flow of acidic solution (pH ~2), alkaline solution (pH ~13) or BaP solutions of high concentrations (0.1–10 ppm). However, a same chip could be reused, because the antibody was found to be removed by exposing to the flow of a 100 ppm pepsin solution (Pepsin A from Porcine stomach mucosa; 460 U mg^{-1}) in glycine/HCl buffer (0.2 mol dm^{-3}; pH 2.0). The concentration, the pH and the duration of exposure of the pepsin solution were controlled so that only the antibody was removed from the sensor surface.

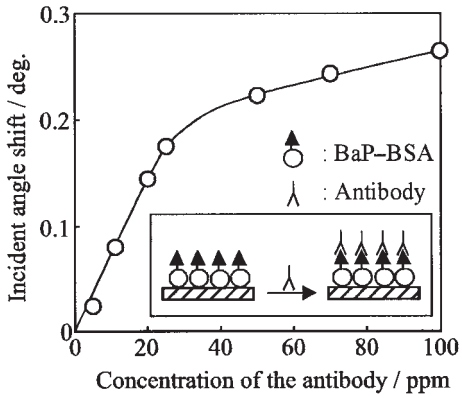

Figure 1. The dependence of the incident angle shift of the BaP–BSA immobilized immunoprobe on the antibody concentration.

The monoclonal antibody solution (22 ppm) was mixed with BaP of various concentrations and was incubated for 5 min at room temperature prior to flowing over the BaP–BSA immobilized sensor chip. The observed incident angle *vs.* time plots are shown in Figure 2. In the absence of BaP, the incident angle increased by 0.155 ± 0.005 degree. With the addition of BaP to

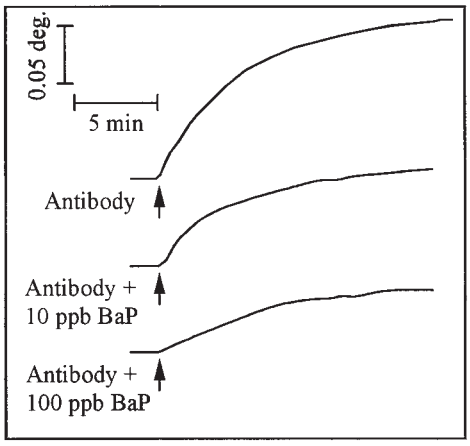

Figure 2. Response transients of incident angle of the BaP–BSA immobilized immunoprobe to BaP solutions of various concentration. (The antibody coexists in BaP solutions at the concentration of 20 ppm).

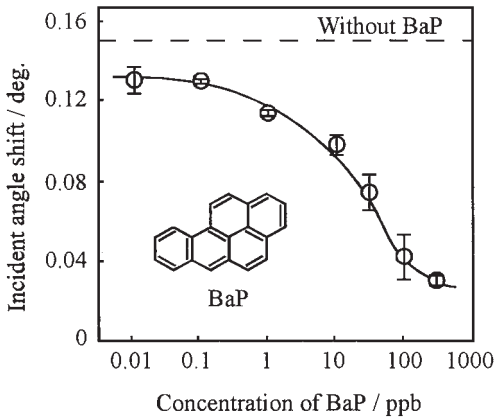

Figure 3. The dependence of the incident angle shift of the BaP–BSA immobilized immunoprobe on the concentration of BaP under the fixed concentration (22 ppm) of the antibody.

the antibody solution, the incident angle shift decreased depending on the concentration of BaP added, due to the inhibition effect of BaP. The incident angle shift was plotted against the concentration of BaP (Figure 3). The error bars indicate the deviation of the incident angle shift in three independent measurements. The incident angle shifts were reproducible within ±7% on repeated measurements (n = 3). A same sensor chip was found to be reused more than 10 times by washing off the bound antibody using the pepsin solution.

As shown in Figure 3, the incident angle shift was sensitive to the concentration of BaP in the range of 0.01–300 ppb. From this figure, the present sensor was confirmed to estimate BaP sensitively in the concentration range of 0.1–300 ppb. The large difference in MW between the BaP–BSA antibody and BaP and the strong association of the antibody with the antigen seem to furnish such a high sensitivity. Using the currently developed SPR immunosensor, BaP could be determined as low as 0.1 ppb with a response time of about 15 min. This is the first time such a sensitive detection of BaP using a SPR sensor is reported.

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