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Detection of *Escherichia coli* O157:H7 Using Immunosensor Based on Surface Plasmon Resonance

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Abstract An immunosensor based on surface plasmon resonance (SPR) with a self-assembled protein G layer was developed for the detection of *Escherichia coli* O157:H7. A self-assembled protein G layer on a gold (Au) surface was fabricated by adsorbing the mixture of 11-mercaptoundecanoic acid (MUA) and hexanethiol at various molar ratios and by activating chemical binding between free amine (-NH_2) of protein G and 11-(MUA) using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) in series. The formation of a self-assembled protein G layer on an Au substrate and the binding of the antibody and antigen in series were confirmed by SPR spectroscopy. The surface morphology analyses of the self-assembled protein G layer on the Au substrate, monoclonal antibody (Mab) against *E. coli* O157:H7 which was immobilized on protein G, and bound *E. coli* O157:H7 extracts on immobilized Mab against *E. coli* O157:H7 were performed by atomic force microscopy (AFM). The detection limit of the SPR-based immunosensor for *E. coli* O157:H7 was found to be about 10^4 cells/ml.

Key words: *Escherichia coli* O157:H7, immunosensor, protein G, self-assembled layer, surface plasmon resonance

Escherichia coli O157:H7 is a major food-borne pathogen in humans and is an increasing world-wide concern, since the number of incidences continues to rise [7, 9, 15]. The major impact of diarrhea induced by the bacterial lies on children under the age of 10 and elderly people living in less-developed countries, in which bacterial diarrhea diseases remain a significant public-health problem.

The methods for the detection of *E. coli* O157:H7 based on enzyme-linked immunosorbent assays (ELISA) and polymerase chain reaction (PCR) have been developed and their performance and sensitivity are continuously improving [6, 11]. However, although the PCR based method has the potential to detect a single cell, this method has not been fully accepted, because of the need for relatively expensive equipment and associated specialist skills to perform the analyses. For these reasons, the ELISA method has been used frequently for the detection of *E. coli* O157:H7. However, the ELISA based methods have typically low sensitivities of detecting about 10^6 bacteria per ml and require much time from sample preparation to the final analysis [8]. In order to avoid the problems encountered with these methods, an alternative method with high sensitivity, with a short detection time and simplicity, is needed to detect *E. coli* O157:H7.

Recently, surface plasmon resonance (SPR) based immunosensors have been developed for the measurement of antigens which bound to antibody molecules immobilized on the SPR sensor surface, and they are capable of detecting analytes in complex biological media with high specificity and sensitivity, a short detection time, and simplicity [3, 12, 14]. However, few papers on the immunosensors for detection of *E. coli* O157:H7 have been reported.

The enhanced sensitivity of SPR immunosensors is required to detect biological materials, because analytes in biological systems are in very low concentration. However, the sensitivity of the SPR immunosensor can be increased by controlling the orientation of antibodies immobilized on the sensor surface. When antibodies are immobilized on a solid surface, their activity is usually less than that of dispersed antibodies. The main reason of the reduced activity is due to the randomly oriented array of the antibody molecules on the solid surface. Therefore, development of a highly oriented layer of immobilized antibodies has strongly been suggested [2]. Nevertheless, no attempt has been reported to enhance sensitivity of the SPR immunosensor by controlling 2 dimensional (2D) configuration of immobilized antibody molecules on the sensor surface.

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The objective of this study was to develop a SPR-based immunosensor for detection of *E. coli* O157:H7. In order to endow antibody molecules with orientation on the SPR sensor surface, a self-assembled protein G layer on an Au substrate was fabricated. Protein G, which is a cell wall protein found in most *Streptococci* species, is the most extensively studied of antibody-binding proteins, since it specifically interacts with the F_c portion of immunoglobulin G (IgG) [1]. To minimize steric hindrance by the size of antigen in the binding between antibody and antigen, the 2D configuration of immobilized antibody molecules on solid surface was controlled by varying the molar ratio of 11-(MUA) to hexanethiol. The formation of the self-assembled protein G layer on the Au substrate and the binding of antibody to antigen in series were confirmed by SPR spectroscopy. Morphological analyses of the surface of the self-assembled protein G layer on the Au substrate, monoclonal antibody (Mab) against *E. coli* O157:H7 immobilized on the self-assembled protein G layer, and *E. coli* O157:H7 extracts bound on immobilized Mab against *E. coli* O157:H7 were performed by atomic force microscopy (AFM). Using the above methods, an SPR immunosensor for detection of *E. coli* O157:H7 using a self-assembled protein G layer was developed.

