Enhanced Sensitivity of a Surface-plasmon-resonance (SPR) Sensor for 2,4-D by Controlled Functionalization of Self-assembled Monolayer-based Immunosensor Chip

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A highly sensitive SPR immunosensor has been fabricated by covalent binding of a protein conjugate of 2,4-D (2,4-D-BSA) on SAM of 3-mercaptopropionic acid. With indirect inhibition immunoassay, it demonstrated a low detection limit of 0.1 ppb 2,4-D. Taking advantage of the combination of the SAM technique and immobilization of 2,4-D-BSA at controlled surface coverage, a remarkable enhancement of the low detection limit by 10-fold to 10 ppt with no use of any labels was demonstrated, while keeping the response time constant at 4 min.

Public concern on protection of the environment and the quality of food products has been ever more increasing, because of widespread use of various toxic chemicals and of the increase in the number of environmental pollutants. 2,4-Dichlorophenoxyacetic acid (2,4-D) is a systemic herbicide and has been associated with potential endocrine-disrupting activities.[1] Primary screening methods for 2,4-D detection should be inexpensive and usable for automated analysis of a large number of food and potable water samples. Immunosensors based on specific antigen–antibody interaction are immensely explored for direct determination from complex sample matrices.

SPR immunosensor systems have received wide acknowledgement for applications into various fields, because of their inherent advantages over other type of biosensors in its versatility and compatibility with miniaturization, high throughput analysis and label-free detection.[2,3] Integration of biological receptors with physicochemical transducers plays a very significant role in the fabrication of biosensors, where the reactivity of bioreceptor should not be affected while maintaining high resistivity to irrelevant sample matrices. Various methods employed in the fabrication of functional sensing surfaces include physisorption, self-assembly method, binding of complementary ssDNA units, etc. Physisorption of proteins had been primarily investigated because of its advantages such as simplicity, applicable to various biomolecules, etc. We had fabricated SPR immunosensors with the use of simple physical adsorption of a protein–analyte conjugate, and reliable highly sensitive immunosensor systems had been fabricated for various low-molecular-weight analytes (MW < 500 Da) of biological and environmental interest.[4–6] Another well-investigated approach is the binding of target molecule directly on SAM surfaces, where interference due to nonspecific adsorption was detrimental to sensor performance.[7]

In this communication, we demonstrate the development of a novel SAM based SPR immunosensor chip for 2,4-D detection, where a combination of stable SAM structure using low-cost commercially available materials and 2,4-D-BSA conjugate used for antibody production has been applied. An on-line indirect competitive immunoreaction principle has been investigated, in which the immunoreactivity between 2,4-D and its antibody is modulated by controlled surface modification procedures to enhance the sensitivity of the SPR immunosensor.

SPR experiments were performed using SPR-670 analyzer from NLE, Japan. Au-sputtered glass plates with an inner Cr layer (5-nm Cr layer and 50-nm-Au layer) performed as SPR active sensing platforms. Initially, the Au surface of the SPR sensor chip was exposed to the flow of ethanol to cleanse the sensor surface. After a stable baseline with PBS (pH 7.2), the thin-film Au chip was exposed to the flow of 10 mM MPA in PBS for 20 min. A stable increase in SPR angle was observed indicating irreversible binding of MPA. Carboxyl end groups of the resultant MPA layer were activated for amide bonding by the flow of a mixture of EDC and NHS and were covalently bound to 2,4-D-BSA conjugate. The sensor response observed for the covalent binding process (Figure 1) showed a gradual increase in SPR angle indicating the uniform binding of the conjugate on MPA monolayer. After the immobilization of 2,4-D-BSA, a high concentration of BSA (1000 ppm) was let to flow to block any free active sites available on the sensor chip.

The fabricated sensor chip exhibits a highly specific and sensitive immunoreactivity towards the monoclonal anti-2,4-D antibody (2,4-D-mAb). Flow of 2,4-D-mAb at 15 ppm ($\mu g\,mL^{-1}$) showed a smooth and stable increase in SPR angle by ca. 0.14 deg. (Figure 2a), indicating the binding of 2,4-D-mAb by specific immunoaffinity reaction. Dissociation of the antigen–antibody complex without affecting the beneath 2,4-D-BSA conjugate had been investigated. The antibody bound sensor surface was treated with an acidic glycine–HCl buffer (pH 2.0) for 30 s, and the resonance angle of the sensor chip was found to decrease to the original level, indicating complete removal of the antibody from the sensor surface and the stability

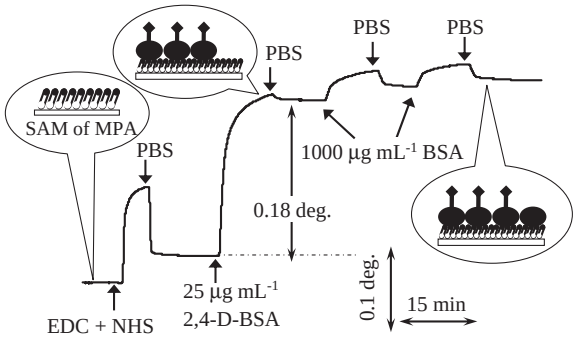

Figure 1. Activation of the SAM of MPA by a mixture of EDC and NHS (2 mg mL^{-1} each) and followed covalent binding of 2,4-D-BSA on sensor surface.

