Surface Plasmon Resonance Immunosensor for Detection of *Legionella pneumophila*

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Abstract An immunosensor based on surface plasmon resonance (SPR) onto a protein G layer by self-assembly technique was developed for detection of *Legionella pneumophila*. The protein G layer by self-assembly technique was fabricated on a gold (Au) surface by adsorbing the 11-mercaptoundecanoic acid (MUA) and an activation process for the chemical binding of the free amine ($-NH_2$) of protein G and 11-(MUA) using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) in series. The formation of the protein G layer by self-assembly technique on the Au substrate and the binding of the antibody and antigen in series were confirmed by SPR spectroscopy. The surface topographies of the fabricated thin films on an Au substrate were also analyzed by using an atomic force microscope (AFM). Consequently, an immunosensor for the detection of *L. pneumophila* using SPR was developed with a detection limit of up to 10^2 CFU per mL.

Keywords: immunosensor, surface plasmon resonance, protein G, self-assembly technique, *Legionella pneumophila*

INTRODUCTION

Legionella pneumophila was first identified as the causative agent of a 1976 outbreak of pneumonia that affected the participants at an American Legion convention in a Philadelphia hotel. The disease, subsequently named Legionnaires' disease, brought panic to the city of Philadelphia, as 182 cases of infection were reported that resulted in 29 deaths [1]. *L. pneumophila* is conventionally detected by using a complicated procedure involving isolation in a selective medium [2]. However, this method has encountered several problems, including the presence of viable yet non-culturable pathogens, loss of viability after collection, and long time required for culture and confirmation, which takes several days. In addition, the detection of *L. pneumophila* in biocontaminated samples is more difficult, as *L. pneumophila* can be inhibited and masked by the rapid or abundant growth of other microorganisms [3]. Therefore, to avoid the problems with conventional methods, an alternative highly sensitive and simple method with a short detection time is needed for detecting *L. pneumophila*. However, there have been no previous reports on the development of immunosensors for the detection of *L. pneumophila*.

Recently, surface plasmon resonance (SPR) immunosensors have been developed to measure the binding of antigens to antibody molecules immobilized on the SPR sensor surface. These immunosensors are capable of detecting analytes in complex biological media with a high specificity and sensitivity [4-6]. This enhanced sensitivity is required to detect biological materials, as the concentrations of analytes in a biological system are extremely low. The sensitivity of an SPR immunosensor is influenced by the surface property available for binding the antibody. Thus a highly oriented antibody molecular layer on solid surfaces can provide ultimate sensitivity in immunosensing [7].

Several methods including physical and chemical adsorption have been proposed for preparing an oriented antibody molecular layer on a solid matrix surfaces [8-10], and self-assembly techniques have attracted particular attention. Since many of the fundamental and central biological recognition and transduction processes required for immunosensing occur on biological surfaces, particularly within cellular mem-branes [11], it is important to fabricate a biomimicking artificial membrane with the best mechanical resistance and functionality of biological molecules.

For the construction of a well-defined antibody surface protein G, a cell wall protein found in most species of *Streptococci*, is used for proper orientation of antibody. Since protein G exhibits a specific interaction with the Fc portion of Immunoglobulin G (IgG), the paratope of IgG can face the opposite side of the protein G-immobilized solid support [12]. As a result, protein G mediated antibody immobilization can lead to a highly efficient immunoreaction.

Accordingly, the objective of the current study was to develop a SPR immunosensor for the detection of *L. pneumophila* using protein G layer by self-assembly

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technique. To provide the orientation of the antibody molecules on the SPR sensor surface, a protein G layer by self-assembly technique was fabricated on an Au substrate. The formation of the protein G layer by self-assembly technique on the Au substrate, and binding of the antibody and antigen were confirmed by SPR spectroscopy. The surface morphologies of the fabricated thin layers were also analyzed using an atomic force microscope (AFM). As a result, an SPR immunosensor for the detection of *L. pneumophila* was developed using a protein G layer by self-assembly technique.

MATERIALS AND METHODS

Materials

The protein G (M.W. 22,600 daltons), purchased from Prozyme Inc. (USA), was a recombinant protein G, capable of binding the Fc portion of IgG. Each protein G molecule could bind 2 molecules of IgG. The *L. pneumophila* (ATCC 33154) was kindly offered by the Korean National Institute of Health. The monoclonal antibody (Mab) against *L. pneumophila* was obtained from Fitzgerald Industries International, Inc. (USA). All other chemicals used in the current study were obtained commercially as reagent grade.

Immobilization of Mab against *L. pneumophila*

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 for self-assembly were performed in the same way as in the cited reference [6].

