J. Microbiol. Biotechnol. (2006), **16**(12), 1968–1976

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

© The Korean Society for Microbiology and Biotechnology

Enhancement of Sensitivity in Interferometric Biosensing by Using a New Biolinker and Prebinding Antibody

PARK, JAE-SOOK, SUNG-HYUK LIM, SANG JUN SIM, HEEYEOP CHAE, HYUN C. YOON[1], SANG SIK YANG[2], AND BYUNG-WOO KIM*

Department of Chemical Engineering, Sungkyunkwan University, Suwon 440-746, Korea
[1]Department of Biotechnology, Ajou University, Suwon 443-749, Korea
[2]School of Electrical and Computer Engineering, Ajou University, Suwon 443-749, Korea

Received: June 28, 2006
Accepted: September 12, 2006

Abstract Recombinant *E. coli* ACV 1003 *(recA::lacZ)* was used to measure low concentrations of DNA-damaging chemicals, which produce β-galactosidase via an SOS regulon system. Very low β-galactosidase activities of less than 0.01 unit/ml, β-galactosidase produced through an SOS response corresponding to the 10 ng/ml (ppb) of DNA damaging chemicals in the environment, can be rapidly determined by using an alternative interferometric biosensor with optically flat thin films of porous silicon rather than by the conventional time-consuming Miller's enzyme assay as well as the ELISA method. In order to enhance the sensitivity in the interferometry, it needs to obtain more uniform distribution and higher biolinking efficiency, whereas interferometric sensing is rapid, cheap, and advantageous in high throughput by using a multiple-well-type chip. In this study, pore size adjusted to 60 nm for the target enzyme β-galactosidase to be bound on both walls of a Si pore and a calyx crown derivative was apllied as a more efficient biolinker. Furthermore, anti-β-galactosidase was previously functionalized with the biolinker for the target β-galactosidase to be specifically bound. When anti-β-galactosidase was bound to the calyx-crown derivative-linked surface, the effective optical thickness was found to be three times as high as that obtained without using anti-β-galactosidase. The resolution obtained was very similar to that afforded by the time-consuming ELISA method; however, the reproducibility was still unsatisfactory, below 1 unit β-galactosidase/ml, owing to the microscopic non-uniform distribution of the pores in the etched silicon surface.

Key words: Si-based biosensor, Fabry-Ferot Fringe, functionalization, β-galactosidase, DNA damaging chemicals

*Corresponding author
Phone: 82-31-290-7246; Fax: 82-31-290-7272;
E-mail: bwkim@skku.ac.kr

An interferometry biosensing system with the sensitivity enhanced in this study was applied to detect low level of β-galactosidase, which was produced by the SOS response of recombinant *E. coli* (*SOS::lacZ* fusion strains) [1, 2] when exposed to a DNA-damaging endocrine disruptor.

The SOS gene product β-galactosidase, however, is difficult to assay, because the assay is time-consuming and the sample pretreatment is tedious. In the previous study, extacellular β-galactosidase activity without using extracting solvents was 5% of that obtained by the conventional Miller's enzyme assay [3] using solvent. Such a low enzyme activity can be readily determined not by the conventional enzyme assay, but by an alternative method, which uses an inexpensive and easily available, optically flat, thin film of porous silicon to detect short oligonucleotides and proteins in a highly sensitive manner.

This method, which deviates the need for the waveguide and microfluidic cell in the surface plasmon resonance method, was used in this study in order to overcome these problems and the disadvantages associated with the conventional enzyme assay or the ELISA method; *i.e.*, time-consuming experimental assay procedures, the consumption of costly biochemicals, and tedious cell collection. This method is based on estimations of changes in effective optical thickness due to the formation of a complete protein monolayer on a functionalized porous silicon surface.

In order to establish the interferometric biosensing system using a porous Si, the following major steps should be done: 1) etching Si with a uniform pore size appropriate to the target biomolecules; 2) surface modification to avoid the signal drift due to oxidation of SH group on the freshly etched Si surface, and surface functionalization to provide an efficient binding of the target biomolecules; and 3)

specific binding of target molecule to the functionalized surface.

Firstly, the optimal etching condition was obtained by investigating the key parameters of HF concentration, etching time, and current density.

Immobilization methods such as physical adsorption, covalent linkage, and affinity capture have been used [4–7]. Physical adsorption has lower linkage efficiency with poor reproducibility, and the covalent method has lower immobilization with loss of bioactivity resulting in non-uniform signal noise. An alternative affinity capture method makes target proteins to be randomly bound with protein denaturation as well as steric hindrance [8].

Another immobilization method using a calyx crown derivative (ProLinker A) [9], instead of the common biotinylation method adopted in the previous study [10], has been shown to achieve higher immobilization efficiencies, without any activity loss or incorrect orientation for the capture protein, a phenomenon that occurs in the affinity interaction method. In this study, in order to remove the nonspecific binding effect induced by other proteins by using the specific Ab-Ag reaction, anti-β-galactosidase was bound to the ProLinker A-silane coupled surface, whereas the previous study did not applied the Ab-Ag reaction.

MATERIALS AND METHODS

Cell Culture and Enzyme Assay

The bacterial strain used in this study was ACV 1003, which was transformed using the plasmids pRecALac1 and pUvrALac2 into the isogenic strain RFM443 [11]. The following genes are among those that express the SOS response: *umuC* and *umuD* (induced mutagenesis), *sulA* (filamentous growth), *uvrA* and *uvrB* (excision repair), *himA* (site-specific recombination), and several of *din* (damage-inducible). All of these genes are members of a regulatory network controlled by the products of the *recA* and *lexA* loci, which are self-inducible. The *lexA* protein functions as a repressor of all of the *din* genes. Damage to cellular DNA or interference with its replication initiates a series of events that lead to activation of protease activity of *recA* protein. The *lexA* protein is then proteolytically cleaved by the *recA* protease, and as the pools of *lexA* protein decrease, various *din* genes begin to be expressed at higher levels. Following DNA damage from exposure to DNA-damaging chemicals, the *SOS::lacZ* fusion strain produces β-galactosidase.

