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Nano-scale Probe Fabrication Using Self-assembly Technique and Application to Detection of *Escherichia coli* O157:H7

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Abstract A self-assembled monolayer of protein G was fabricated to develop an immunosensor based on surface plasmon resonance (SPR), thereby improving the performance of the antibody-based biosensor through immobilizing the antibody molecules (IgG). As such, 11-mercaptoundecanoic acid (11-MUA) was adsorbed on a gold (Au) support, while the non-reactive hydrophilic surface was changed through substituting the carboxylic acid group (-COOH) in the 11-MUA molecule using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrocholide (EDAC). The formation of the self-assembled protein G layer on the Au substrate and binding of the antibody and antigen were investigated using SPR spectroscopy, while the surface topographies of the fabricated thin films were analyzed using atomic force microscopy (AFM). A fabricated monoclonal antibody (Mab) layer was applied for detecting *E. coli* O157:H7. As a result, a linear relationship was achieved between the pathogen concentration and the SPR angle shift, plus the detection limit was enhanced up to 10^2 CFU/mL.

Keywords: self-assembled monolayer, protein G, surface plasmon resonance, immunosensor, *E. coli* O157:H7

INTRODUCTION

Within the context of studying protein functions, the miniaturization of analytical procedures can play an important role in facilitating the rapid detection of target materials, understanding complex biological functions, and novel drug discovery [1]. Bioassays based on a monoclonal antibody (Mab) offer unlimited applicability and specificity, which can be applied to the quantification of an antigen [2]. As such, the conceptual combination of point-of-care testing and a minituarized bioassay based on a Mab will inevitably result in a decreased sensing area and sensitivity.

Although the performance and sensitivity of traditional assay methods, such as an enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR), continue to be developed [3], the potential of single pathogen detection using a PCR-based method has been limited due to the need for relatively expensive equipment and associated specialist skills to perform the analyses. Consequently, ELISAs are frequently used for detecting a pathogen. However, in the case of *Escherichia coli* O157:H7, ELISA-based methods have low sensitivities of about 10^6 bacteria per mL and require much time from the initial sample preparation to the final analysis [4]. This low sensitivity results from the immobilization technique where the antibodies are usually bound on surfaces no less than one hundred millimeters square. As such, to avoid the problems encountered with these analysis methods, an alternative simple detection method is needed with a high sensitivity and short detection time.

With the increased requirements for sensitive assays and antigen sizes, the configuration control of immobilized antibodies has become more important. Over the past few decades, the major trends in antibody-based diagnostics have been related to advances in assay specificity detection technologies and sensitivity. Assay sensitivity and reproducibility are influenced by the surface property available for binding an antibody, where a highly oriented antibody molecular layer on a solid support surface can attain ultimate selectivity as well as sensitivity in immumosensing. As such, several methods, including physical and chemical adsorption, have already been proposed for preparing an oriented antibody molecular layer on solid matrix surfaces [5-7].

Recently, self-assembly (SA) techniques have also attracted increasing attention as a method of creating an antibody molecular layer on a solid surface. Since many fundamental and central biological recognition and transduction processes required for immunosensing occur at biological surfaces, particularly within cellular membranes [8], it is important to fabricate a biomimicking artificial membrane with the best mechanical resistance and functionality of biological molecules.

Molecular self-assembly is a nano-fabrication strategy for generating monolayers of biological molecules on various substrates [9]. Such monolayers are extremely versatile and can facilitate the *in vitro* development of

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biosurfaces that are able to mimic naturally occurring molecular recognition processes. In addition, self-assembled monolayers (SAMs) fabricated on a nano-scale also permit reliable control of the packing density and immobilized environment with a recognition center or multiple centers at the substrate surface [10].

For the construction of a well-defined antibody surface, protein G, a cell wall protein found in most species of *Streptococci*, is used as the binding material. Since protein G has a specific interaction with the F_c portion of Immunoglobulin G (IgG) [11], 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.

