Bioelectrocatalytic Signaling From Immunosensors With Back-Filling Immobilization of Glucose Oxidase on Biorecognition Surfaces

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Abstract: We report a novel method of electrochemical signaling from antigen–antibody interactions at immunoelectrodes with bioelectrocatalyzed enzymatic signal amplification. For the immunosensing surface construction, a poly(amidoamine) G4-dendrimer was employed not only as a building block for the electrode surface modification but also as a matrix for ligand functionalization. As a model biorecognition reaction, the dinitrophenyl (DNP) antigen-functionalized electrode was fabricated and an anti-DNP antibody was used. Glucose oxidase (GOX) was chosen to amplify electrochemical signal by enzymatic catalysis. The signal amplification strategy introduced in this study is based on the back-filling immobilization of biocatalytic enzyme to the immunosensor surface, circumventing the use of an enzyme-labeled antibody. The non-labeled native antibody was biospecifically bound to the immobilized ligand, and the activated enzyme (periodate-treated GOX) reacted and "back-filled" the remaining surface amine groups on the dendrimer layer by an imine formation reaction. From the bioelectrocatalyzed signal registration with the immobilized GOX, the surface density of biospecifically bound antibody could be estimated. The DNP functionalization reaction was optimized to facilitate the antibody recognition and signaling reactions, and ≈6% displacement of surface amine to DNP was found to be an optimum. From quartz crystal microbalance measurement, immunosensing reaction timing and the surface inertness to the nonspecific biomolecular binding were tested. By changing the surface functionalization level of DNP in the calibration experiments, immunosensors exhibited different dynamic detection ranges and limits of detection, supporting the capability of parameters modulation for the immunosensors. For the anti-DNP antibody assay, the fabricated immunosensor having 65% functionalization ratio exhibited the linear detection range of 10^{-4} to 0.1 g/L protein and a limit of detection around 2×10^{-5} g/L.

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INTRODUCTION

Immunoassay, the measurement of antibody or antigen concentrations based on biospecific recognition interactions, has been considered a major analytical method in the fields of clinical diagnostics and environmental analysis (Ohmura et al., 2003; Pillot, 1996). And more sophisticated analytical devices for immunoassay, so-called *immunosensors*, have been studied on the basis of various signal generation principles from complex interactions between antibodies and antigens (Delehanty and Ligler, 2002; Lim et al., 2002; Morozov and Morozova, 2003). These methods are fundamentally based on the immobilization of biomolecules on the sensing surface, following their interaction with the target counterparts in the sample, and resultant physicochemical changes such as mass change, electrical difference, heat generation, and direct or indirect optical variation occurrence (Hock, 1997, Luppa et al., 2001). Recently, immunosensor technology has evolved as a of "lab-on-a-chip" (LOC) to minimize human intervention in sample pretreatment and to overcome the drawbacks of conventional assay methods that are time-consuming and labor-intensive (Ko et al., 2003; Liu et al., 2003; Sato et al., 2002).