Other strains such as GW1010, 1030, and 1040 based on GW1000 [12], made by using the Mu *d*(Ap *lac*) bacteriophage to generate *lac* operon fusions to various *din* genes [13], were also used. However, ACV 1003 was the most effective in terms of its response to the DNA-damaging chemicals.

Luria-Bertani medium was used for liquid and plate cultures. Cells were cultured to an optical density of 0.2, and tributyl tin was then added. Samples were prepared for conventional β-galactosidase assay as described by Miller [3] and the previous study [10].

In order to compare the resolution of our developed interferometric method with that afforded by the ELISA technique, the following procedures were performed [14, 15]. β-Galactosidase (G-5635, Sigma Aldrich GmbH) solution (100 μl) at à given concentration, obtained by diluting with 0.05 M carbonate-bicarbonate buffer at pH 9.6, was added to each well in an ELISA plate (Greiner Bio-One Co, CA, U.S.A.) and incubated at 37°C for 90 min. After washing three times with 0.15 M phosphate buffer solution (PBS) containing 100 μl of 0.1% (v/v) Tween-20, anti-β-galactosidase (B-0271, Sigma Aldrich GmbH; 50 μg/ml in 0.04 M PBS-HCl containing 0.1% bovine serum albumin, BSA) was added to the wells and incubated at 37°C for 90 min. After additional washing (as mentioned above), the well surface was bound at 37°C for 90 min with another antibody, namely horseradish peroxidase (HRP)-conjugated anti-mouse IgG, which was diluted in 0.04 M PBS-HCl containing 0.1% BSA. After washing three times, color was developed by adding 100 μl of 0.3 μg *o*-phenylenediamine (Sigma, U.S.A.)/ml in a 0.15 M citrate-phosphate buffer (pH 5.0) containing 0.01% H_2O_2. The amount of bound HRP-conjugated IgG was estimated by the addition of the color solution. Each well was then incubated at 37°C for 15–30 min. After this incubation, 100 μl of stopping solution (0.5 M H_2SO_4) was added to each well. The amount of bound enzyme was determined at 490 nm using a microplate reader (E-MAX, Molecular Devices Co., CA, U.S.A.). The conventional assay [3] of enzyme expressed by the reporter gene and the ELISA method require more than six hours analysis time, which needs expensive biochemicals and a much larger cell harvest in order to meet the detection limit of the UV-visible spectrometer. However, the described interferometry system requires less than one hour, small amounts of biochemicals, and only a few cells.

Si-Wafer Etching and Analysis of the Wavelength Shift

Si wafers [B-doped, orientation (100)] were used to make porous Si by anodic etching in ethanolic HF solution (HF:EtOH=1:1, v/v). Prior to the etching, the Si wafers were rinsed with EtOH and dried under a stream of nitrogen gas. Si wafers with an exposed area 3.1 cm^2 were contacted on the back side with a thin copper plate (as an anode) and mount in a Teflon etching cell. A platinum mesh served as the counter cathode to provide a homogeneous electrical field by using a DC power supply. The Si layer thickness was adjusted by the illumination of a 60 W UV lamp with a peak emission at 254 nm.

Interferometric reflectance spectra of porous Si were recorded by using a spectrometer (S2000, Ocean Optics, Inc., Dunedin, Florida, U.S.A.) fitted with a fiber optic probe including six illumination fibers and one reader fiber, so that the wavelength shifts in the Fabry-Perot fringes were analyzed, and then the corresponding β-galactosidase concentrations were determined by the calculation of effective optical thickness.

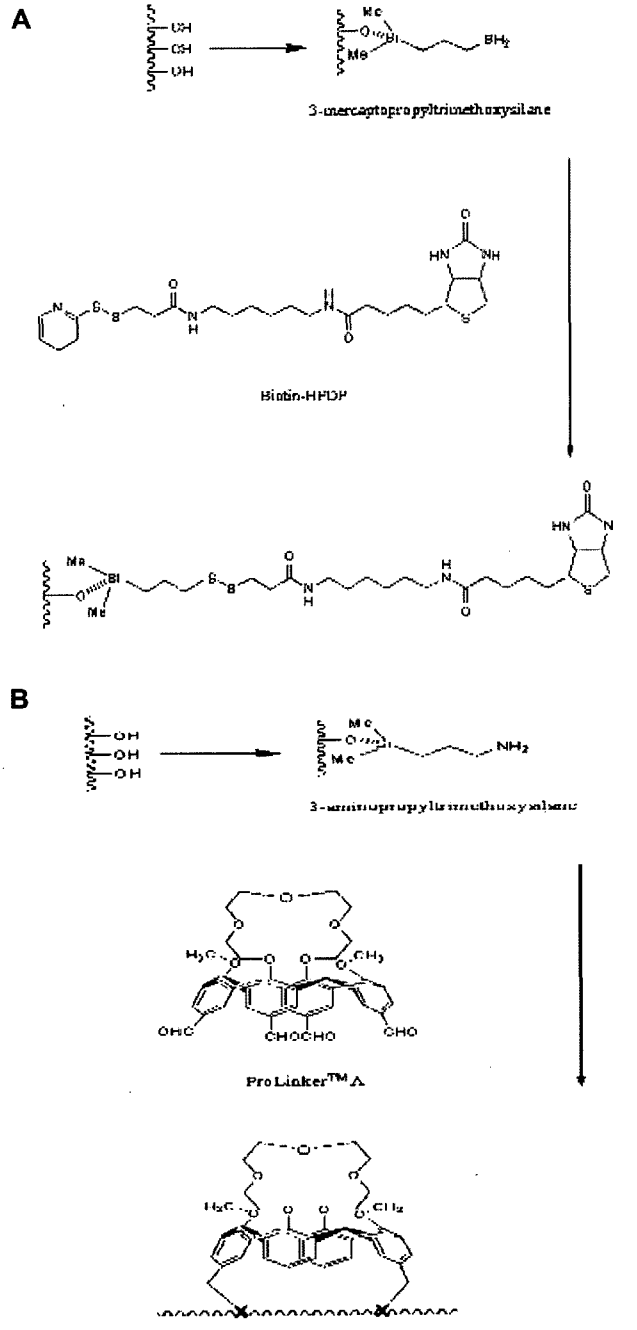

Fig. 1. Functionalization of a porous silicon surface using biotin with a mercaptopropyltrimethoxyl group (**A**) and by using ProLinker A (**B**).