MATERIALS AND METHODS

Materials

Protein G (M.W. 22,600 Da) was purchased from Prozyme Inc. (U.S.A.). This is a recombinant protein G which is capable of binding the F_c portion of IgG. *E. coli* O157:H7 (ATCC 43895), *Salmonella* spp. (ATCC 9150), and *Yersinia* spp. (ATCC 700823) were kindly donated by the American Type Culture Collection (U.S.A.). *Shigella* spp. (KCTC 2517) and *Vibrio* spp. (KCTC 2715) were kindly donated by the Korean Collection for Type Cultures (Korea). Mab against *E. coli* O157:H7 was obtained from Fitzgerald Industries International, Inc. (U.S.A.). Other chemicals of reagent grade used in this study were obtained commercially.

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

BK 7 glass plate (18 mm×18 mm, Superior, Germany) was used as the solid support, and Au was sputtered on the BK 7 glass surface. Before sputtering Au, chromium (Cr) was sputtered on the glass slide to promote adhesion of Au. The Au and Cr films had a thickness of 50 nm±1 nm and 2 nm, respectively. The Au surface was cleaned with pirahna solution (30 vol% H_2O_2 and 70 vol% H_2SO_4) at 60°C for 5 min, and then rinsed with ethanol and deionized water.

A thin layer of 11-(MUA) on the Au surface was prepared by submerging the Au substrate into glycerol/ethanol (1:1, v/v) solution containing 150 mM 11-(MUA) for at least 12 h [16]. For chemical binding between 11-(MUA) adsorbed on Au and free amine of protein G, the carboxyl group of 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 self-assembled protein G layer was fabricated by incubating the activated Au substrate in 10 mM phosphate buffer (PBS, pH 7.4) containing 10 mg/l protein G, 0.14 M/l NaCl, and 0.02% (w/v) thimerosal (PBS) at room temperature for 2 h. In addition, the self-assembled protein G layer on the Au substrate was incubated with 0.1% Tween 20 for 20 to 30 min and then washed with PBS buffer. Before immobilization of the antibody, the self-assembled protein G layer on the Au substrate was blocked by inactivating the residual carboxyl group of 11-(MUA) with 1 M ethanolamine.

For immobilization of the antibody, a solution containing antibodies (50 pmol/ml Mab against *E. coli* O157:H7) in PBS buffer was applied to the self-assembled protein G layer. After 2 h of incubation, the surface was washed with PBS buffer and incubated for 20 min with PBS buffer containing 0.1% Tween 20 in order to provide antigen accessibly to the binding site of the antibody by separating the antibody molecules clustered on the surface or around other antibody molecules. Finally, it was washed with PBS buffer.

To minimize the steric hindrance by antigen size in the binding characteristic between antibody and antigen, the 2D configuration of immobilized antibody molecules on the solid surface was controlled by the variation of molar ratio of 11-(MUA) and hexanethiol in the range of 1:0 to 1:5.

SPR Spectroscopy

The schematic diagram of SPR spectroscopy (Multiskop™, Optrel GbR, Germany) is shown in Fig. 1. A He-Ne laser

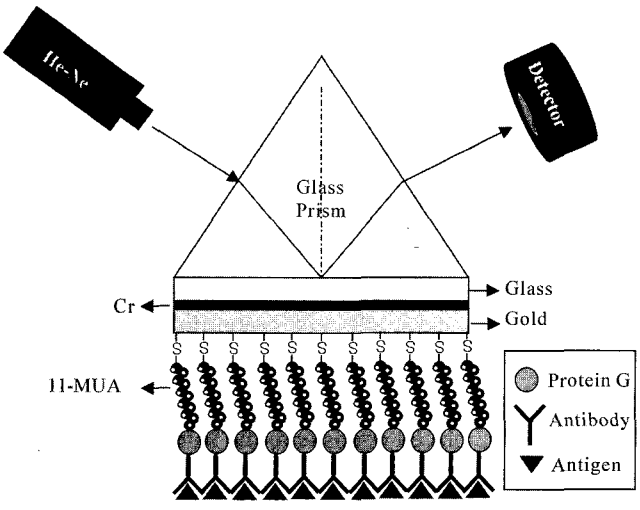

Fig. 1. Schematic diagram of SPR spectroscopy.

