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Surface plasmon resonance immunosensor using self-assembled protein G for the detection of *Salmonella paratyphi*

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Abstract

A surface plasmon resonance (SPR) based immunosensor using self-assembled protein G was developed for the detection of *Salmonella paratyphi*. In order to endow a solid substrate binding affinity to protein G, the free amine ($-NH_2$) of protein G was substituted into thiol (–SH) using 2-iminothiolane. Thus, self-assembled protein G was fabricated on gold (Au) substrate. The formation of protein G layer on Au surface, and the binding of antibody and antigen in series were confirmed by SPR spectroscopy. The surface morphology analysis of the protein G layer on Au surface was performed by atomic force microscope (AFM). Consequently, an immunosensor based on SPR for the detection of *S. paratyphi* using self-assembled protein G was developed with a detection range of 10^2–10^7 CFU/ml. The current fabrication technique of a SPR immunosensor for the detection of *S. paratyphi* could be applied to construct other immnosensors or protein chips.

Keywords: Immunosensor; Surface plasmon resonance; Self-assembled protein G; 2-Iminothiolane; *Salmonella paratyphi*

1. Introduction

Antibodies, a protein that binds to the target antigen with extremely high specificity, have been used extensively as diagnostic tools in many different formats. Antibody-based immunoassays are the most commonly used type of diagnostic assay and still one of the fastest growing technologies for the analysis of biomolecules. Although the antibody is not the only molecule that can be used for quantification of antigens, bioassays based on whole cells, receptors and enzymes do not, in many instances, offer the same unlimited applicability and specificity. Owing to antibodies' high specificity to the target antigen, immobilized antibodies on various solid-phase surfaces have been widely used in many fields such as purification of materials, diagnostic immunoassays, and immunosensors (Choi et al., 2001a; Disley et al., 1998; Koch et al., 2000; O'Brien et al., 2000).

Recently, antibody based immunosensors using surface plasmon resonance (SPR) have been developed for the measurement of antigens binding to antibody

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immobilized on the SPR sensor surface, which are capable of detecting analytes in complex biological media with high specificity and sensitivity, with a short detection time, and with simplicity as a local increase in the refractive index (Darren et al., 1998; Oh et al., 2002; Sakai et al., 1998; Toyama et al., 1998). Therefore, the analyte's presence can be determined directly without the use of labeled molecules. However, as the concentrations of analytes in a biological system are extremely low, the enhancement of sensitivity is required to detect biological materials.

The sensitivity of a SPR immunosensor for the detection of antigens with a very low concentration can be increased by control of the orientation of antibodies immobilized on the SPR sensor surface. When antibodies are immobilized on a solid-phase surface, their binding activity is usually less than that of soluble antibodies. One of the main reasons for this reduction of binding activity is due to steric hindrance of the molecules in the solid-phase compared with that in solution. Another possible reason is attributed to the random orientation of the antibody molecules on the solid-phase surface. Therefore, the development of the immobilization method for antibodies in a highly oriented manner is strongly required.

Several methods have been proposed to prepare oriented antibody molecule on the solid matrix surfaces. For instances, Langmuir–Blodgett (LB) technique and self-assembly technique were applied for the fabrication of protein thin film (Breen et al., 1999; Choi et al., 2001b; Damrongchai et al., 1995; Duan and Meyerhoff, 1994; Kanno et al., 2000). And, in order to enhance the sensitivity of antibody-based immunoassay, the distance between the transducer surface and the immobilized film of protein ligands should be minimized and the monolayer on the surface should possess a two-dimensional order. Among these technique, self-assembly technique which utilizes the thiol–gold interaction have extensively used for preparing antibody molecules monolayer on solid surface, since controlling the orientation of protein molecules by LB technique is rather difficult because hydrophobic and hydrophilic parts of protein molecules are not separated precisely.

In order to construct a well-defined antibody surface, protein G, a cell wall protein found in most species of *Streptococci*, can be used for proper orientation of antibody. Since protein G exhibits a specific interaction with the Fc portion of Immunoglobulin G (IgG) (Boyle and Reis, 1987), the paratope of IgG can face the opposite side of the protein G-immobilized solid support. As a result, protein G mediated antibody immobilization can lead to a highly efficient immunoreaction.