Oxidation and Functionalization of the Si Surface

The Si surface after the etching was hydride-terminated and then sensitive to oxidation and hydrolysis in the aqueous solution. Therefore, the porous layer was oxidized prior to functionalization by the thermal oxidation (heat treatment) in a furnace. Without this oxidation, optical signal drift can result in the unwanted oxidation in the aqueous solution.

The second step involved the coupling of the sulfhydryl-terminal with the succinimide group in order to change the carboxyl group to the amine reactive NHS group (N-hydroxy succinimide ester). The third step was performed to eliminate nonspecific binding; *i.e.*, the coupling the biotinylated HPDP (hexyl-3'-2'-pyridyldithiopropion amide) to the NHS-modified p-Si surface. -NH-C=O- groups were observed to be present in two different places in the final functional group. In Fig. 1B, ProLinker A molecules are bound with 3-aminopropyltrimethoxysilane directly on the porous silicon surface, instead of 3-mercaptopropyltrimethoxysilane being bound with biotin-HPDP. ProLinker A, which is a calyx crown derivative, containing CHO groups for binding to amine groups on the Si substrate, provides effective high-density protein immobilization without activity loss or incorrect orientation of the capture protein [9].

Instrumental Analysis

The porous Si substrate morphology (pore size and porous layer thickness) was checked using SEM (Scanning Electronic Microscopy, JSM6700F, FESEM II, JEOL, Japan). Chemical analysis to confirm the formed silane layer on the Si surface was done by XPS (X-ray Photoelectron Spectroscopy, ESCA 2000, VG Microtech, England). The biotin group after the functionalization process was investigated by using spectrometry FT-IR (Fourier Transform Infrared Spectrometer, FT/IR-660 Plus, Jasco, Japan). The bulk Si surface after each functionalization was investigated by AFM (Atomic Force Microscopy, CP Research, Thermo Microscopes, California, U.S.A.). Enzyme assays were done using a microplate reader (E-MAX, Molecular Devices Co., California, U.S.A.) in the ELISA method and using UV/Vis spectrophotometry (2120UV, Optizen, Korea) in the conventional Miller's assay.

Results and Discussion

In the present study, β-galactosidase activity released from cells was determined, not by the Miller's method requiring several experimental steps that are time consuming, but by using a rapid and cheap interferometric biosensing method. Extracellular β-galactosidase activity measured without using extracting solvent was 51 unit/ml, which was 5% of the level measurable using solvents in the

previous study [16], where TBT (tributyl tin), TPT (triphenyl tin), and DMSO with the concentrations of 0.01–1 μg/ml were used as the DNA-damaging chemicals. This low enzyme activity was rapidly determined using an alternative interferometric biosensor, rather by the time-consuming and tedious conventional enzyme assay.

Etching Condition to Obtain Appropriate Pore Size

Since the applicability of an interferometric sensor to the small biomolecules (DNA fragments or oligonucleotides) was shown by Lin and Motesharei [17], several works have been done by numerous researchers [8, 13, 18–24]. In order to establish the interferometric biosensing system, there are major steps such as etching the Si wafer to get an appropriate fringe pattern, surface modification and functionalization, and further antibody binding if needed to avoid nonspecific binding of unwanted biomolecules.

In the first etching step, the key parameters for formation of porous Si are the electrolyte type, the HF concentration, the doping level and type (n, n+, p, p+), temperature, and the current density.

The pore size of p-type porous Si can be increased by increasing the concentration of the dopant and decreasing the HF concentration, but low current densities result in a random orientation of highly interconnected filament-like micropores. Large and cylindrically shaped pores can be obtained with higher current densities near the electropolishing region. If the current density increases fro 150 to 600 mA/cm^2, the pore radius increases from 5 to 600 nm [8]. Anodization of p-type Si at a current density of 600 mA/cm^2 resulted in a flat mat surface, due to increased light scattering that occurred at the surface of the upper porous Si layer.

Pores that formed in p-type Si typically show much smaller diameters than n-type ones for the same formation conditions [21]. Dancil *et al.* [18] demonstrated that etching p+ wafers generally could produce pores on the order of 5 nm in diameter.

The pore size has to be large enough to allow target biomolecules to enter the pores, but small enough to retain optical reflectivity of the porous Si surface. In this study, the target biomolecule is β-galactosidase, with a typical size of 6×8×11 nm [25]. Therefore, the pore diameter should be at least above 50–60 nm for both walls of a pore to form a single layer of enzyme sufficiently.

Investigation of the effect of the etching time on the change in the fringe pattern in the reflectance spectrum strongly suggested that the optimal etching time was 70 sec at a current density of 25 mA/cm^2, resulting in the maximum number of fringes. The number of fringes initially decreases and then the fringes disappears altogether when the etching time exceeds 120 sec. The average pore size of the etched Si surface at 70 sec was estimated to be approximately 60–70 nm by SEM analysis, which enabled our requirement for the target biomolecules to enter the pore.