was used as a light source to make a monochromatic light with a wavelength of 632.8 nm. The p-polarized light beam by the polarizer was used as a reference and the intensity of the reflected beam was measured by photo multiplier tube (PMT) sensor. A 90° glass prism (BK 7, n=1.5168) was used as a Kretschmann ATR coupler [5]. The plane face of the 90° glass prism was coupled to a BK 7 glass slide via index matching fluid. The resolution of the angle reading of the goniometer was 0.001°.

Topological Analysis by AFM

Surface topography of the self-assembled protein G layer on Au substrate, the immobilized Mab against *E. coli* O157:H7 on self-assembled protein G layer, and *E. coli* O157:H7 extracts bound on the immobilized Mab against *E. coli* O157:H7 were investigated with AFM (Autoprobe CP, PSI, U.S.A.) in contact mode at room temperature under air conditioning. Images were acquired at a scan rate of 1.5 Hz with a silicon cantilever (Ultralever 06B, PSI, U.S.A.).

Enzyme-Linked Immunosorbent Assay (ELISA)

E. coli O157:H7 was cultivated in a 250-ml flask containing 100 ml of Luria Bertani (LB) medium at 37°C under aerobic conditions. Wells of a microtiter plate were coated with cell extracts (100 µl/well) of *E. coli* O157:H7 ($OD_{600\ nm}$=1) and then dried overnight in a 40°C oven. The wells were washed three times with PBS buffer containing 0.05% Tween 20 (PBS-T; pH 7.4), treated with 300 µl/well 5% bovine serum albumin (BSA)-PBS at 37°C for 1 h for blocking, and then washed with PBS-T. Serial dilutions of Mab against *E. coli* O157:H7 in PBS-T (1:1,000 to 1:10,000) were prepared and Mab dilutions (50 µl/well) were added to wells. After incubation at room temperature for 1 h, they were then washed three times with PBS-T. Diluted secondary antibody horseradish peroxidase (HRP) conjugate was added to each well. The mixture was incubated at room temperature for 1 h and then washed five times with PBS-T. Two hundred ml of substrate solution (0.4 mg/ml of tetramethylbenzidine) was added. The reaction was stopped by the addition of 2 N sulfuric acid, and the optical density at 495 nm was measured in a microplate reader [4].

Results and Discussion

Fabrication of Self-Assembled Protein G Layer on Au Substrate

Ideally, F_{ab} fragments of antibody molecules (especially, IgG) should be placed opposite to the solid surface for the SPR immunosensor to have high sensitivity. However, the probability that F_{ab} fragments of IgG molecules are posed in opposition to the SPR surface is realistically low. For this reason, protein G was used for immobilization of antibody molecules on the SPR surface in a highly oriented manner.

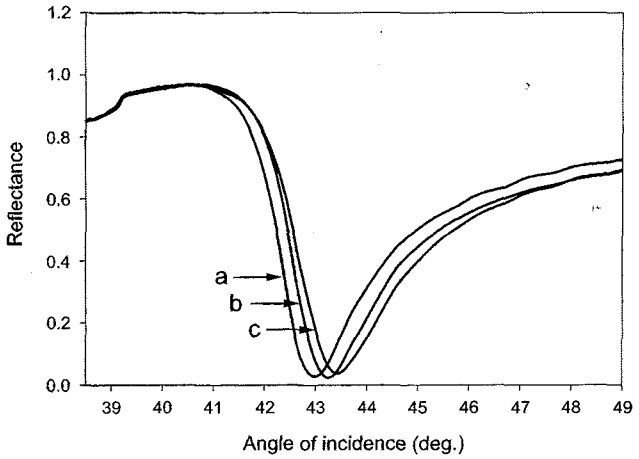

Fig. 2. The changes of the SPR curve by adsorbing 11-(MUA) and by binding protein G onto the Au substrate in series.
Lines; a: bare gold, b: 150 mM 11-(MUA), c: protein G.