Salmonella is of major significance as a pathogenic microorganism in food-borne infections in humans, causing diarrhea, fever, and abdominal pain. In the United State, about 76 million illnesses per year are estimated to cause by food-borne pathogens (Mead et al., 1999) and the number has increased more than five fold since 1942. Detection of *Salmonella* contamination of food, therefore, is very important for public health protection (Pathirana et al., 2000; Wong et al., 2002). However, conventional microbiological culture methods used for the detection of microorganism are labour-intensive and requires several days to obtain results and may be unsatisfactory to respond in a timely manner in cases of contamination. Many immunoassay techniques are widely attempted for the detection of bacteria, but they are usually expensive and require time-consuming and complex sample pretreatment procedures. For example, enzyme-linked immunosorbent assay (ELISA) is the most frequently used immunochemical approach to detect pathogens with detection limits ranging from 10^4 to 10^6 colony-forming units (CFU) per ml requiring enrichment usually for 16–24 h (De Boer and Beumer, 1998). Therefore, alternative methods to detect pathogens in contaminated environment with high sensitivity, with a short detection time, and with simplicity may be need. Various immunosensor systems for the detection of *Salmonella* spp. have been reported (Jongerius-Gotemaker et al., 2002; Koubovà et al., 2001; Si et al., 2001). But, SPR immunosensor using self-assembled protein G to improve the sensitivity of immunosensor by controlling the orientation of immobilized antibody molecules for the detection of *Salmonella paratyphi* has not been reported.

The objective in this study is to develop a SPR based immunosensor using self-assembled protein G for detection of *S. paratyphi*. Protein G is introduced to improve the sensitivity of sensor by exposing the binding site of antibody molecule to *S. paratyphi*. Because protein G is a protein lacking cysteine residues, the thiol (–SH) group was substituted at the surface or terminus of protein G using 2-iminothiolane to form the

self-assembled layer of protein G. The surface morphology analysis of the protein G layer on Au surface was performed by atomic force microscope (AFM). The formation of protein G layer on Au surface and the binding of antibody and antigen in series were confirmed by SPR spectroscopy. And, the detection limit of the proposed SPR immunosensor system was investigated.

2. Materials and methods

2.1. Materials

Protein G (MW 22,600 Da) was purchased from Prozyme Inc. (USA). This is a recombinant protein G which is capable of binding the Fc portion of IgG. Each protein G molecule can bind two molecules of IgG. *Salmonella paratyphi* (ATCC 9150), *Escherichia coli* O157:H7 (ATCC 43895) and *Yersinia* spp. (ATCC 700823) was kindly donated from the American Type Culture Collection (USA). *Legionella* spp. (ATCC 33154) was kindly offered from National Institute of Health in Korea. Monoclonal antibody (Mab) against *S. paratyphi* was obtained from Biogenesis Ltd. (USA). Other chemicals including 2-iminothiolane used in this study were obtained commercially as the reagent grade.

2.2. Immobilization of antibody

A BK 7 type cover glass plate (18 mm × 18 mm, Superior, Germany) was used as the solid support. The metal coating and substrate cleaning were performed in the similar way as in the cited reference (Oh et al., 2003). In order to chemically binding of protein G on Au surface by self-assembly technique, the thiol (–SH) group was substituted at the surface or terminus of the protein G using 2-iminothiolane and the procedures are as following (Bush and Winkler, 1989). A 500 nM protein G in degassed 10 mM phosphate buffer, pH 7.4 containing 0.14 mol/L NaCl and 0.02% (w/v) thimerosal (PBS) allowed to react with a 10 M excess of 2-iminothiolane for 30 min at 0 °C under nitrogen gas. Excess 2-iminothiolane was immediately removed by spin column (Vivaspin 500, Vivascience, Germany). Self-assembled protein G layer was prepared by adding a PBS solution containing thiolated protein G to a freshly cleaned Au surface. In order to prepare highly oriented antibody layer, a solution of antibody (50 pmol/mL Mab against *S. paratyphi*) in PBS buffer was applied to the self-assembled protein G layer. After 4 h incubation at 4 °C, the surface was washed with PBS buffer.

In order to provide antigen access to the binding site of antibody by separation of antibody molecules clustered around preferred points on the surface or around other antibody molecules, the antibody layer on the self-assembled protein G layer was incubated for about 20 min with PBS containing 0.1% Tween 20, followed by washing with PBS buffer.

2.3. SPR setup

The bimolecular interactions were monitored using a SPR spectroscope (Multiskop^{TM}, Optrel GbR, Germany) (Harke et al., 1997). The instrumental configuration of the laser light source, polarizer, photo multiplier tube (PMT), and attenuated total reflection (ATR) coupler (Kretschmann, 1971) were the same as in the cited reference (Oh et al., 2003). The resolution of the angle reading of the goniometer was 0.001°.

2.4. Culture condition

Salmonella paratyphi was cultivated in a 250 ml flask with 100 ml of medium (medium composition: pancreatic digest of casein 10 g, NaCl 5 g in 1 L deionized water, pH 7.4 ± 0.2 at 25 °C) at 37 °C with shaking at 200 rpm.