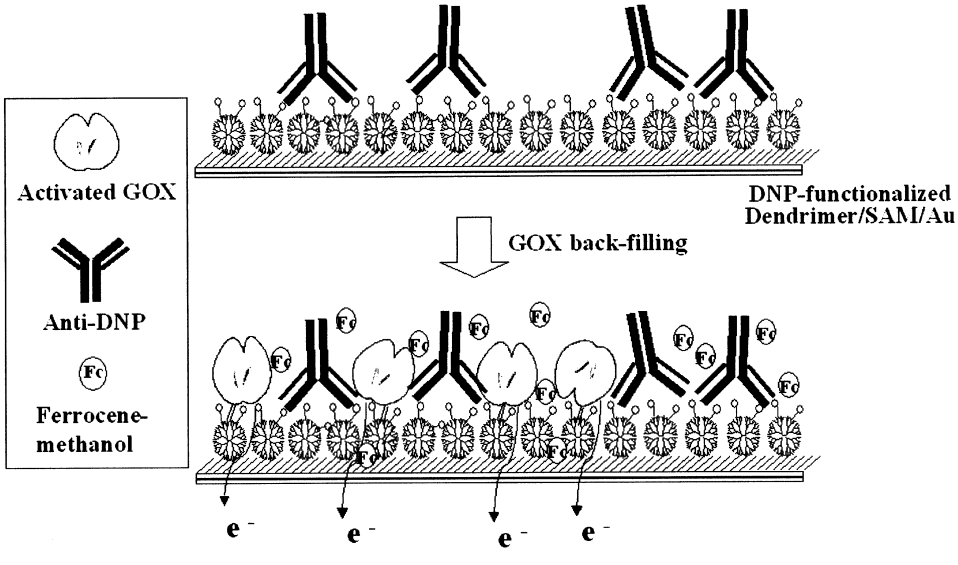
Currently, surface plasmon resonance (SPR), quartz crystal microbalance (QCM), optical detection methods including fluorescence and chemiluminescence, and electrochemical method are being widely employed in biomolecular detections and immunoassays (Darain et al., 2004; Feng et al., 2000; Kim et al., 2004; Oh et al., 2004; Sand et al., 2003). Among them, the electrochemical method has many advantages including simple instrumentation that enables device miniaturization and easy signal

quantification (Bakker and Telting-Diaz, 2002; Ghindilis et al., 1997; Kojima et al., 2003; Thévenot et al., 2001, Wang et al., 2002; Zhang et al., 2000). However, the electrochemical method, as well as the optical method based on fluorescence and chemiluminescence, do have one significant limit on the label-molecule requirement such as enzyme and fluorophore/luminophore to the biospecifically interacting molecules. Therefore, analytical devices based on SPR or QCM theory that do not require labeling steps are widely used despite their complex instrumentation and slow signaling. In this regard, the immunosensing strategy that is based on the electrochemical principle and does not require the antibody-labeling reaction would be valuable, compared to conventional ELISA (enzyme-linked immunosorbent assay) tests.

Another important issue in immunosensor development resides in the design and fabrication of immunosensing interfaces. To detect target biomolecules, the target-specific ligands must be effectively immobilized on the electrode surface (Zen et al., 2003). The analytes must be able to access their immobilized recognition couple under satisfactory frequency without severe steric hindrance and nonspecific binding. Thus, the design and implementation of a unique sensing surface for facile ligand functionalization and biospecific interaction are crucial. Dendrimer is a unique monodisperse polymer that has a globular shape and branched structure, and its molecular size and the number of surface functional groups are controllable during synthetic processes (Tully and Fréchet, 2001). For example, the fourth-generation poly(amidoamine) dendrimer has 64 surface amine groups and a molecular weight of 14,215. Based on its molecular characteristics, dendrimers have been widely applied as building blocks for bottom-up multilayers and nanostructures, DNA and drug delivery vehicles, and chemical sensing materials (Kukowska-Latallo et al., 1996; Vögtle et al., 2000; Yoon and Kim, 2000).

There have been a number of studies describing the uses of poly(amidoamine) dendrimer as a constituent of self-assembled monolayers (SAM) on solid surfaces for biosensing interface construction and biomolecular micropatterning (Benters et al., 2001; Degenhart et al., 2004; Hong et al., 2003; Pathak et al., 2004). With such an approach, the highly functionalized SAM-like dendrimer layer could be constructed and its surface functional group density was high enough to derive biochemical signals from biosensors and to achieve protein micropatterns.