The changes of the SPR curve due to adsorption of 150 mM 11-(MUA) and chemical binding of protein G in series on the Au substrate are shown in Fig. 2.

The SPR minimum position was shifted significantly from 43.002°±0.03 to 43.257°±0.04 by the adsorption of 150 mM 11-(MUA) on the Au surface. Also, the SPR minimum position was shifted from 43.257°±0.04 to 43.437°±0.03 by chemical binding between protein G and the activated carboxyl group of 11-(MUA) with EDAC. In principle, a surface plasmon is a bound electromagnetic wave propagating at the metal-dielectric interface. The external laser field drives the free electron gas of metal in a distinct mode. The spatial charge distribution creates an electric field which is localized at the metal-dielectric interface. Hence, the plasmon resonance is extremely sensitive to the interfacial architecture. An adsorption process leads to a shift in the plasmon resonance and allows monitoring of the mass coverage at the surface with a high accuracy [13]. Therefore, the shift in the SPR minimum position verified that a thin layer of 11-(MUA) on the Au surface was formed and protein G molecules were well bound with 11-(MUA) adsorbed on the Au substrate.

AFM images of the 11-(MUA) layer on the Au substrate and the self-assembled protein G layer on the 11-(MUA) layer in comparison with that of bare gold are shown in Fig. 3.

Since 11-(MUA) has a long alkyl chain, which can provide van der Waals attractive forces among the molecules, it can form a strong and close-packed 2D molecular array [10]. Although a blurred image was obtained, a fairly well organized molecular array could be observed in nm scale. Also, it was observed that protein G molecules were adsorbed onto the 11-(MUA) modified Au substrate as an