There are several advantages to IgG deposition onto a protein G sublayer when compared with direct deposition onto a solid substrate. In the case of direct deposition, the F_{ab} fragments of IgG molecules can sometimes be physically adsorbed on the Au surface, thereby drastically decreasing both the sensitivity and the selectivity of the immunosensor. In contrast, the ability to control the IgG orientation increased the sensitivity of the proposed immunosensor [11]. Furthermore, the protein G sublayer protected the IgG molecules from the effects of the substrate, which can denature the protein molecules and control the direction of the IgG molecules, and explains why protein G was able to immobilize the antibody molecules on the SPR surface in a highly oriented manner.

In the current study, to detect the pathogen *E. coli* O157:H7, surface plasmon resonance (SPR) spectroscopy is introduced for a bimolecular interaction analysis. SPR spectroscopy is a highly sensitive method for monitoring the properties of biomolecules immobilized on a metal surface, and since it can also perform a real-time *in situ* analysis of a dynamic surface, it is capable of defining the adsorption and desorption rates for a range of surface interactions [12,13]. Therefore, an immunosensor based on SPR spectroscopy enables analytes to be detected with a high specificity and short detection time in complex biological media [13].

In parallel with SPR spectroscopy, the surface topographies of the fabricated molecular films are also investigated using atomic force microscopy (AFM). Similar to scanning tunneling microscopy (STM), the high resolution of AFM has already been shown to reach molecular levels (nano-scale), demonstrating its ability to study a wide range of biological specimens, such as whole cells, proteins, and nucleic acids [14]. Accordingly, the above methods were combined to develop an SPR-based immunosensor with a nano-fabricated protein G layer for detecting *E. coli* O157:H7.

MATERIALS AND METHODS

Materials

E. coli O157:H7 (ATCC 43895), a major food-borne pathogen in humans with an increasing incidence that is causing worldwide concern, was chosen as the target pathogen [15-17]. The protein G (M.W. 22,600 Daltons), purchased from Prozyme Inc. (USA), was a recombinant protein G capable of binding the F_c portion of IgG. The Mab against *E. coli* O157:H7 was obtained from Fitzgerald Industries International, Inc. (USA). A 500 nM protein G in 10 mM phosphate buffer saline containing NaCl 0.138 M; KCl 0.0027 M (PBS, pH 7.4) was used to immobilize the protein G. All other chemicals used in the current study were of reagent grade and obtained commercially.

Immobilization of Mab against *E. coli* O157:H7

A BK 7 type cover glass plate (18 mm × 18 mm, Superior, Germany) was used as the solid support. The method used to coat the metals and substrate cleaning for self-assembly were all performed in the same way as in the cited reference [18].

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 11-(MUA) for at least 12 h [19]. To chemically bind the 11-(MUA) adsorbed on the Au and the free amine of the protein G, the carboxyl group of the 11-(MUA) was activated by submerging the Au substrate modified with 11-(MUA) into a solution of 10% 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) in water/ethanol (10/1, v/v) for 2 h. at room temperature. The protein G was then left to covalently bond on the activated 11-MUA surface at room temperature for 2 h. Thereafter, the self-assembled protein G layer on the Au substrate was incubated with 0.1% Tween 20 for 20 to 30 min and washed with a PBS buffer Before immobilizing the antibody, the self-assembled protein G layer on the Au substrate was blocked by inactivating the residual carboxyl group of the 11-(MUA) with 1 M of ethanolamine.

To immobilize the Mab, the self-assembled protein G layer was immersed in a solution containing antibodies (50 pmol/mL Mab against *E. coli* O157:H7) in a PBS buffer. After 4 h. of incubation at 4°C, the surface was rinsed with the PBS buffer and incubated for 20 min with PBS containing 0.1% Tween 20 to provide the antigen access to the binding sites of the antibody through separating the antibody molecules clustered around preferred points on the surface or around other antibody molecules followed by washing with the PBS buffer.

SPR Spectroscopy

The bimolecular interactions were monitored using an SPR spectroscope (Multiskop™, Optrel GbR, Germany) [20]. The instrumental configurations for the laser light source, polarizer, photo multiplier tube (PMT), and attenuated total reflection (ATR) coupler [21] were the same as in the cited reference [18]. When the incidence angle of the laser light was varied from 38° to 50°, all the samples were monitored at room temperature. The resolution of the angle reading of the goniometer was 0.001°.