In this study, we have developed an electrochemical immunosensing strategy of which the sensor signal is amplified by an enzymatic catalytic reaction at the interface. The unique point in this method is that the proposed signal amplification strategy does not require the routine and cumbersome process such as antibody labeling or the use of a labeled secondary antibody (Yoon et al., 2002). As shown in Scheme 1, the proposed signaling strategy is based on the back-filling of immunosensing surface with biocatalytic enzyme, GOX, circumventing the use of enzyme-labeled antibody. For the affinity sensing, nonlabeled native antibody was reacted and biospecifically bound to the functionalized antigen or ligand group, and the remaining surface amine groups on the dendrimer layer was covalently covered with periodate-treated GOX ("back-filled"). From the bioelectrocatalytic reaction with the stably immobilized GOX and electrochemical signal registration, the surface density of biospecifically bound antibody could be quantified. As a model antigen functionality, 2,4-dinitrophenyl (DNP) group was immobilized on the gold electrode surface using a poly(amidoamine)



Scheme 1. Schematic representation of the electrochemical immunosensing with the "back-filling" immobilization of enzyme (GOX) and bioelectrocatalysis. The dimensions of the components are not drawn to scale for simplicity.

dendrimer layer as an interfacing monolayer. Anti-DNP antibody was employed as the target molecule in affinity biosensing. Details are reported herein.

MATERIALS AND METHODS

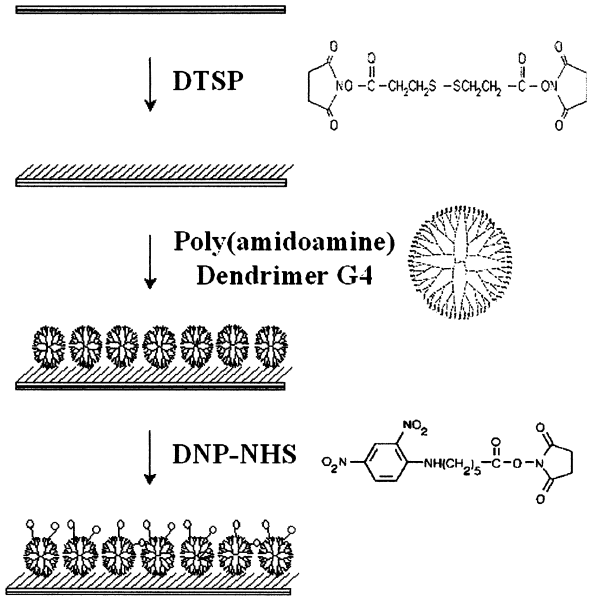
Apparatus and Reagents

3-3′-Dithio-bis(propionic acid *N*-hydroxysuccinimide ester) (DTSP), glucose oxidase (GOX, from *Aspergillus niger*), and β-D-glucose were purchased from Sigma. Poly(amidoamine) fourth-generation dendrimer, ferrocenemethanol, 4-nitrobenzaldehyde, and sodium periodate were obtained from Aldrich. 6-(2,4-dinitrophenyl) aminohexanoic acid succinimidyl ester (DNP-NHS) as a ligand and anti-dinitrophenyl-KLH antibody (anti-DNP, rabbit IgG fraction) as a target protein were supplied from Molecular Probe and used without further purification. All other materials used were of the highest quality available and purchased from regular sources. For solutions, doubly distilled and deionized water with a specific resistance over 18 MΩ · cm was used throughout the study.

Electrochemical measurements were carried out with an electrochemical analyzer model 630B (CH Instruments) that was connected to a laptop computer. A standard three-electrode configuration with an evaporated gold working electrode, a platinum auxiliary electrode, and an external Ag/AgCl (3 *M* NaCl, BAS) reference electrode was used. Quartz crystal microbalance (QCM) study was conducted with the QCN-1000 system (BioMechatron), whose mass sensitivity was 4.2 ng/Hz · cm^2 when 9-MHz quartz crystal was used.

Electrode Fabrication

The DNP-functionalized gold electrode was formed as follows. Freshly evaporated gold surfaces were prepared by the resistive evaporation of 200 nm of Au (99.999%) onto titanium-primed (20 nm Ti) Si[100] wafers, and were used as base substrates for the fabrication of immunosensing surfaces. The procedures used for the construction of the DNP-modified electrode surfaces are depicted in Scheme 2. Prior to the bottom-up layer formation process, the evaporated gold surfaces were cleaned by immersing them in piranha solution for 5 min. (*Caution*: piranha solution reacts violently with most organic materials and must be handled with extreme care.) Amine-reactive SAM was formed by dipping the surfaces into a 5 m*M* DTSP solution in DMSO for 2 h. After a washing step with DMSO and ethanol, the electrodes were transferred to the 0.5% (w/w) poly(amidoamine) G4 dendrimer solution in ethanol for 30 min. After a thorough rinsing with ethanol, the electrodes were immersed in a 0.03 mg/mL DNP-NHS solution in DMSO for a predetermined time duration from 5 min to 2 h. After all the steps, the electrodes were dipped in PBS (0.1 *M*, pH 7.2) and stored before the immunosensing step.