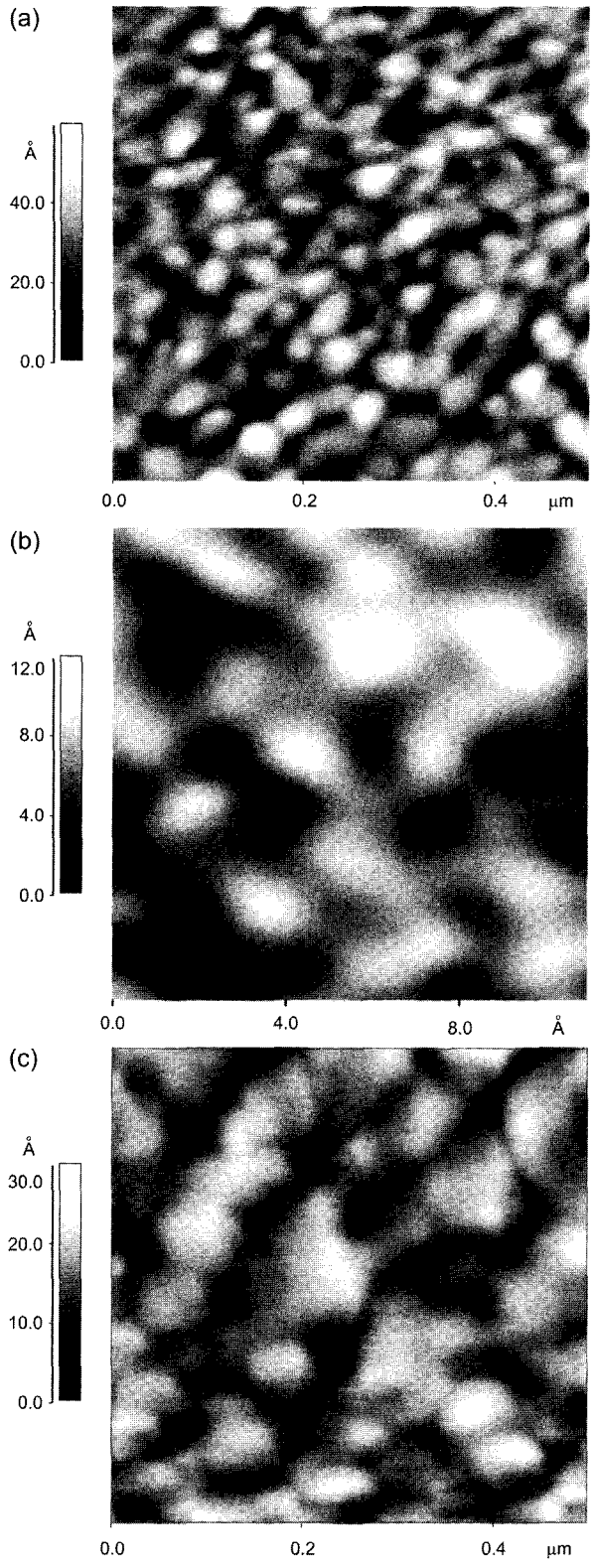

Fig. 3. AFM images of 11-(MUA) layer on Au and the self-assembled protein G layer on 11-(MUA) layer in comparison with that of bare Au.
(a) Bare Au (scan size 0.5 μm×0.5 μm), (b) 11-(MUA) (scan size 1 nm× 1 nm), (c) self-assembled protein G layer (scan size 0.5 μm×0.5 μm).

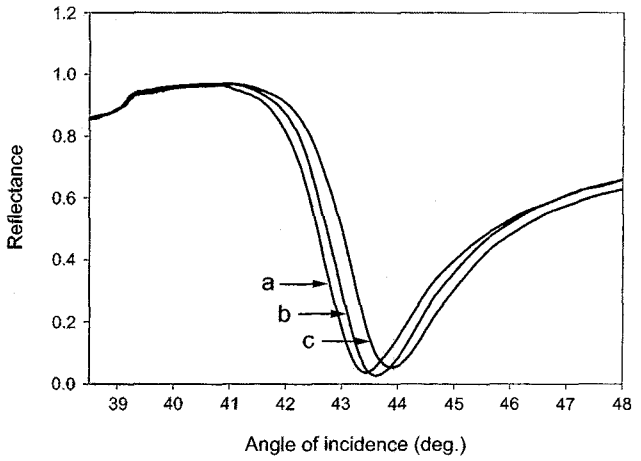

Fig. 4. Changes of the SPR curve by binding of Mab against *E. coli* O157:H7 and by formation of Mab against *E. coli* O157:H7-*E. coli* O157:H7 complex onto the self-assembled protein G layer in series.
Lines; a: self-assembled protein G layer, b: Mab against *E. coli* O157:H7, c: *E. coli* O157:H7 extract.

aggregated pattern in solid-like state, keeping its random cloud-like structure as in bulk solution. The above results confirmed that the self-assembled protein G layer was fabricated on the Au substrate.

Immobilization of Mab Against *E. coli* O157:H7 on Self-Assembled Protein G Layer

The changes of the SPR curve by adsorbing Mab against *E. coli* O157:H7 (50 pmol/ml Mab against *E. coli* O157:H7) on the self-assembled protein G layer and by forming of immobilized Mab against *E. coli* O157:H7 - *E. coli* O157:H7 extracts complex are shown in Fig. 4.

As shown in Fig. 4, the SPR minimum position was shifted significantly from 43.437°±0.03 to 43.607°±0.03 by the binding of the Mab against *E. coli* O157:H7 on the self-assembled protein G layer, and the SPR minimum position was shifted from 43.607°±0.03 to 43.907°±0.05 by a complex formed between the immobilized Mab against *E. coli* O157:H7 and *E. coli* O157:H7 extracts, since a shift of the SPR minimum position resulted from the adsorption of dielectric materials on the SPR sensor surface.

AFM images of the immobilized Mab against *E. coli* O157:H7 molecules and *E. coli* O157:H7 extracts bound on the Mab against *E. coli* O157:H7 molecules layer are shown in Fig. 5.

It was also observed that the Mab against *E. coli* O157:H7 was adsorbed onto the self-assembled protein G layer as an aggregated pattern in solid-like state and *E. coli* O157:H7 extracts were bound to the immobilized Mab against *E. coli* O157:H7 in a similar pattern. The aggregated pattern size of *E. coli* O157:H7 extracts was smaller than that of the Mab against *E. coli* O157:H7 molecules.