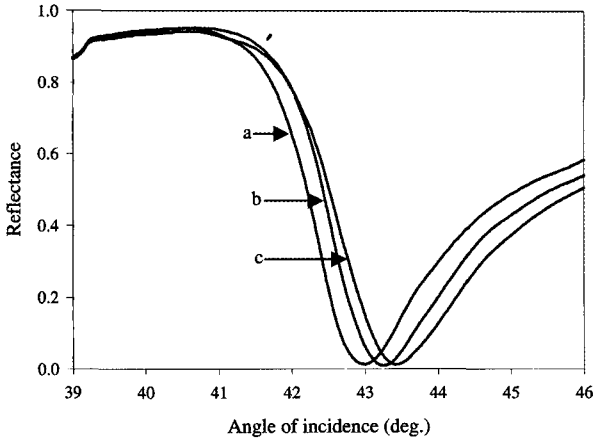

Fig. 1. Changes in SPR curve after adsorbing 11-(MUA) and binding protein G onto Au substrate, respectively. (Lines; a : bare gold, b : 150 mM 11-(MUA), c : protein G).

Topological Analysis Using AFM

The surface topography of the self-assembled protein G layer on the Au substrate, Mab immobilized against *E. coli* O157:H7 on the self-assembled protein G layer, and *E. coli* O157:H7 bound to Mab immobilized against *E. coli* O157:H7 was investigated using an AFM (Autoprobe CP, PSI, USA) in contact mode at room temperature under air conditioning. The images were acquired at a scan rate of 1.5 Hz using a silicon cantilever (Ultralever 06B, PSI, USA).

RESULTS AND DISCUSSION

Fabrication of Self-assembled Protein G Layer on Au Substrate

Fig. 1 shows the SPR spectra of the clean Au substrate, 11-MUA, and self-assembled protein G layer. When 150 mM 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 shifted again from 43.257°±0.04 to 43.437°±0.03 after the chemical binding of protein G with the activated carboxyl group of 11-(MUA) using EDAC. In principle, a surface plasmon is a bound electromagnetic wave propagated at a metal-dielectric interface, and an external laser field will drive the free electron gas of a metal in a distinct mode. The spatial charge distribution then creates an electric field localized at the metal-dielectric interface. As such, the plasmon resonance is extremely sensitive to the interfacial architecture. An adsorption process leads to a shift in the plasmon resonance, thereby allowing the mass coverage at the surface to be monitored with a high accuracy [22]. Consequently, the shift in 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.