Scheme 2. Schematic illustration of the procedure employed in the bottom-up construction of dinitrophenyl-functionalized affinity sensing interface. The dimensions of the components are not drawn to scale for simplicity (DTSP: 3-3′-Dithio-bis(propionic acid *N*-hydroxysuccinimide ester, DNP-NHS: 6-(2,4-dinitrophenyl) aminohexanoic acid succinimidyl ester).

Determination of the Functionalized Ligand Density on the Electrode Surface

To confirm the surface functionalization and to estimate the immobilized ligand density, the amine density on the electrode surface was quantified by the 4-nitrobenzaldehyde reaction that was developed by Moon and colleagues (Moon et al., 1997). DNP-functionalized surfaces, which were formed by dipping the dendrimer-modified surfaces in a DNP-NHS solution for various times, were further reacted by immersing them in a 1 mg/mL 4-nitrobenzaldehyde solution in ethanol at 50°C for 6 h. During the reaction, Schiff's bases were formed between aldehyde groups from 4-nitrobenzaldehyde and remaining amine groups on the dendrimer layer. After a washing step with ethanol and sonication, electrodes whose surface amines were reacted with nitrobenzoyl groups were dipped into a 0.2% aqueous acetic acid at 30°C for 1 h to hydrolyze Schiff's bases. From spectrophotometric measurements, the amount of hydrolyzed 4-nitrobenzaldehyde was quantified, having direct proportionality to the surface amine density and inverse proportionality to the functionalized DNP surface density. The molar extinction coefficient of 4-nitrobenzaldehyde (ϵ_{max}) was 1.45×10^4 $M^{-1}cm^{-1}$ in 0.2% acetic acid at 265 nm. The DNP displacement ratio was predicted from the calculated amine density by taking the amine density from a nonmodified surface as 100%.

Validation of the Anti-DNP Recognition to the Surface With Quartz Crystal Microbalance (QCM)

To confirm the occurrence of biospecific interaction between the antibody analyte and the immobilized antigen and the inertness of the prepared surface to the nonspecific protein binding, QCM measurement was performed. The dendrimer-modified SAM layer was formed on the gold-coated quartz crystal and DNP was functionalized on the dendrimer layer by the same process described above. The DNP-modified electrode was installed at the QCM cell and the system was driven until the base line was stabilized. Then, the anti-DNP sample in PBS was injected to the sample loop to the final concentration of 0.01 mg protein/mL, and the interaction between anti-DNP and immobilized DNP group on the surface was investigated. As a control, QCM chips whose surfaces were modified with nonfunctionalized dendrimer were prepared and applied to the same analysis.

Anti-DNP Electrochemical Immunosensing With the "Back-Filling" Assay

For the back-filling immobilization of GOX on the antibody-reacted and dendrimer-modified electrode surface, the enzyme was rendered to be amine-reactive by the oxidation of surface carbohydrates with sodium periodate. GOX and sodium meta-periodate was dissolved in PBS with 2:3 (w/w) and reacted at 4°C for 1 h. After the oxidation process, the activated GOX was purified and concentrated with ultrafiltration (molecular weight cut-off 30,000, Centricone). After a purification step, the activated GOX was used immediately to avoid protein self-aggregation.