From these results, it could be confirmed that the Mab against *E. coli* O157:H7 layer was well formed on the self-

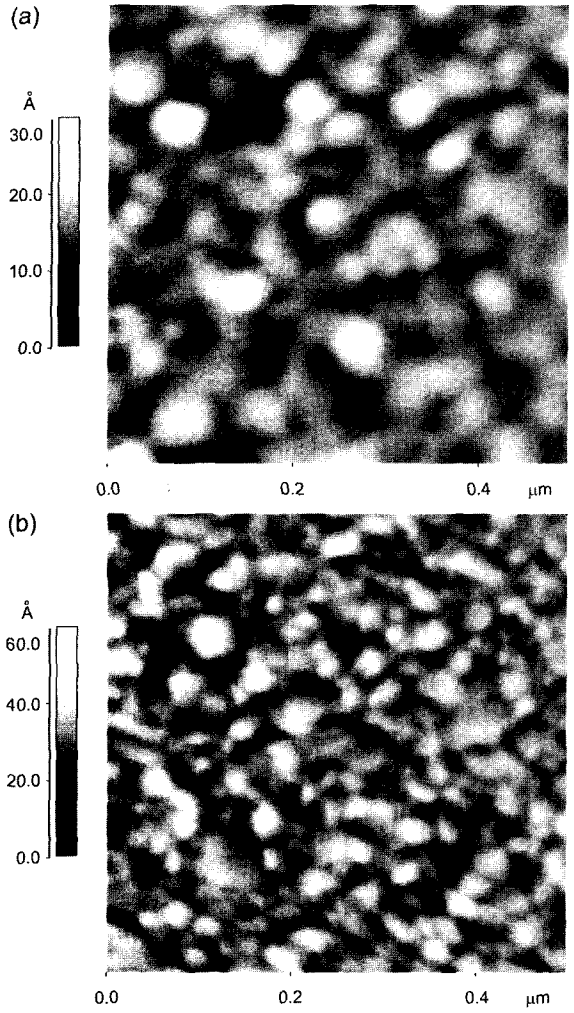

Fig. 5. AFM images (scan size 0.5 μm×0.5 μm) of immobilized Mab against *E. coli* O157:H7 molecules on the self-assembled protein G layer and *E. coli* O157:H7 extract bound on the immobilized Mab against *E. coli* O157:H7 molecules.
(a) Mab against *E. coli* O157:H7, (b) *E. coli* O157:H7 extracts.

assembled protein G layer and that the binding activation of the Mab against *E. coli* O157:H7 continued during the immobilization process. Also, it was considered that F_{ab} fragments of IgG molecules were posed in opposition to the SPR surface, since recombinant protein G used in this study has 2 domains that can bind to the F_c portion of IgG, which is at the junction of the CH2 and CH3 domains of the heavy chain.

Control of 2D Configuration of Immobilized Mab against O157:H7 Molecules

The changes of the SPR minimum position shift by binding between the immobilized Mab against *E.coli* O157:H7 on the self-assembled protein G layer with various molar ratios of 11-(MUA), hexanethiol, and *E.coli* O157:H7 extracts are shown in Fig. 6.

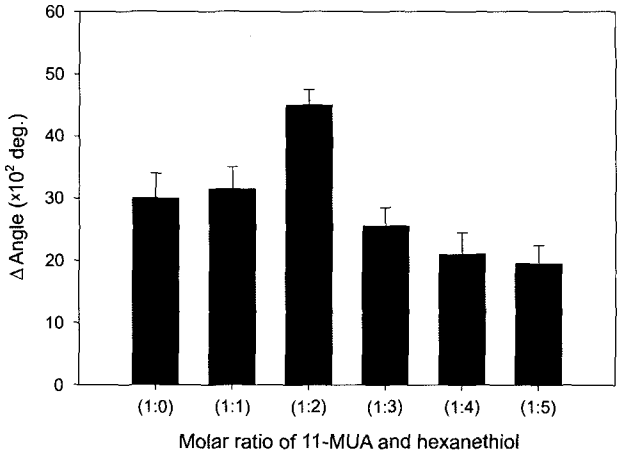

Fig. 6. Changes of the SPR minimum position shift by binding between immobilized Mab against *E. coli* O157:H7 on the self-assembled protein G layer with various molar ratios of 11-(MUA), hexanethiol, and *E. coli* O157:H7 extracts.