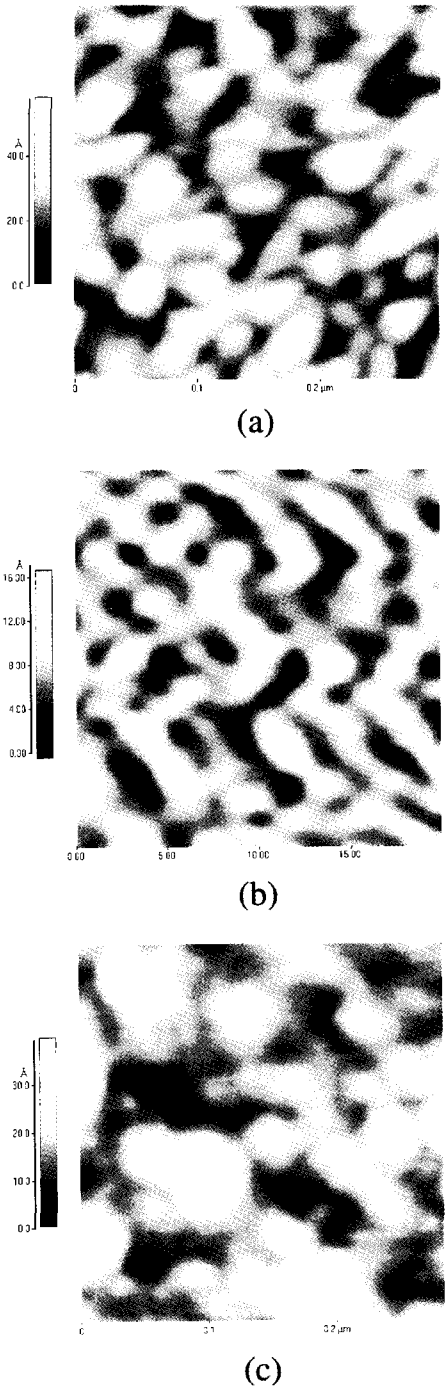

Fig. 2. AFM images of 11-(MUA) layer on Au, self-assembled protein G layer on 11-(MUA) layer, and bare Au; (a) bare Au (scan size 0.3 μm × 0.3 μm), (b) 11-(MUA) (scan size 2 nm × 2 nm), (c) self-assembled protein G layer (scan size 0.3 μm × 0.3 μm).

Fig. 2 shows AFM images of the 11-MUA layer on the Au substrate and self-assembled protein G layer on the 11-MUA layer in comparison to an AFM image of bare gold.

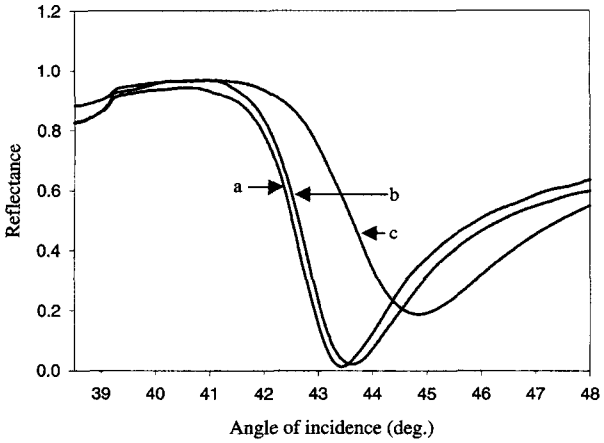

Fig. 3. Changes in SPR curve after binding of antibody and formation of Mab against *E.coli* O157:H7 – *E.coli* O157:H7 complex on self-assembled protein G layer, respectively. (Lines; a : self-assembled protein G layer, b : Mab against *E.coli* O157:H7, c : *E.coli* O157:H7).

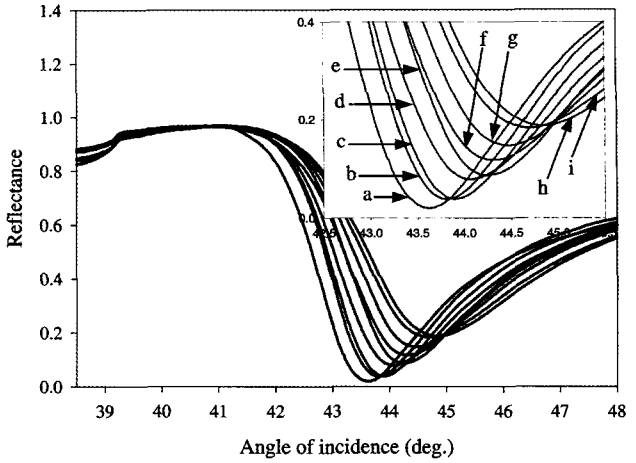

Fig. 4. Changes in SPR curve with various concentration *E. coli* O157 : H7 bound to Mab immobilized against *E. coli* O157:7 on self-assembled protein G layer ((Lines; a : Mab against *E.coli* O157:H7, b: 10^2 CFU/mL, c : 10^3 CFU/mL, d : 10^4 CFU/mL, e : 10^5 CFU/mL, f : 10^6 CFU/mL, g : 10^7 CFU/mL, h : 10^8 CFU/mL, I : 10^9 CFU/mL).

Since 11-MUA has a long alkyl chain, which can provide a van der waal attractive force among the molecules, a strong and close-packed 2 D molecular array can be formed [23]. Despite a blurred image, a fairly well organized molecular array was observed on an nm scale. In addition, the adsorption of the protein G molecules onto the Au substrate modified with 11-(MUA) exhibited an aggregated pattern in a solid-like state, in contrast to the random cloud-like structure in the bulk solution, indicating the fabrication of a self-assembled protein G layer on the Au substrate.