Prior to the DNP assay, fabricated immunosensors were clamped to homemade Teflon holders, which were designed to expose the active electrode area of 0.148 cm^2. Then, the biospecific affinity reaction was performed with anti-DNP antibody. Aliquots of anti-DNP antibody samples (50 μL) of predetermined concentrations were prepared in PBS and applied for 20 min at the immunosensor surface. After rinsing with PBS, the antibody-associated surfaces were subjected to the back-filling step with activated GOX solution (0.2 mg/mL, in 0.1 *M*, pH 7.2 PBS) for 20 min. After another washing with PBS, electrochemical signaling was conducted to register current from the GOX bioelectrocatalysis at the affinity sensing electrodes. Anodically generated current from the redox reaction of covalently attached GOX that is mediated by ferrocene mediators was registered by cyclic voltammetry. Voltammetric measurements were conducted in PBS in the presence of 0.1 m*M* ferrocenemethanol as an electron transferring mediator and 10 m*M* glucose substrate under the potential sweep rate of 5 mV/s. Because the resulting cyclic voltammograms exhibited stable current around +400 mV vs. Ag/AgCl (anodic), differences in anodic currents at +400 mV between the GOX-amplified and background voltammograms were collected and registered as the sensor signals.

RESULTS AND DISCUSSION

Effect of the Functionalized Ligand Density to the Biorecognition and Signaling Reactions

After the formation of DTSP SAM to make the sensor surface amine-reactive, poly(amidoamine) dendrimer was employed as a building block of platform surface for ligand functionalization and signal generation. The dendrimer-assisted immunosensing interface was selected to have the merits of an organized monolayer and the ligand functionalization with high density and accessibility. These advantages are likely to originate from a structural feature of dendrimer monolayers such as a surface-exposure of derivatized ligands and a corrugated surface (Hong et al., 2003). Then, a NHS-ester activated antigenic ligand to the target antibody (DNP-NHS) was reacted with amine groups of dendrimer for various time durations to follow the change in functionalized ligand density correlating to the reaction time. To quantify the amount of remaining amine groups on DNP-modified electrodes, 4-nitrobenzaldehyde was reacted with surface amines to form Schiff's bases, hydrolyzed by the acid treatment, and quantified spectrophotometrically. Changes in absorbance (at 265 nm) and calculated amine concentrations during the reaction are shown in Figure 1. The decrement in amine density indicates the progress of DNP functionalization reaction on the electrode surface. The unreacted surface amine concentration decreased rapidly as the DNP functionalization reaction went on for 30 min. After that, the surface reaction slowed down and reached the maximum level of modification at 1 h. The slight increase in absorbance after the 1 h reaction was due to the hydrolysis of surface reacted DNP.

With this result, the level of surface functionalization with DNP ligand could be calculated. Figure 2 shows the displacement ratio between surface amine and DNP as a function of reaction time. The level of surface modification

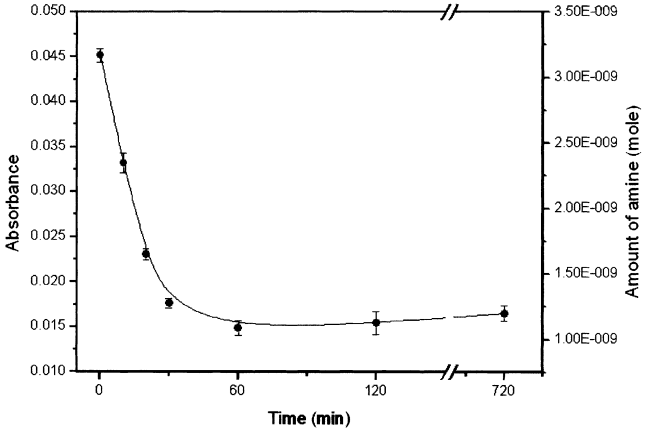


Figure 1. Change in the amount of surface-remaining amine groups as a function of reaction time for DNP-functionalization. The calculated amounts of amine groups at the surface from the absorbance data are shown in the right ordinate. Error bars represent 95% confidence limits for three tests.