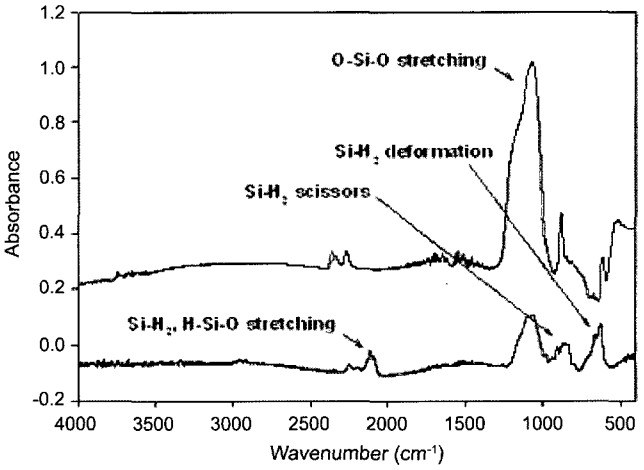

Fig. 2. FT-IR spectra of anodically etched and thermally oxidized porous Si surface (bottom, porous silicon; upper, thermally oxidized porous silicon).

Thermal Oxidation to Prevent Signal Drift

Freshly etched hydride-terminated porous Si is unstable and tends to be oxidized easily in an aqueous solution. The lack of reactivity of pre-thermally oxidized sample is due to the absence of free silane groups on the thermally treated porous Si surface, which protect the porous Si surface from hydrolysis and provide a modifiable chemical species for the subsequent protein-ligand immobilization [8].

Fig. 2 shows the FT-IR spectra of the anodically etched and thermally oxidized porous silicon surface. As explained in the functionalization procedure, there are three major steps involved in this process. Firstly, the bulk silicon is etched and thermally oxidized to reduce the signal drift caused by oxidation of the pSi surface in an aqueous solution. The Si-H_2 peaks are shown at 640, 870, and 2,100 cm^{-1} and the Si-O-H peak is shown at 2,250 cm^{-1} [26].

As the etched silicon surface is oxidized thermally, the Si-O-Si peak intensity increases considerably, whereas the Si-H_2 peak deforms at 870 cm^{-1}.

Characterization of the Functionalized Porous Si Surface

The functionalization with biotin and ProLinker A was done using a self-assembly method [27, 28], after the thermal preoxidation and then the reduction of the S-S to SH group with thio- and amino- groups.

The spectral band profile at each surface modification and functionalization step was analyzed by X-ray photoelectron spectroscopy (XPS), and the results are shown in Fig. 3, where the black, blue, green, and red

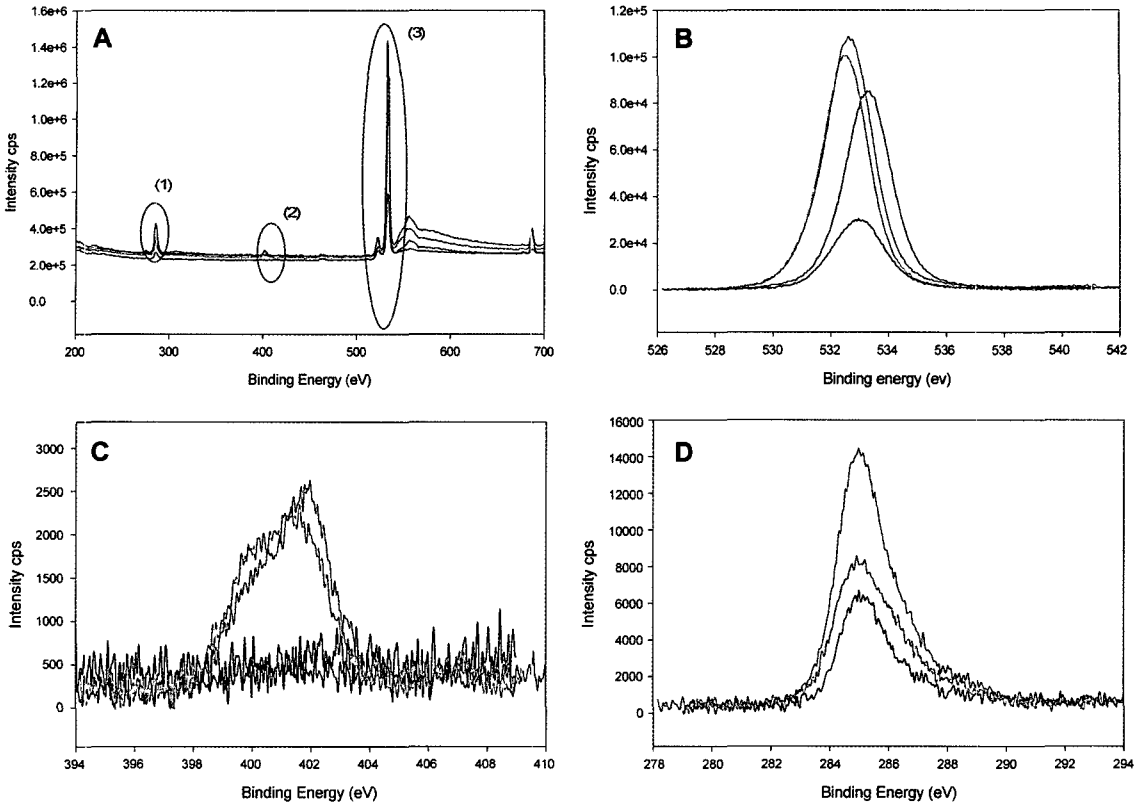

Fig. 3. Spectral band profiles at each surface modification and functionalization step (———, bare Si; ———, thermally oxidized Si; ———, functionalized Si with 3-aminopropyl trimethoxysilane; and ———, functionalized Si with ProLinker A).
A. Wide spectral band profile; **B**. C 1s spectral band profile (285 eV); **C**. N 1s spectral band profile (399.5 eV); and **D**. O 1s spectral band profile (533 eV).

spectra refer to the bare Si, thermal-oxidized Si, amine-functionalized Si, and ProLinker-bound Si surfaces, respectively. The binding energies of the significant elements were 258, 398, and 533 eV for carbon, nitrogen, and oxygen, respectively (Fig. 3A).