3. Results and discussion

3.1. Preparation of self-assembled protein G layer

Protein G used in this study, which is capable of binding the Fc portion of IgGs, is a monomeric protein lacking cysteine residues and contains the terminus which has quite lysine-rich. Because protein G lacking cysteine could not be chemically bound onto Au surface, it was necessary to substitute the lysine-rich terminus of protein G into any other functional groups such as a thiol (–SH). Just in case that the thiol (–SH) group was substituted at the terminus of the protein

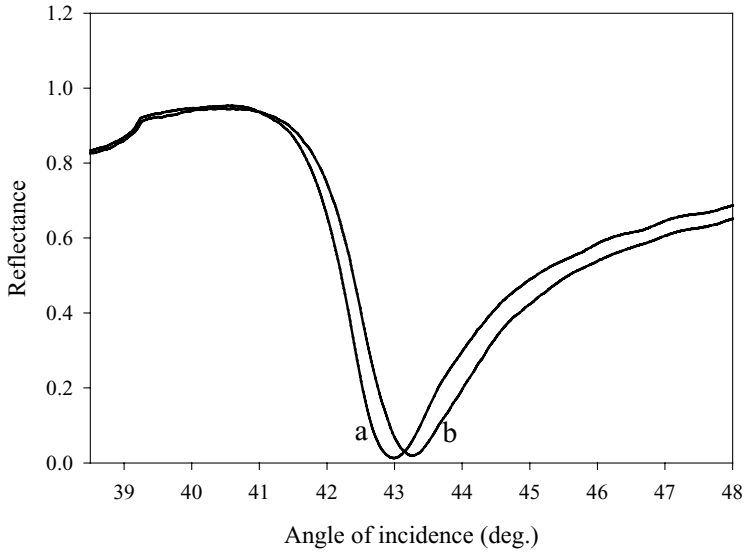

Fig. 1. The variation of SPR curves by adsorbing the thiolated protein G onto Au surface; (a) gold, (b) self-assembled protein G.

G using 2-iminothiolane, protein G layer could stably be fabricated by using self-assembly technique on Au surface.

The change of SPR curves by the adsorption of the thiolated protein G by using 2-iminothiolane on Au surface is shown in Fig. 1. As a result, the SPR angle was shifted significantly from 43.002 ± 0.02 to 43.257 ± 0.015° by the adsorption of the thiolated protein G on Au surface. In principle, a surface plasmon resonance is extremely sensitive to the interfacial architecture. An adsorption process leads to a shift in the plasmon resonance and allows monitoring the mass coverage at the surface with a high accuracy (Fagerstam et al., 1992; Lundstrom, 1994; Matsubara et al., 1988; Salmon et al., 1997). Therefore, the shift in the SPR angle verified that the thiolated protein G molecules were well bound on Au substrate.

AFM images of the self-assembled protein G layer on Au surface in comparison with that of bare gold are shown in Fig. 2. It could be observed that protein G molecules are adsorbed onto the Au substrate as an aggregated pattern in solid-like state with keeping its random cloud-like structure as in bulk solution. From the above results, it can be concluded that the self-assembled protein G layer was fabricated on the Au substrate.

3.2. Antibody immobilization on self-assembled protein G layer

The change of SPR curve by adsorbing antibody (50 nM of Mab against *S. paratyphi*) and its antigens in series on self-assembled protein G layer is represented in Fig. 3. As shown in Fig. 3, the SPR angle was shifted significantly from 43.257 ± 0.015 to 43.512 ± 0.015° by the immobilization of antibody on self-assembled protein G layer and from 43.512 ± 0.015 to 44.142 ± 0.045° by its antigen binding to immobilized antibody layer, respectively, because a shift of SPR angle resulted from the adsorption of dielectric materials on SPR sensor surface. From these results, it could be confirmed that antibody layer was well formed on the self-assembled protein G layer and the binding activation of antibody molecules continued during the immobilization process.