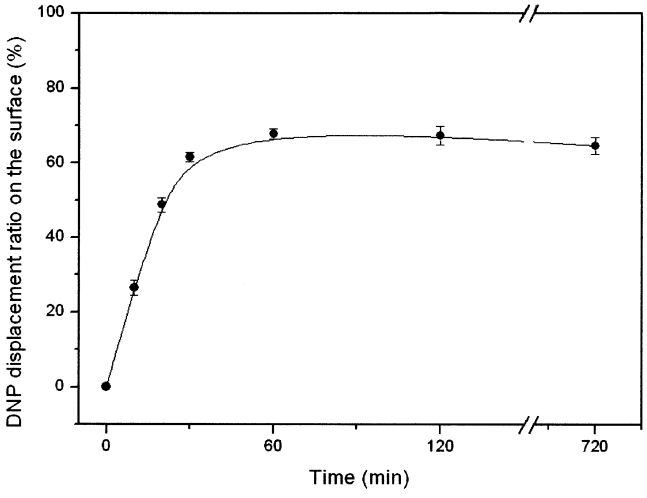


Figure 2. Dependence of the displacement ratio between surface amine and DNP on the ligand-functionalization reaction time.

with DNP increased significantly in the early stages of the reaction, slowed down gradually, and reached its maximum displacement ratio of ~65% at 1 h reaction time. It should be noted that the observed displacement ratio was not based on the total amine but on the amount of surface amines that are available for functionalization from the surface-confined dendrimers. A limited part of the surface amines from the dendrimer monolayer is available for DNP and 4-nitrobenzaldehyde reactions because some amines are consumed in the surface attachment and there is a steric limitation for the access of chemicals. Therefore, we used 4-nitrobenzaldehyde that has a similar molecular size to DNP as the labeling chemical, and the data from the DNP functionalization reaction were expressed in ratio units.

For the electrochemical signal generation and amplification from the fabricated DNP immunosensors, activated GOX enzyme should be efficiently immobilized to the electrode surface at the sites where anti-DNP antibody was not bound. In addition to this back-filling reaction, the immobilized enzymes should have a bioelectrocatalytic reaction to generate an amperometric sensor signal effectively. To confirm the GOX binding to the affinity-sensing surface, periodate-oxidized GOX was reacted to the functionalized surfaces that had various DNP densities. And the voltammetric signals produced by the bound GOX under ferrocene-mediated bioelectrocatalysis were registered for each immunosensor having a different DNP density. In this experiment, an anti-DNP antibody reaction step was omitted to find the maximum binding of activated GOX to the immunosensor surface. Figure 3 shows the maximum signals collected at + 400 mV vs. Ag/AgCl from respective voltammograms and depicts the change comparison with the DNP-functionalization ratio on the immunosensor surface. Although the DNP-functionalization ratio increased gradually in the early stages of 10 ~30 min and leveled off around 1 h reaction time at the ratio of ~65%, the maximum signals by the GOX bioelectrocatalysis steeply decreased under 5 min DNP reaction time at the functionalization ratio of as low as 13%. Based on the voltammetric condition that the concentrations of ferrocenemethanol and glucose are not rate limiting for the GOX bioelectrocatalysis, the collected signals directly correlate to the amount of active enzyme immobilized at the electrode surface. Thus, in Figure 3, the amount of actively bound GOX to the DNP-modified surface was lower than that observed on the nonmodified, bare dendrimer layer by about 50%, although the DNP-functionalization ratio was only 13%. The attached GOX density was further decreased to about 37% of the initial level when the DNP ratio was 65% and maintained. From this observation, it seems that the density of the bound GOX and the immunosensor signal magnitude are mainly dependent on the density of immobilized DNP groups and the characteristics of the functionalized interface. The steric hindrance from the crowded surface DNP groups would exclude the access and binding of GOX molecules (molecular weight 160 kD) to the interface. So, choices should be made to obtain the desired immunosensing capability with respect to the ligand density and to the maximum loading of signaling enzyme. By changing the surface functionalization level of DNP, the dynamic detection range for anti-DNP antibody immunosensing would be modified. Likewise, the limit of detection from the sensor signal is dependent on the amount of signaling enzyme and is controlled by the ligand functionalization ratio. From the results shown in Figure 3, the signal window for the immunosensor having 65% DNP ratio was 0 ~3.10 μA; the window would be widened by lowering modification levels.