The optimal molar ratio of 11-(MUA) to hexanethiol for the formation of Mab against *E. coli* O157:H7 - *E. coli* O157:H7 extracts complex was 1 to 2 [50 mM 11-(MUA) to 100 mM hexanethiol]. Ideally, binding the antibody in exposing the paratope is favored by dense packing of antibody molecules on the SPR surface. Dense packing by itself, however, is not sufficient for optimal antibody–antigen complex formation. Antigens need lateral access to the antibody paratope for unimpaired binding, and a larger antigen needs more space between antibodies to access the paratope than a smaller antigen. Thus, the effect of the steric hindrance by antigen size in the binding characteristics

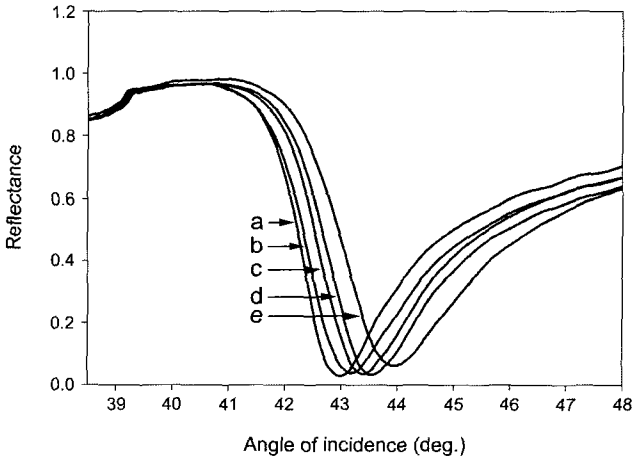

Fig. 7. Changes of the SPR curve by adsorbing the mixture of 11-(MUA) and hexanethiol (molar ration 1:2), protein G, the Mab against *E. coli* O157:H7 and *E. coli* O157:H7 extracts in series.
Lines; a: bare gold, b: the mixture of 11-(MUA) and hexanethiol, c: self-assembled protein G, d: Mab against *E. coli* O157:H7, e: *E. coli* O157:H7 extracts.

between antibody and antigen must be minimized to develop an SPR immunosensor with high efficiency.

In optimal conditions (molar ratio of 1:2), the changes of the SPR curve by adsorbing the mixture of 11-(MUA) and hexanethiol, protein G, the Mab against *E. coli* O157:H7, and *E. coli* O157:H7 extracts in series are shown in Fig. 7.

As a result, the SPR minimum position was shifted from 43.002°±0.03 to 43.187°±0.02 by adsorbing a mixture of 11-(MUA) and hexanethiol (molar ratio 1:2) on the Au surface. The SPR minimum position was shifted from 43.187°±0.02 to 43.382°±0.03 by chemical binding between protein G and the activated carboxyl group of 11-(MUA) with EDAC. The SPR minimum position was shifted from 43.382°±0.03 to 43.517°±0.03 by the binding of the Mab against *E. coli* O157:H7 on the self-assembled protein G layer, and it was shifted from 43.517°±0.03 to 43.967°± 0.02 by the formation of immobilized Mab against *E. coli* O157:H7-*E. coli* O157:H7 extracts complex.

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

The selection of an antibody with high specificity is important in developing the SPR based immunosensor for the detection of *E. coli* O157:H7, because the specificity for the measurement of analytes in all immunosensor systems is dependent on the antibody used. In the present study, commercial Mab against *E. coli* O157:H7 was used in developing the immnosensor for the detection of *E. coli* O157:H7. The Mab against *E. coli* O157:H7 has specificity to lipopolysaccharide (LPS) in *E. coli* O157:H7. For the investigation of the cross-reaction between commercial Mab against *E. coli* O157:H7 and related pathogens existing in contaminated water, indirect ELISA was performed (Fig. 8).

The result in Fig. 8 shows that the selected Mab against *E. coli* O157:H7 had high specificity toward *E. coli*

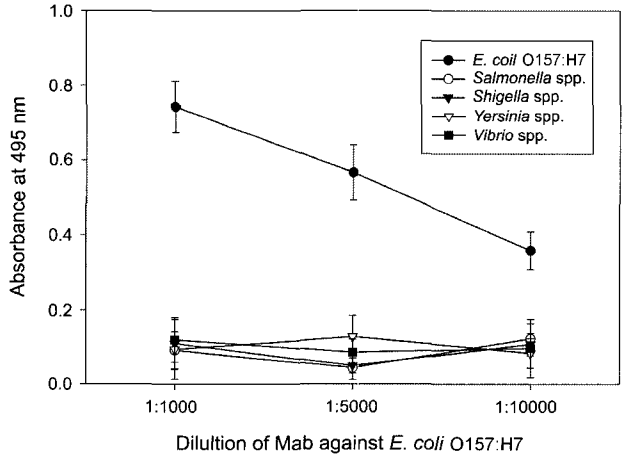

Fig. 8. The result of indirect ELISA for the Mab against *E. coli* O157:H7 with various pathogens.