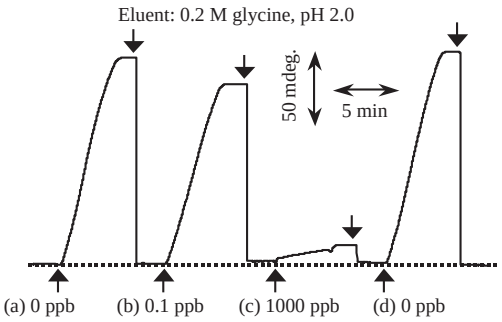

Figure 2. SPR responses to the flow of 15 μg mL^{-1} 2,4-D-mAb in the absence and presence of 2,4-D of various concentrations. Flow speed 50 μL min^{-1}, sample vol 200 μL, response time 4 min.

of the SAM-based immunosensor chip for multiple analysis. This regeneration ability enabled a single sensor chip for 30 measurement cycles with only a marginal decrease (totally 6%) in the sensor response.

With the detection principle of the immunosensor is indirect competitive immunoassay,[4] the sensor surface is exposed to the flow of a mixture of 2,4-D and 2,4-D-mAb, where the 2,4-D-BSA on sensor surface will compete with 2,4-D in solution for binding to antibody. For the purpose, 2,4-D solutions of various concentrations (0.01–1000 ppb (ng mL^{-1})) were incubated with a fixed concentration of 2,4-D-mAb for 5 min and exposed to 2,4-D-BSA bound SPR immunosensor chip. Resulting to the competitive immunoreaction, immunobinding of 2,4-D-mAb on the sensor surface would be inhibited by 2,4-D present in solution and thus the sensor response will decrease with increasing concentrations of 2,4-D. SPR sensorgrams observed were consistent to the sensor principle. A remarkable decrease in SPR angle shift was evident with the addition of 2,4-D as low as 0.1 ppb (Figure 2b). In the presence of 1000 ppb 2,4-D, the increase in SPR angle to mere 10% of the original response implies high specificity of the immunosensor response. SPR angle shifts observed at different concentrations of 2,4-D were plotted against 2,4-D concentration (Figure 3a). Low detection limit of the immunosensor is estimated to be 0.1 ppb, where

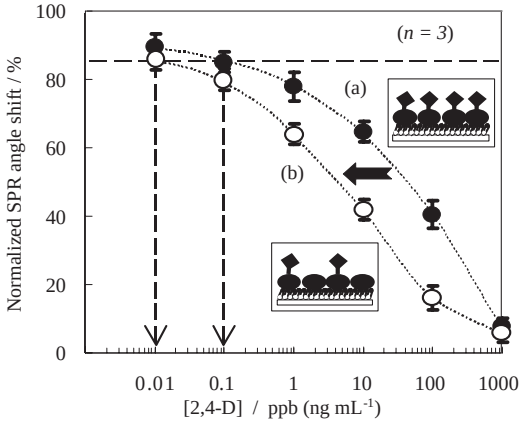

Figure 3. Calibration curves of normalized SPR angle shift vs. 2,4-D concentration determined using 2,4-D-BSA bound SAM sensor chip with different surface concentrations of 2,4-D-BSA, a) 180 ng cm^{-2} and b) 80 ng cm^{-2}. SPR angle shift of 0.01 deg. = 10 ng cm^{-2}.[10] [2,4-D-mAb] = 15 μg mL^{-1}.

the sensor response is inhibited to 85% of the original level.

Enhancement of the sensitivity of SPR based immunosensor systems is reported recently with the use of various multistep sandwich immunoassay techniques employing high molecular-weight labels such as secondary antibodies, colloidal gold, liposomes, etc.[8,9] Recently, binding of gold nanoparticles coupled with secondary antibodies in an additional signal-enhancement procedure to the sensor surface had been shown to improve the low detection limit of the SPR immunosensors for progesterone and human complement factor by as much as 8–20-fold.

Taking advantage of the combination of 2,4-D-BSA binding over SAM in controlled manner and the competitive immunoassay principle, the immunoaffinity interaction of 2,4-D-mAb with 2,4-D could be significantly modulated by changing the surface concentration of 2,4-D-BSA on sensor surface. With decreased surface concentration of 2,4-D-BSA, the relative affinity interaction of the sensor surface with 2,4-D-mAb decreased, increasing further the sensitivity of the immunosensor system towards the analyte 2,4-D. A fresh sensor surface was fabricated by covalent binding of 2,4-D-BSA to only half of its original coverage level. 2,4-D-BSA solution of 5 ppm was let to flow over the EDC-NHS activated MPA monolayer surface for 10 min, and the increase in SPR angle (0.08 deg.) was nearly half to that observed in Figure 1. The remaining active sites of the sensor chip were blocked using a high concentration BSA solution.

With the new SPR sensor surface, the SPR angle shifts observed at different concentrations of 2,4-D in the indirect competitive immunosensing method were plotted in Figure 3b. The low detection limit of the sensor system is 10 ppt (pg mL^{-1}), which is 10-fold better than that obtained earlier with fully packed sensor system. The present low detection limit (10 ppt) is very much better than those reported previously (500 ppt or 3 ppb) for 2,4-D detection with different SPR immunosensor systems.[11,12] Besides a comparable enhancement of the sensitivity, the present sensor system has employed a single-step immunoassay procedure, contrary to multistep sensitivity-enhancement sandwich immunoassay procedures investigated previously,[8,9] and a short response time of only 4 min.

Advantages of the combination of such simple SAM strategy and the protein–analyte conjugate in the fabrication of immunosensor chips will facilitate the development of rapid, high performance analytical tools for small molecular analytes in biomedical, and environmental applications.

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