Amplification of the Immunosensor Signal Via Back-Filling Immobilization of Enzyme and Bioelectrocatalysis

Prior to the signal acquisition step with the proposed bioelectrocatalytic strategy, a QCM measurement was con-

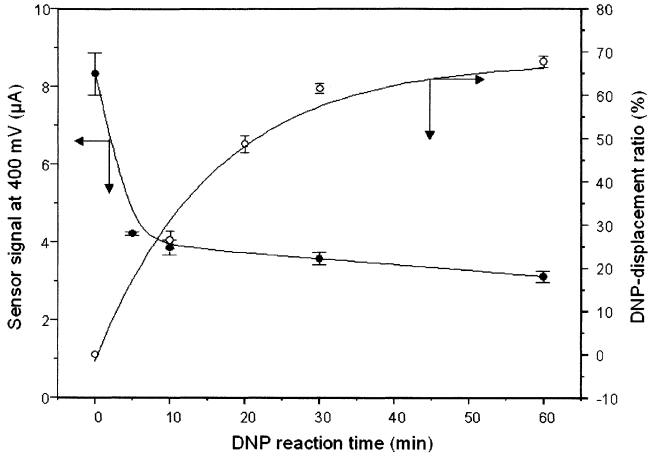


Figure 3. Comparison between the DNP displacement ratio (open circle) and the bioelectro-catalyzed signals from the back-filled GOX (closed circle) as a function of reaction time for DNP-functionalization. Error bars represent 95% confidence limits for three tests.

ducted to verify the biospecific interaction between the target antibody and the surface immobilized DNP ligand. Figure 4 shows the frequency shifts for 9-MHz QCM chips by the interaction of an anti-DNP antibody to the immunosensing surface. The (A) in Figure 4 is a sensogram for the biospecific interaction between anti-DNP antibody to the DNP-functionalized surface, exhibiting a rapid frequency decrement of about 7 Hz during the first 10 minutes and during signal stabilization. Frequency traces for QCM chips whose surfaces were not modified with DNP were obtained and are depicted in (B) of Figure 4, indicating that nonspecific binding of protein was not significant. Thus, anti-DNP antibody could bind specifically to the immobilized antigen ligand, and the nonspecific binding to the platform dendrimer surface could be ignored for at least 30 min of reaction time, which is required for the immunosensor operation.

Next, the amplification of the immunosensor signal via back-filling immobilization of GOX on the biorecognition interface and the bioelectrocatalysis were conducted. We prepared two types of DNP-functionalized immunosensors having the displacement ratio of 13% and 65%. The fabricated immunosensors were tested with protein samples containing various concentrations of anti-DNP antibody. Calibration curves from each immunosensor, obtained by GOX back-filling immobilization and electrocatalyzed signaling steps are shown in Figure 5. Anodically amplified currents at + 400 mV vs. Ag/AgCl from respective voltammograms were registered and employed as immunosensor signals. The calibration curve (A) was obtained from the DNP immunosensor having a 13% DNP functionalization ratio, and curve (B) was from another immunosensor having a 65% DNP functionalization ratio. The curve (A) exhibits a linear detection range from 10^{-7} to 10^{-5} g/L anti-DNP antibody concentration. While the other curve (B) shows a linear detection range shifted to the higher concentration ranging from 10^{-4} to 0.1 g/L anti-DNP antibody. It should be noted that the linear and dynamic detection range was extended for curve (B), obtained from the immunosensor having a high DNP-functionalization ratio. In addition, the signal sensitivity was higher for the 13% DNP-functionalized immunosensor than the 65% case, exhibiting a wider signal window. This result suggests that by changing the DNP-functionalization ratio, the sensor sensitivity and the detection range could be modulated. The sensitivity (and signal magnitude) is dependent on the density of bound GOX on the surface, so the low DNP-functionalization ratio was advantageous because of the limited accessibility of enzyme to the surface due to the steric hindrance. However, the immunosensing surface having a high density of the ligand group was desirable with regard to the detection range (linear and dynamic). Therefore, comparatively, the immunosensing surface (A) exhibited a high sensitivity and a narrow detection range, while the surface (B) showed a low sensitivity with a wide detection range.