The self-assembled monolayer of 11-(MUA) on the Au surface was fabricated by submerging the prepared Au substrate into a glycerol/ethanol (1:1, v/v) solution containing 150 mm of 11-(MUA) for at least 12 h [13]. For chemical binding between the 11-(MUA) adsorbed on the Au substrate and the free amine from the protein G, the carboxyl group in 11-(MUA) was activated by submerging the Au substrate modified with 11-(MUA) into a solution of 10% EDAC in water/ethanol (10/1, v/v) for 2 h at room temperature. Thereafter, the protein G was covalently bound by itself on the activated 11-(MUA) surface at room temperature for 2 h, then the protein G layer by self-assembly technique on the Au substrate was incubated with 0.1% Tween 20 for 20 to 30 min. and washed with a PBS buffer. Before the immobilization of the antibody, the protein G layer by self-assembly technique on the Au substrate was blocked by inactivating the residual carboxyl group in 11-(MUA) with 1 M of ethanolamine for 2 h.

To immobilize the Mab, the protein G layer by self-assembly technique was immersed in a solution containing antibodies (50 pmol/mL Mab against *L. pneumophila*) in a PBS buffer. After 4 h of incubation at 4°C, the surface was rinsed with a PBS buffer and incubated for 20 min with PBS containing 0.1% Tween 20, thereby providing the antigen with access to the binding site of the antibody based on separating the antibody molecules clustered around preferred points on the surface or around other antibody molecules, followed by washing with a PBS buffer [7].

SPR Spectroscopy

The bimolecular interactions were monitored using a SPR spectroscope (Multiskop^{TM}, Optrel GbR, Germany) [14]. The instrumental configuration of the laser light source, polarizer, photo multiplier tube (PMT), and attenuated total reflection (ATR) coupler [15] were the same as in the cited reference [6]. The resolution of the angle reading of the goniometer was 0.001°.

Topological Analysis by AFM

The surface topographies of the 11-(MUA) layer on the Au substrate and protein G layer by self-assembly technique on the Au surface modified with 11-(MUA) were compared with that of Au using an AFM (Autoprobe CP, PSI, USA) in contact mode at room temperature with air conditioning. The images were acquired at a scan rate of 1.5 Hz using a silicon cantilever (Ultralever 06B, PSI, USA).

Culture Condition and Enzyme-linked Immunosorbent Assay (ELISA)

L. pneumophila was cultivated in a 250 mL flask with 100 mL of medium (medium composition: yeast extract 20 g, L-cysteine · HCl 0.4 g and $Fe(NO_3)_3 \cdot 9H_2O$ 0.1 g in 500 mL deionized water, pH 6.85-7.0 at 25°C) at 37°C under 5% CO_2 condition.

A sensitive indirect ELISA with *L. pneumophila* was performed for the investigation of cross reaction between Mab against *L. pneumophila* and various related pathogens as the cited reference [16].

RESULTS AND DISCUSSION

Fabrication of Protein G Layer by Self-assembly Technique on Au Substrate

Fig. 1 shows the SPR spectra of the clean Au substrate, 11-(MUA) attached surface, and protein G layer by self-assembly technique. When the 150 mm of 11-(MUA) was immobilized on the Au substrate, the SPR angle shifted significantly from 43.002°±0.03 to 43.257°±0.04. The SPR angle also shifted from 43.257°±0.04 to 43.437°±0.03 based on chemical binding between the protein G and the activated carboxyl group in 11-(MUA) with EDAC.

In principle, a surface plasmon is a bound electromagnetic wave propagating at the metal-dielectric interface. The attenuated total reflectance configuration by Kretchmann, which is widely used as the design for SPR instruments, relies on the phenomenon of total internal reflection. The external laser field drives the free electron

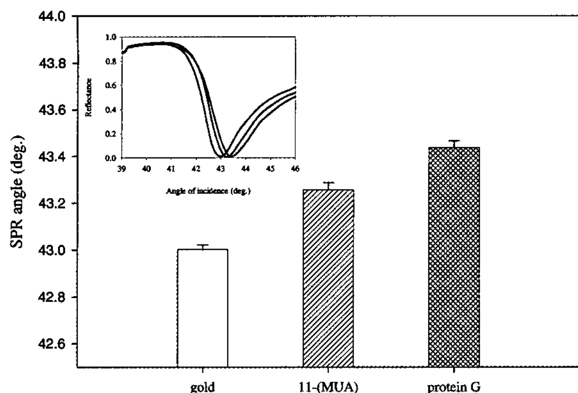

Fig. 1. Changes in SPR angle due to adsorption of 11-(MUA) and binding of protein G onto Au substrate in series.