The effect of protein G about the binding interaction between antibody and antigen was previously investigated in comparison with shift degree of SPR angle by binding of antigen to immobilized antibody on Au substrate without/with protein G (Oh et al., 2004). Compared with the shift degree of SPR angle by binding interaction between antibody and antigen, the shift degree of SPR angle in case of immobilized antibody on solid surface using protein G is larger than that of

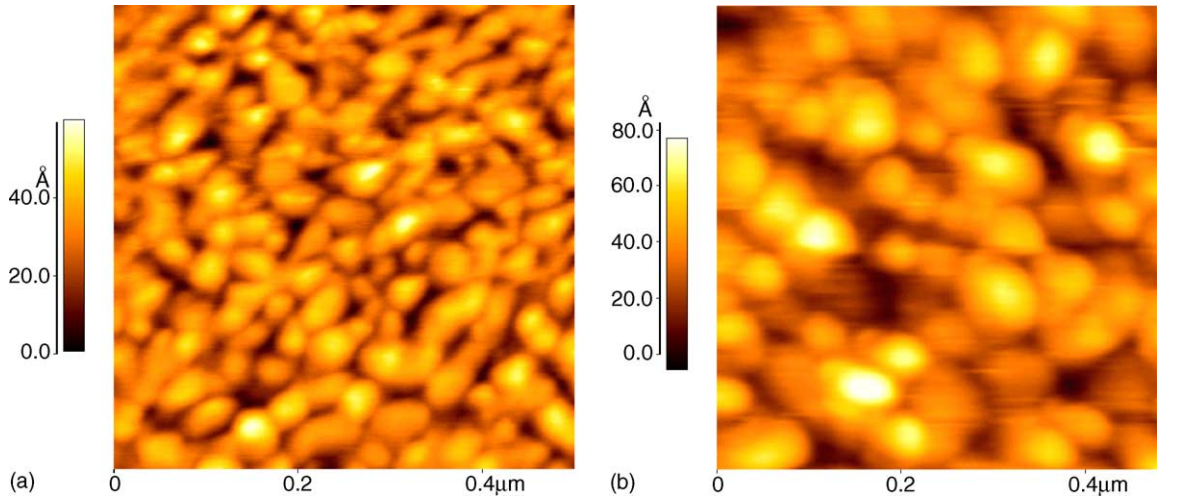

Fig. 2. AFM images of self-assembled protein G on Au surface in comparison with that of bare gold; (a) gold, (b) self-assembled protein G.

SPR angle in case of directly immobilized antibody on solid surface without protein G. It is meant the binding efficiency of antigen to the antibody immobilized on Au surface was improved by using protein G because the binding site of immobilized antibody on solid surface is exposed to the medium of the analytical system, since recombinant protein G used in this study has two domains that can bind to the Fc portion of IgG which is at the junction of CH2 and CH3 domains of the heavy chain.

3.3. Surface plasmon resonance immunosensing for detection of S. paratyphi

The selection of antibody with high specificity is important in developing the SPR immunosensor for

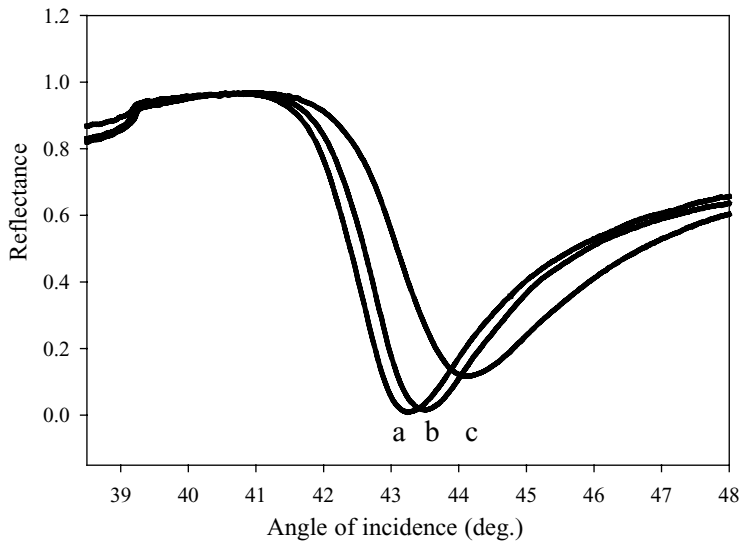

Fig. 3. The variation of SPR curve by adsorbing Mab against *S. paratyphi* and *S. paratyphi* in series on self-assembled protein G layer; (a) self-assembled protein G, (b) Mab against *S. paratyphi*, (c) *S. paratyphi*.