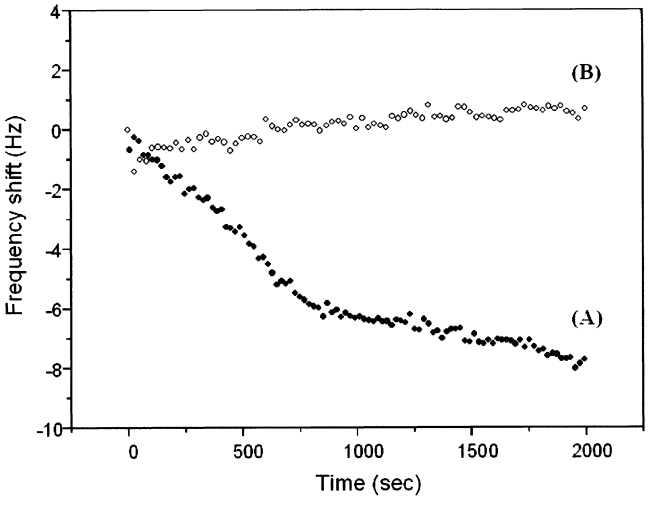


Figure 4. QCM sensorgrams for the biospecific binding of anti-DNP antibody on the DNP-functionalized electrode surface (A) and for the nonspecific antibody binding on the unmodified surface (B). Nonspecific protein binding was tested with the dendrimer/SAM-associated surfaces that had not been functionalized by DNP.

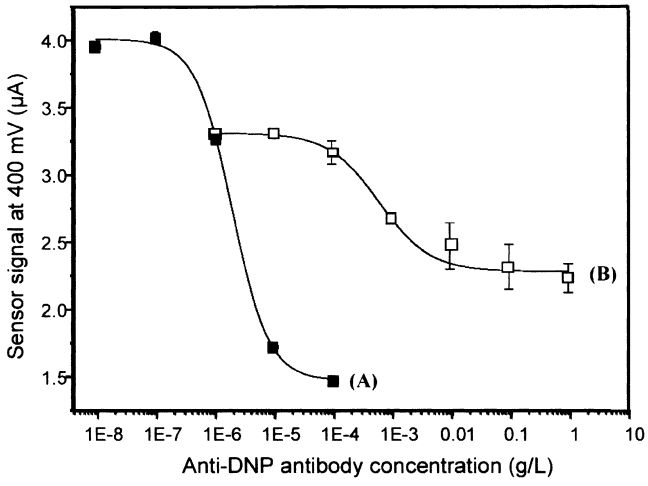


Figure 5. Calibration curves for the DNP/anti-DNP IgG affinity biosensors as a function of target protein concentration. Two types of immunosensors were tested, having different DNP-functionalization ratios of 13% (A) and 65% (B). Error bars represent 95% confidence limits for three measurements.

CONCLUSIONS

In this article, we have developed a novel signaling method from electrochemical immunosensors via the back-filling immobilization of enzyme with bioelectrocatalysis and have shown its application to anti-DNP antibody affinity sensing. The results suggest that the developed sensing interface adopting poly(amidoamine) dendrimer would be useful for a biospecific affinity reaction and for enzyme-catalyzed signaling. Furthermore, we have shown that the ligand density on the interface is a critical factor in terms of analyte and enzyme accessibility, and it determines

the detection range and the electrochemical signal range of the enzymatic back-filling assay. Thus, this immunosensing method, which has the capability of sensing parameter modulation and does not require labeling procedures, could be applied to other antigen–antibody or ligand-receptor couples. Further studies are under progress in our laboratory to implement the current method into microsystem-based immunoassays.

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