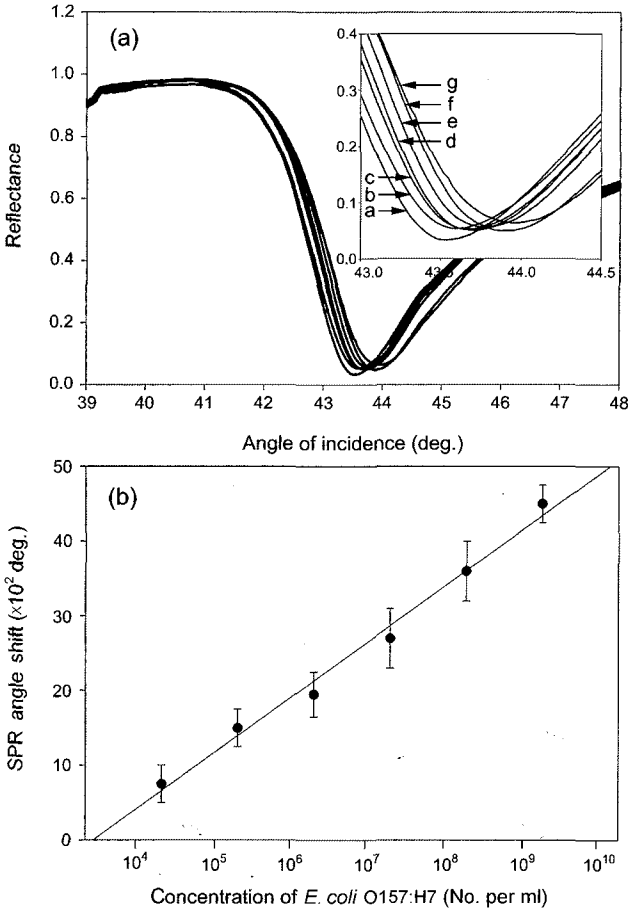

Fig. 9. The SPR response by binding between the immobilized Mab against *E. coli* O157:H7 and *E. coli* O157:H7 extracts.
(a) The changes of the SPR curve by binding *E. coli* extracts (Lines; a: Mab, b: 10^4 cells/ml, c: 10^5 cells/ml, d: 10^6 cells/ml, e: 10^7 cells/ml, f: 10^8 cells/ml, g: 10^9 cells/ml), (b) changes of the SPR minimum position shift by binding of various concentrations of *E. coli* O157:H7 extracts.

O157:H7 and did not react with various pathogens present in the water contaminated with *Salmonella* spp., *Yersinia* spp., *Shigella* spp., and *Vibrio* spp. These results indicated that the commercial Mab against *E. coli* O157:H7 used in this study was adequate as an antibody for developing an SPR based immunosensor for the detection of *E. coli* O157:H7.

The change of the SPR curve and the degree of the SPR minimum position shift by binding between the immobilized Mab against *E. coli* O157:H7 and various concentrations of *E. coli* O157:H7 (LPS) are shown in Figs. 9(a), 9(b), respectively.

As shown in Fig. 9(a), the minimum position of the SPR curve in a high concentration of *E. coli* O157:H7 (LPS) was shifted more than that of a low concentration of *E. coli* O157:H7 (LPS). As shown in Fig. 9(b), the degree of shift of the SPR minimum position also increased linearly, as the concentration of cells increased. The lowest detection limit of the SPR based immunosensor was 10^4 cells/ml and

the assay was two orders of magnitude more sensitive than a standard ELISA. Based on these results, it could be concluded that an immunosensor based on SPR can be used to monitor *E. coli* O157:H7 in wastewater. The fabrication technique of the SPR immunosensor used in this study for the detection of *E. coli* O157:H7 can be applied to construct other immunosensors or protein chips with high efficiency.

Acknowledgments

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