After thermal oxidation of the bare silicon, the corresponding spectrum (blue color) increased considerably at 533 eV in comparison with the spectrum of the bare Si, which is due to the formation of O-Si-O groups at the surface (Fig. 3B). When the oxidized Si surface was functionalized with 3-aminopropyl trimethoxysilane, the peak energy band of N shifted from 398 to 401 eV, owing to the formation of the Si-NH_2 linkage, and increased considerably (Fig. 3C). As the CHO in the ProLinker A bound to the amine group existing in the previously functionalized surface with 3-aminopropyl trimethoxysilane, the relative intensity increased remarkably at 285 eV (Fig. 3D).

When the bare Si was thermally oxidized, the oxygen content increased from 15.6 to 36.3%, whereas the Si content decreased from 41.4 to 36.3%, because SiH was converted to SiO_2. After thermal oxidation, the Si surface was functionalized with 3-aminopropyl trimethoxysilane, forming a NH_2 group, which resulted in an increase in the nitrogen content from 0 to 1.55%, whereas the Si content decreased from 41.4 to 27.7%. During the next functionalization step with ProLinker A, the NH_2 group on the Si surface was bound with the CHO group in ProLinker A. Therefore, this step resulted in an increase in the C content from 8.4 to 11.5%, whereas the Si content decreased slightly from 27.7 to 22.4% and the variation in the nitrogen content was negligible.

When the protein of interest is an antibody, it is desirable to biotinylate the protein in a manner that will maintain the immunological activity. Many biotin derivatives react with primary amine groups and can interfere with antigen binding. This problem can be overcome because biotin-HPDP (hexyl-3'-2'-pyridyldithiopropionamide) is reactive towards sulfhydryls.

The biotinylation agent, Biotin-HPDP, is sulfhydryl reactive and cleavable by reducing agents, which can regenerate the starting protein in its original unmodified form. Therefore, it has an advantage over other cleavable reagents such as NHS (N-hydroxysuccinimide)-SS-biotin, which cannot regenerate the original protein after cleavage from biotin. Each original reacted with theNH_2 group using the NHS-SS-biotin, a target protein, with NHS-SS-biotin changed to an -NH_2-$(CH_2)_2$-SH residue.

In the case of ProLinker A, it is involved in the host-guest interaction in an amine group of the capture proteins

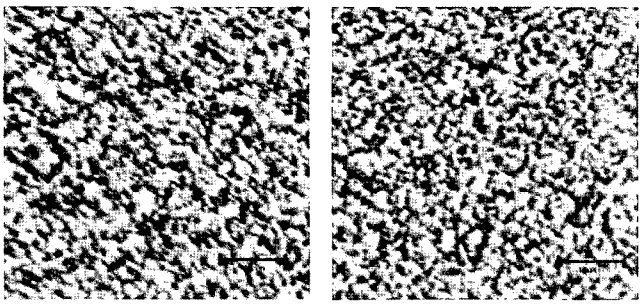

Fig. 4. AFM image of the functionalized silicon surface of 1×1 μm^2 [**A**. (3-Aminopropyl) trimethoxysilane-coupled silicon surface; and **B**. ProLinker A-silane coupled silicon surface].

with a crown moiety. Hydrophobic interactions between the hydrophobic residues of the protein and the methoxy group of the linker molecule might also be involved in protein immobilization with ProLinker A.

Fig. 4 shows the AFM (Atomic Force Microscopy) images of the biotin-silane coupled Si surface (A) and the ProLinker A-silane coupled Si surface (B). The dense formation of a biotin-silane layer was observed at 2–3 nm intervals on the porous Si surface in (A). The formation of a ProLinker A group was a lttle denser in (B) than in the biotin group. Even though the density is similarly shown, the ProLinker A-silane coupled surface enhances a higher binding capacity with a target biomolecule, which leads to an increase of the refractive index, since the ProLinker A has more binding sites than the biotin.

Blue Shift of Fringe Pattern with Bound β-Galactosidase on the Si Surface

The optical spectrum from a thin porous Si film is estimated by the Fabry-Perot relationship. The wavelength of a peak in the reflecting spectrum is given by $m\lambda=2$ nl, where m is the spectral order of the optical fringe, λ the wavelength, n refractive index of the film, and l its thickness [19]. The product nl is the quantity referred to as optical thickness. Any change of the refractive index n will induce a proportional shift of the position of the interference fringe position λ. The bind of a target biomolecule to the linker on the porous Si surface increases the optical thickness as Δnl, which is defined as the effective refractive index of the layer medium.

Fabry-Perot fringe pattern is dependent on the etching condition, such as current density and etching time at an HF concentration, since the porosity, pore size, and porous layer depth change very sensitively. Usually, the pattern is generated in a thin (1 to 5 μm) layer with pore radius less than 1 μm.

As the current density (or etching time) increases (or decreases), the thickness increases, which results in the enhanced sensitivity. However, much higher current density