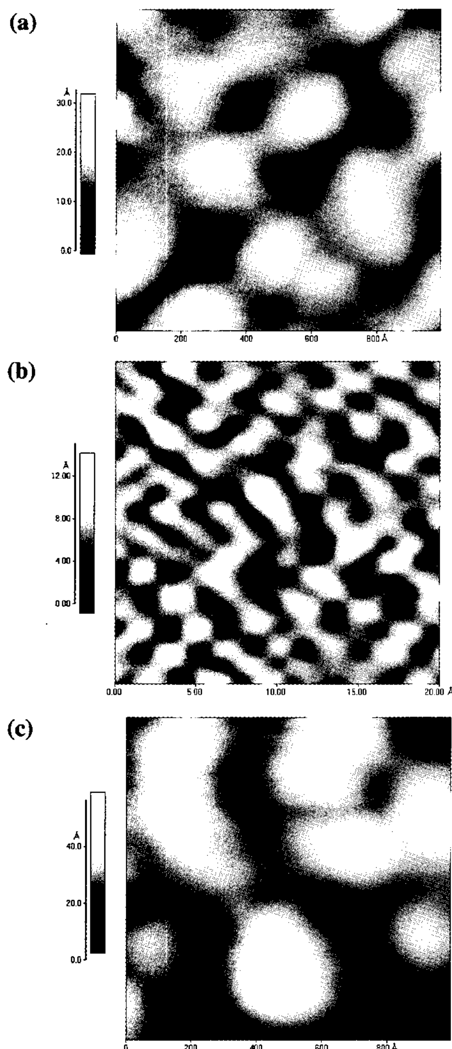

Fig. 2. AFM images of 11-(MUA) layer on Au and protein G layer by self-assembly technique on 11-(MUA) layer in comparison with that of bare Au; (a) bare Au (scan size 0.1 μm ×0.1 μm), (b) 11-(MUA) (scan size 2 nm×2 nm), (c) protein G layer by self-assembly technique (scan size 0.1 μm×0.1 μm).

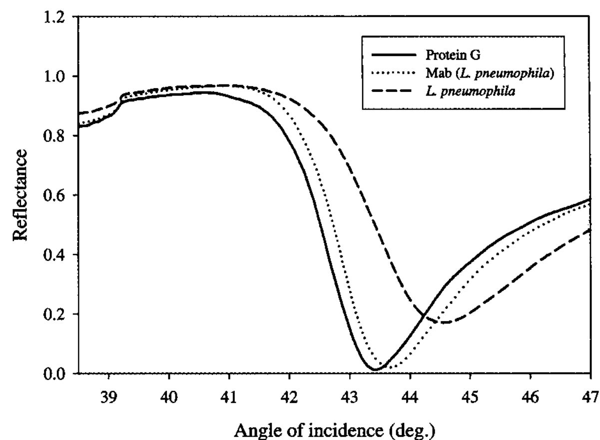

Fig. 3. Changes in SPR curve relative to binding of antibody and formation of Mab against *L. pneumophila* – *L. pneumophila* complex on protein G layer by self-assembly technique in series.

gas of metal in a distinct mode. The spatial charge distribution creates an electric field which is localized at the metal-dielectric interface. So, the plasmon resonance is extremely sensitive to the interfacial architecture. Consequently, an adsorption process leads to a shift of SPR minimum in a SPR curve and it allows monitoring the mass coverage at the surface with a high accuracy [15 17-19].

As such, in the current study, the shift of the SPR angle verified that a thin layer of 11-(MUA) had been formed on the Au surface and that the protein G molecules were well bound with the 11-(MUA) adsorbed on the Au substrate.

Fig. 2 shows AFM images of the 11-MUA layer on the Au substrate and protein G layer by self-assembly technique on the 11-MUA layer in comparison with bare gold.

Since 11-MUA has a long alkyl chain that can provide a van der waals attractive force among the molecules, it can form a strong and close-packed 2 D molecular array [20]. In the current study, although a blurred image was obtained, a fairly well organized molecular array was detected on an nm scale. Also, the protein G molecules were observed as adsorbed onto the Au substrate modified with 11-(MUA) in an aggregated pattern in a solid-like state, while retaining its random cloud-like structure as in the bulk solution. Therefore, these results confirmed the fabrication of a protein G layer by self-assembly technique on the Au substrate.