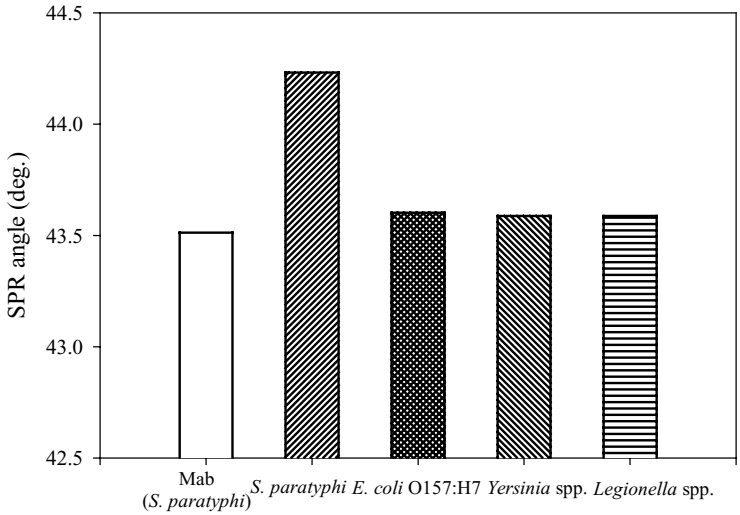

Fig. 4. The changes of SPR angle by non-specific binding between Mab against *S. paratyphi* immobilized on self-assembled protein G and various pathogens.

the detection of *S. paratyphi*, because the specificity for the measurement of analytes in all immunosensor system is dependent on used antibody. In this study, commercial Mab against *S. paratyphi* was used in developing the SPR immnosensor for detection of *S. paratyphi*. In order to investigate the cross-reaction between commercial Mab against *S. paratyphi* and related pathogens existed in contaminated water, indirectly ELISA was performed. As a result, it was observed that Mab against *S. paratyphi* had the high specificity with *S. paratyphi* and did not react with various pathogens in contaminated water (data not shown). As such, the commercially available Mab against *S. paratyphi* was deemed to be appropriate as

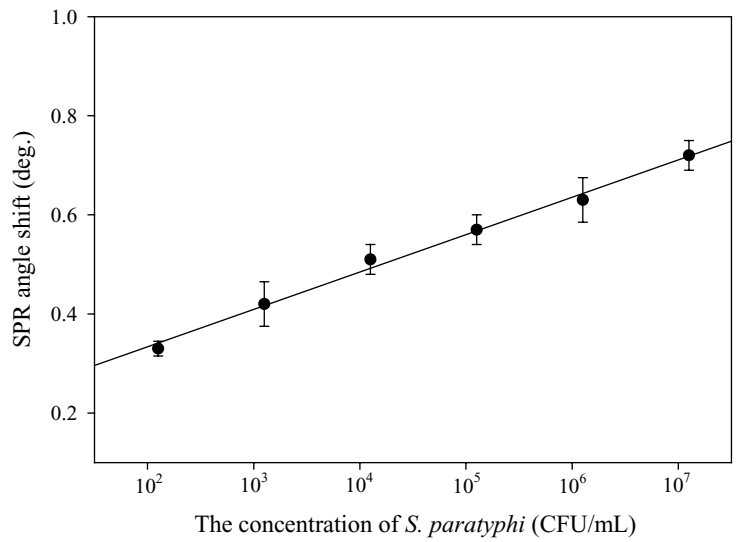

Fig. 5. The changes of the SPR angle shift by binding between Mab against *S. paratyphi* immobilized on self-assembled protein G and various concentrations of *S. paratyphi*.

an antibody for developing an immunosensor for the detection of *S. paratyphi* based on SPR.

The changes of SPR angle by non-specific binding between Mab against *S. paratyphi* on self-assembled protein G layer and various pathogens such as *E. coli* O157:H7, *Yersinia* spp., and *Legionella* spp. is shown in Fig. 4, compared with that of specific binding between Mab against *S. paratyphi* on self-assembled protein G layer and *S. paratyphi*. It was observed that the shift of SPR angle by non-specific binding between Mab against *S. paratyphi* and various pathogens such as *E. coli* O157:H7, *Yersinia* spp., and *Legionella* spp. was much lower than that of SPR angle by specific binding between Mab against *S. paratyphi* and *S. paratyphi*. As a result, it was concluded that SPR surface modified with self-assembled protein G and Mab against *S. paratyphi* had the high specificity with *S. paratyphi* and did not react with various pathogens in contaminated water.

The signal relationship with respect to the pathogen concentration is presented in Fig. 5. As shown in Fig. 5, the shift in the SPR angle was also increased in proportion to the concentration of *S. paratyphi*, thereby presenting a linear relationship between the concentration of *S. paratyphi* and the SPR angle shift. The lowest detection limit for the immunosensor based on SPR was 10^2 CFU/mL, plus the assay was four orders of magnitude more sensitive than a standard ELISA (Kim et al., 1999). Accordingly, it was concluded that an immunosensor based on SPR can be used to monitor *S. paratyphi*. The current fabrication technique of a SPR immunosensor for the detection of *S. paratyphi* could also be applied to construct other immnosensors or protein chips with a high efficiency.

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