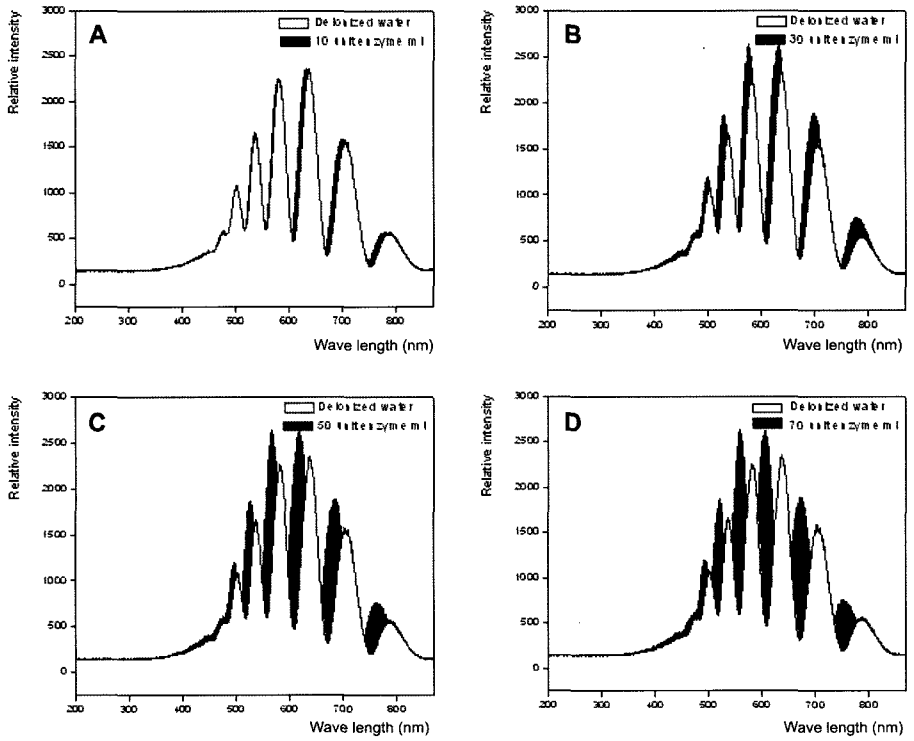

Fig. 5. Interference pattern and difference spectra with β-galactosidase solution when ProLinker A was used as the biological binder (**A**. 10 units; **B**. 30 units; **C**. 50 units; and **D**. 70 units of β-galactosidase per ml solution).

(several hundred mA/cm^2) tends to bring disappearance of the fringe pattern owing to the electropolishing effect. Therefore, fewer fringes can be obtained at the much higher current density.

With the porous Si (depth: 1.5 μm and average pore radius of 60–70 g μm) formed at a DC current density of 20 mA/cm^2 in this study, seven fringes were obtained that enabled to estimate well the change in the optical thickness (Fig. 5).

The exterior surface area of the porous silicon was 1 cm^2. The changes in the fringe patterns were measured from the same porous silicon sample after immersing it in a β-galactosidase solution at various concentrations. These changes were compared with those of the fringe obtained with pure deionized water on porous silicon as a reference. The β-galactosidase was diluted with deionized water in order to obtain various concentrations from 0.01 to 150 enzyme unit/ml.

When the target biomolecule β-galactosidase, produced by recombinant *E. coli* responding to DNA-damaging chemicals in the environment, is bound onto the functionalized porous Si surface, a change in the refractive index occurs.

Determination of Effective Optical Thickness ΔnL with Enzyme Concentration

As shown in Fig. 5, the wavelength in the fringe pattern shifted to the left-hand side which is referred to as blue shift. It is because bound enzymes increase the optical thickness of nl as the refractive index increases. If a biomolecule is bound onto the porous Si surface that may result in the decrease of the optical thickness, the wavelength shifts to the right-handed side, which is referred to as red shift.

Effective optical thickness Δnl can be analyzed from the slope of peak order m *vs.* 1/λ curve, by using a general peak-finding algorithm.

The change in the effective optical thickness (ΔEOT) was obtained by subtracting the value of the EOT with only deionized water from that of EOT with β-galactosidase. The changes in the effective optical thickness observed with the ProLinker A-coupled Si surface were 55, 73, 120, 160, and 200 nm in 30, 50, 70, 100, and 150 units of β-galactosidase/ml, respectively, whereas those obtained with biotin-coupled Si surface were 19, 37, 74, 84, 97, 96, 137, and 165 nm in 10, 20, 30, 50, 60, 70, 100, and 140 units of β-galactosidase/ml, respectively. When anti-β-galactosidase was bound onto the ProLinker A-coupled Si surface, the ΔEOT increased considerably compared with the above two cases, resulting in values of 170, 250, 312, 395, and 470 nm in 30, 50, 70, 100, and 150 units of β-galactosidase/ml, respectively. Fig. 6 shows these changes in the ΔEOT as a function of the β-galactosidase activity.

The biotin-coupled porous Si surface can reduce the nonspecific binding, whereas the self-assembled alkenethiol and silane monolayer on the porous Si surface has a simple

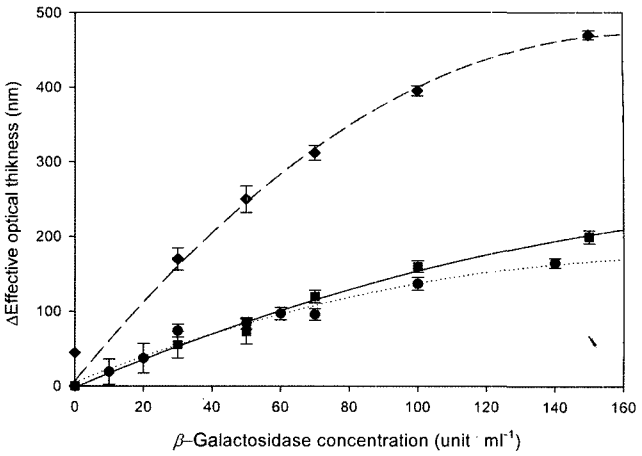

Fig. 6. Effective optical thickness as a function of β-galactosidase concentration when biotin, ProLinker A, and anti-β-galactosidase with ProLinker A were used as the biological binders.
A. ◆ (-----): immobilization of β-gal using anti-β-gal with ProLinker A; **B.** ■ (——): immobilization of β-gal using ProLinker A; and **C.** ● (.....): immobilization of β-gal using biotin.

functionality, such as C- or N-terminals. Fig. 6A shows the saturated pattern in the concentration range of β-galactosidase *vs.* ΔEOT of the above 140 units of β-galactosidase/ml, because the β-galactosidase molecules occupy the adsorption sites of the porous Si surface. The self-assembled monolayer of ProLinker A showed larger increases in ΔEOT with increasing enzyme concentration up to 150 unit/ml than that observed with the biotin-coupled surface. This is because it allows for the tight binding of β-galactosidase to the crown moiety of the linker molecules without any further surface modification (see Fig. 6B).