Fabrication of Mab Layer against *E. coli* O157:H7 on Self-assembled Protein G Film

Fig. 3 shows the SPR spectra when an Mab against *E. coli* O157:H7 (50 pmol/mL Mab against *E. coli* O157 : H7) was adsorbed on the self-assembled protein G layer through the formation of an Mab immobilized against *E. coli* O157:H7 - *E. coli* O157:H7 complex. The SPR angle shifted significantly from 43.437°±0.03 to 43.607°± 0.03 with the binding of the Mab against *E. coli* O157:H7 to the self-assembled protein G layer, while the SPR minimum position shifted from 43.607°±0.03 to 44.832°±0.157 with the formation of the Mab immobilized against *E. coli* O157:H7 - *E. coli* O157:H7 complex, due to the adsorption of the dielectric materials on the SPR sensor surface.

Accordingly, the effective formation of the Mab against *E. coli* O157:H7 layer on the self-assembled protein G layer was confirmed, along with the continued binding activation of the Mab against *E. coli* O157:H7 during the immobilization process. It would also appear that the F_{ab} fragments of the IgG molecules were positioned in opposition to the SPR surface, since the recombinant protein G used in the current study had 2 domains that could bind to the F_c portion of IgG, located at the junction of the CH2 and CH3 domains in the heavy chain.

Detection of *E. coli* O157:H7 Using Surface Plasmon Resonance

The selection of an antibody with a high specificity was critical in developing an immunosensor based on SPR for the detection of *E. coli* O157:H7, as regards the specificity of the analyte measurements. The commercial Mab used in the current study did not have any cross reactivity with other pathogens, such as *Salmonella* spp. *Yersinia* spp., *Shigella* spp., and *Vibrio* spp. [18], making it an appropriate antibody for an immunosensor for the detection of *E. coli* O157:H7 based on SPR.

The shifts in the SPR angle position after binding the Mab immobilized against *E. coli* O157:H7 with various concentrations of *E. coli* O157:H7 are shown in Fig. 4, while Fig. 5 shows the signal relationship relative to the pathogen concentration.

As shown in Fig. 4, the minimal point of the SPR spectrum exhibited a larger shift with a high concentration of *E. coli* O157:H7 than with a low concentration. As shown in Fig. 5, the shift in the SPR angle also increased in proportion to the concentration of *E. coli* O157:H7 and a linear relationship was exhibited between the concentration of *E. coli* O157:H7 and the SPR angle shift. The lowest detection limit of the immunosensor based on SPR was 10^2 CFU/mL and the assay was four orders of magnitude more sensitive than a standard ELISA. Therefore, it was concluded that an immunosensor based on SPR could be used to monitor *E. coli* O157:H7 in wastewater. Plus, the fabrication technique used in the current study to create an SPR immunosensor for the detection of *E. coli* O157:H7 could also be effectively applied to construct other immnosensors or protein chips.

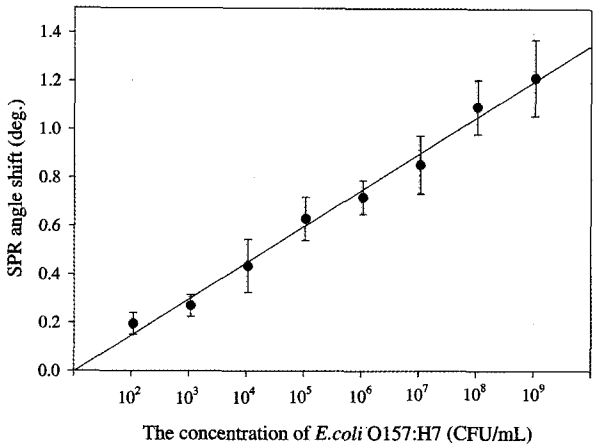

Fig. 5. Changes in SPR minimum position shift with various concentrations of *E.coli* O157:H7.

Accordingly, the current results indicate that the fabrication of analytical devices based on nanotechnology are likely have a significant impact on all aspects of diagnostic testing beyond traditional immunoassay technology.

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