Fabrication of Mab Layer against *L. pneumophila* on Protein G Layer by Self-assembly Technique

Fig. 3 shows the SPR spectra resulting from the adsorption of the Mab against *L. pneumophila* (50 pmol/mL Mab against *L. pneumophila*) on the protein G layer by self-assembly technique, based on the formation of an immobilized Mab against *L. pneumophila* - *L. pneumophila* complex. The SPR angle shifted significantly from

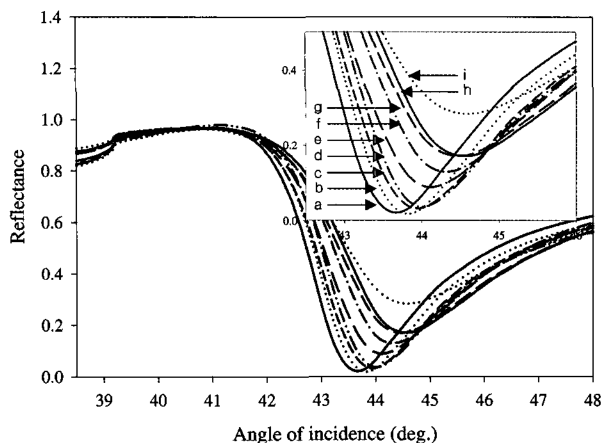

Fig. 4. Changes in SPR curve relative to various concentrations of *L. pneumophila* binding to immobilized Mab against *L. pneumophila* on protein G layer by self-assembly technique (Lines; a: Mab against *L. pneumophila*, b: 10^2 CFU/mL, c: 10^3 CFU/mL, d: 10^4 CFU/mL, e: 10^5 CFU/mL, f: 10^6 CFU/mL, g: 10^7 CFU/mL, h: 10^8 CFU/mL, i: 10^9 CFU/mL).

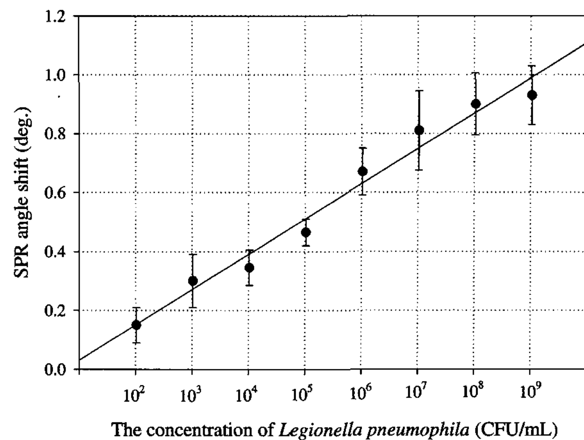

Fig. 5. Changes in SPR minimum position shift relative to binding of various concentrations of *L. pneumophila*.

43.437°±0.03 to 43.662°±0.045 when the Mab against *L. pneumophila* was bound to the protein G layer by self-assembly technique, while the SPR minimum position shifted from 43.662°±0.045 to 44.562°±0.105 with the formation of the immobilized Mab against *L. pneumophila* - *L. pneumophila* complex, as the shift in the SPR minimum position resulted from the adsorption of dielectric materials on the SPR sensor surface.

Accordingly, it was confirmed that the Mab against *L. pneumophila* layer was well formed on the protein G layer by self-assembly technique and the binding activation of the Mab against *L. pneumophila* continued during the immobilization process. In addition, it would appear that the Fab fragments of the IgG molecules were posed in opposition to the SPR surface, since the recombinant protein G used in the current study had 2 domains that could bind to the Fc portion of IgG at the junction of the CH_2 and CH_3 domains in the heavy chain.

Detection of *L. pneumophila* Using Surface Plasmon Resonance

The selection of an antibody with a high specificity is important when developing an immunosensor based on SPR for the detection of *L. pneumophila*, because in all immunosensor system the specificity for measuring the analytes is dependent on the used antibody. The commercial Mab used in the current study does not exhibit any cross reactivity with other pathogens, such as *E. coli* O157:H7, *Salmonella* spp, *Yersinia* spp., *Shigella* spp., and *Vibrio* spp. [16]. As such, the commercially available Mab against *L. pneumophila* was deemed to be appropriate as an antibody for developing an immunosensor for the detection of *L. pneumophila* based on SPR.

The shift in the SPR angle position due to the binding between the immobilized Mab against *L. pneumophila* and various concentrations of *L. pneumophila* is shown in Fig. 4, while the signal relationship with respect to the pathogen concentration is presented in Fig. 5.

As shown in Fig. 4, the minimal point of the SPR spectrum with a high concentration of *L. pneumophila* exhibited a larger shift than that with a low concentration of *L. pneumophila*. As shown in Fig. 5, the shift in the SPR angle was also increased in proportion to the concentration of *L. pneumophila*, thereby presenting a linear relationship between the concentration of *L. pneumophila* 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 [21]. Accordingly, it was concluded that an immunosensor based on SPR can be used to monitor *L. pneumophila* in wastewater. The current fabrication technique of a SPR immunosensor for the detection of *L. pneumophila* could also be applied to construct other immnosensors or protein chips with a high efficiency.

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