The interference effect, in a real application of this interferometric sensing system, can arise from nonspecific interactions of other proteins in the Luria-Bertani culture media with the adsorption site. Interference from nonspecific adsorption has been solved previously by binding an antibody, anti-β-galactosidase, onto the porous Si surface [8, 10].

Comparison experiments were carried out for ΔEOT with pure β-galactosidase and β-galactosidase released from *E. coli* in the culture media. As expected, the ΔEOT for *E. coli*, which released the β-galactosidase along with other proteins, increased to levels that were 85% and 25% higher than those for the pure β-galactosidase at concentrations of 50 and 100 units/ml [10].

In order to reduce this nonspecific binding, anti-β-galactosidase was bound to the ProLinker A-silane coupled pSi surface. When the bound anti-β-galactosidase was combined with β-galactosidase, the ΔEOT increased drastically at a concentration of >50 units of β-galactosidase/ml, increasing to a value three times higher than that observed for the enzyme monolayer on the biotinylated or ProLinker-

bound surface. In the case of the anti-β-galactosidase bound Si surface, Δn differed from the above value of 0.09 and the number of binding sites increased considerably. Furthermore, the self-assembled monolayer of ProLinker A does not allow sufficient space for the nonspecific binding of other proteins. When anti-β-galactosidase molecules were immobilized on a glass substrate at a density of 6.4×10^{12} per cm^2, the number of bound β-galactosidase molecules was estimated to be 9.6×10^{12} by QCM, with a binding ratio of 1.5 [9].

Enhancement of Specific Binding by Using Ab-Ag Reaction

In order to compare the resolution obtained by the interferometric biosensing with that obtained by the well-known ELISA method, the absorbance afforded by the ELISA method and the ΔEOT by interferometry were plotted alongside the enzyme activity at the very low concentration ranges (Fig. 7), which were much lower than those used in the case of Fig. 6.

Thus, the detection limit was extended to 0.01 units of β-galactosidase/ml, which was below the absorbance obtained by the ELISA method. As shown in Fig. 7, the correlation was linear, even though there was some variation in the ΔEOT, due to the non-uniformity in the pore size distribution on the Si surface. The above results afforded by the interferometric biosensing method confirms its strong applicability to the detection of protein analytes in the concentration range of 1–10 ng/ml, if the pore size distribution is sufficiently uniform.

1) Since the target biomolecule has a typical size of 6×8×11 nm, the pore diameter should be at least above 50–60 nm for both walls of a pore to form a single layer of enzyme sufficiently. Investigation of the effect of the etching time on the change in the fringe pattern in the reflectance spectrum strongly suggested that the optimal etching time was 70 sec at a current density of 25 mA/cm^2, resulting in the maximum number of fringes. The average pore size obtained in this condition was estimated to be approximately 60–70 nm by SEM analysis, which enabled our requirement for the target biomolecules to enter the pore.

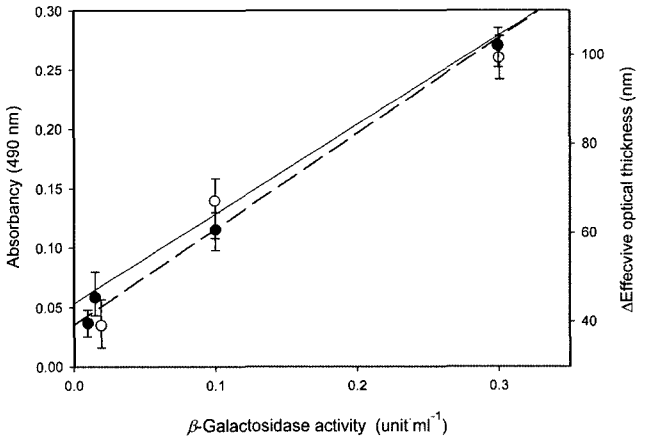

Fig. 7. The absorbance in the ELISA (--●--) *vs.* EOT (–○–) in the interferometry experiments.

2) Denser linker formation by using ProLinker A rather than biotin was confirmed by the AFM image. A higher binding capacity with the target biomolecule can result in an increase in the refractive index, which enables the sensitivity to increase.

3) In order to reduce nonspecific binding by other proteins in the medium, anti-β-galactosidase was bound to the ProLinker A-silane coupled pSi surface. When the bound anti-β-galactosidase was combined with β-galactosidase, the ΔEOT value increased drastically at concentrations of >50 units of β-galactosidase/ml, attaining a level that was three times higher than that obtained using a biotinylated surface.

4) In order to compare the resolution afforded by the interferometric biosensing method with that afforded by the well-known ELISA method, the absorbance measured by the ELISA method and the ΔEOT value by interferometry were compared with the enzyme activity at very low concentration ranges down to 0.01 unit β-galactosidase/ml, below that which the absorbance could be detected by the ELISA method. The relationship was linear, even though some variation in ΔEOT was observed, due to the non-uniformity of the pore size distribution on the Si surface.

5) The above results obtained by the interferometric biosensing method confirm its strong applicability to the detection of protein analytes at concentrations ranging from 1–10 ng/ml, if the pore size distribution is sufficiently uniform. In order to improve the reproducibility to <1 unit β-galactosidase/ml, which resulted from the non-uniform distribution of the pores in the etched silicon surface, the substrate will need to be replaced with an anodized aluminum oxide substrate, because this produces a more regular and uniform pore distribution.

Acknowledgment

This work was supported by grant No. RTI04-03-05 from the Regional Technology Innovation Program of the Ministry of Commerce, Industry and Energy (MOCIE).

References

1. Walker, G. C. 1984. Mutagenesis and inducible responses to deoxyribonucleic acid damage in *E. coli. Microbiol. Rev.* **48:** 60–93.
2. Heitman, J. and P. Model. 1991. SOS induction as an *in vivo* assay of enzyme-DNA interactions. *Gene* **103:** 1–9.

3. Miller, J. H. 1972. *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, N.Y.
4. Ziauddin, M. and D. M. Sabatini. 2001. Microarrays of cells expressing defined cDNAs. *Nature* **411:** 107–110.
5. Sandres, G. H. S. and A. Manz. 2000. Chip-based microsystems for genomic and proteomic analysis. *Trends Anal. Chem.* **19:** 364–378.
6. Kim, S. Y., B. Y. Kwak, Y. Y. Shim, and D. H. Shon. 2004. Detection of chitooligosaccharides in Korean soybean paste by tandem immunoaffinity-ELISA. *J. Microbiol. Biotechnol.* **14:** 256–261.
7. Kwak, B. Y., B. J. Kwon, C. H. Kweon, and D. H. Shon. 2004. Detection of *Aspergillus*, *Penicillium*, and *Fusarium* species by sandwich enzyme-linked immunosorbent assay using mixed monoclonal antibodies. *J. Microbiol. Biotechnol.* **14:** 385–389.
8. Janshoff, A., K. P. S. Dancil, C. Steinem, D. P. Greiner, V. S. Y. Lin, C. Gurtner, K. Motesharei, M. J. Sailor, and M. R. Ghardiri. 1998. Macroporous p-type silicon Fabry-fringe layers. Fabrication, characterization, and application in biosensing. *J. Am. Chem. Soc.* **120:** 12108–12116.
9. Lee, Y., E. K. Lee, Y. W. Cho, T. Matsui, I. C. Kang, T. S. Kim, and M. H. Han. 2003. ProteoChip: A highly sensitive protein microarray prepared by a novel method of protein immobilization for application of protein-protein interaction studies. *Proteomics* **3:** 1–16.
10. Lim, S.-H. and B.-W. Kim. 2004. Application of an interferometric biosensor chip to biomonitoring an endocrine disruptor. *Biotechnol. Bioproc. Eng.* **9:** 118–126.
11. Drolet, M., P. Phoenix, R. Menael, E. Masse, L. F. Liu, and R. J. Crouch. 1995. Overexpression of RNAase H partially complements the growth defect of an *E. coli ΔtopA* mutant. *Proc. Natl. Acad. Sci. USA* **92:** 3267–3630.
12. Kenyon, C. J. and G. C. Walker. 1980. DNA-damaging agents stimulate gene expression at specific loci in *E. coli*. *Proc. Natl. Acad. Sci. USA* **77:** 2819–2823.
13. Krueger, J. H., S. J. Elledge, and G. Walker. 1983. Isolation and characterization of Tn5 insertion mutations in the *lexA* gene of *E. coli*. *J. Bacteriol.* **153:** 1368–1378.
14. Watanabe, E., H. Eun, K. Baba, T. Arao, Y. Ishii, S. Endo, and M. Ueji. 2004. Rapid and simple screening analysis for residual imidacloprid in agricultural products with commercially available ELISA. *Anal. Chem. Acta* **521:** 45–51.
15. Ouyang, W., J. Xue, J. Liu, W. Jia, Z. Li, X. Xie, X. Liu, J. Jian, Q. Li, Y. Zhu, A. Yang, and B. Jin. 2004. Establishment of an ELISA system for determining soluble LAIR-1 levels in sera of patients with HFRS and kidney transplant. *J. Immunol. Methods* **292:** 109–117.
16. Cha, J. M. and B. W. Kim. 2002. Bioassay of environmental endocrine disruptors TBT and DMSO using recombinant *E. coli*. *Bioproc. Biosyst. Eng.* **24:** 405–410.
17. Lin, Victor S.-Y. and K. Motesharei, K. 1997. A porous silicon-based optical interferometric biosensor. *Science* **278:** 840–843.
18. Dancil, K. P. S., D. P. Greiner, and M. J. Sailor. 1999. A porous silicon optical biosensor: Detection of reversible binding of IgG to a protein A-modified surface. *J. Am. Chem. Soc.* **121:** 7925–7930.
19. Gao, J., Y. Y. Li, and M. J. Sailor. 2002. Vapor sensors based on optical interferometry from oxidized microporous silicon films. *Langmuir* **18:** 2229–2233.
20. Sohn, H., S. Letant, M. J. Sailor, and W. C. Trogler. 2000. Detection of fluorophosphonate chemical warfare agents by catalytic hydrolysis with a porous silicon interferometer. *J. Am. Chem. Soc.* **122:** 5399–5400.
21. Lillis, B. *et al.* 2005. Microporous silicon and biosensor development: Structural analysis, electrical characterisation and biocapacity evaluation. *Biosens. Bioelect.* **21:** 282–292.
22. Mathew, F. P and E. C. Alocilja. 2005. Porous silicon-based biosensor for pathogen detection. *Biosens. Bioelect.* **20:** 1656–1661.
23. Rotiroti, L. *et al.* 2005. Optical microsensors for pesticides identification based on porous silicon technology. *Biosens. Bioelect.* **20:** 2136–2139.
24. Bessueille, F., V. Dugas, V. Vikulov, J. P. Cloarec, E. Souteyrand, and J. R. Martin. 2005. Assessment of porous silicon substrate for well-characterized sensitive DNA chip implement. *Biosens. Bioelect.* **21:** 908–916.
25. Protein Data Bank, http://www.rcsb.org/pdb.
26. Waltenburg, H. N. and S. T. Yates. 1995. Surface chemistry of silicon. *Chem. Rev.* **95:** 1589–1673.
27. Pavlovic, E., A. P. Quist, U. Geliusm, and S. Oscarsson. 2002. Surface functionalization of silicon oxide at room temperature and atmospheric pressure. *J. Colloid Interf. Sci.* **254:** 200–203.
28. Schwartz, D. K. 2001. Mechanism and kinetics of self-assembled monolayer formation. *Annu. Rev. Phys. Chem.* **52:** 107–137.