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Evaluation of Signal Stability from Electrochemical Immunoanalysis Based on the Enzyme 'back-filling' Immobilization to Biorecognition Interfaces

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Abstract

The signal stability from electrochemical immunosensors based on the enzymatic back-filling strategy was investigated. The presence of an analyte, especially antibody, which is bound to the surface-immobilized capture molecule, could cause a significant signal loss during subsequent handling procedures and signaling due to its limited affinity /stability. Compared to the typical method of detection, the signal from the back-filling immunoassay is generated differently from the covalently immobilized ('back-filled') glucose oxidase (GOX) to the sensing surface, not from the bound analyte that is usually labeled with signaling molecules. Therefore, an enhancement of signal stability would be expected with the back-filling immunoassay. To evaluate the merits of the enzymatic back-filling method, various antibody dissociation conditions from immunosensor surfaces were compared in terms of signal stability and sensitivity. As a model immunosensing reaction, the 2, 4-dinitrophenyl (DNP) group was functionalized to the biorecognition surface on a thin-film gold electrode, and anti-DNP antibody was employed as the target analyte. Cyclic voltammetry was used for immunosensor signal registration and to trace the protein adsorption/dissociation process. The back-filling method was found to be suitable for the analysis of immune reactions without labeled-antibody molecules, and the signal stability was comparable to the affinity sensor employing biotin/avidin couple.

Keywords: Immunosensor, Electrochemistry, Back-filling, Dinitrophenyl, Glucose oxidase

Introduction

Immunosensing has evolved in terms of the detection of immune-related molecules with minute concentration ranges and signaling with an acceptable quantification capacity. In general, immunosensing is based on the recognition of affinity between target analytes and their surface-immobilized couples (ligands)[1]. Detection devices such as the surface plasmon resonance (SPR) spectroscope[2,3] and the quartz crystal microbalance (QCM)[4,5] can recognize the concentration of bound biomolecules through changes in mass and/or surface density (molecules/mm^2) at interfaces. However, they are not appropriate for application in the portable biosensor format. In relation to handheld biosensors, electrochemical methods entered the spotlight due to facile device-miniaturization and sensitive signal quantification[6-8].

In a conventional immunoassay that is widely being utilized, supplementary label molecules such as enzymes should be employed to generate electrochemical signals. Therefore, the signaling process requires pre-treatment with ligands or expensive commercial reagent to activate reporter molecules such as secondary antibodies because the signal generation is dependent on the enzymatic labeling reaction[9,10]. In addition, the signal stability from immunosensors usually is not sufficient because of the limited affinities of biomolecules. The known binding constants between antigens and antibodies are diverse, and are several orders of magnitude lower than the well-known model of biotin-streptavidin ($K_a = 1.0 \times 10^{15}\ M^{-1}$) that exhibits the highest affinity in biorecognition. This suggests that the antibody-bound surfaces undergo gradual changes, with a possibility of antibody detachment and signal variation.

Therefore, a detection strategy for electrochemical immunosensors, with which the signal is not dependent on the stability of bound antibody molecules to the sensing interface, is important. In this regard, we have recently reported a method of signal amplification that utilizes electrochemical immunosensors[11]. The unique point is that the strategy does not require the routine and cumbersome process of antibody labeling or the use of labeled reporter molecules such as secondary antibodies and proteins A/G. The sig-

naling method is based on the back-filling covalent immobilization of enzymes onto the immunosensor surface, circumventing the use of enzyme-tagged antibody and alleviating the signal instability caused by low affinity binding. In addition, the electrochemical signal could be maintained despite the dissociation of the target analyte from its limited affinity with immobilized ligand because the signal generating enzyme is separated from the analyte itself.

In this study, we demonstrated the advantage of electrochemical immunosensing by using the enzymatic back-filling technique and evaluating the signal stability. As a model biorecognition reaction, the DNP antigen-functionalized immunosensor surface was fabricated and the anti-DNP antibody was used as a target analyte. The biotin/avidin/biotin-GOX functionalized sensor surface was also examined in the conventional sandwich immunoassay format to compare and evaluate sensor stability. The signal stability from each method was registered by using cyclic voltammetry. Artificial conditions for antibody dissociation and/or protein inactivation, including the pH change, the addition of a chaotropic reagent, and the biospecific displacement with the free antigen, were employed for the stability analysis.

Results and Discussion

Back-filling Immunoassay for Anti-DNP Antibody Detection

We have developed an electrochemical signaling method from antigen-antibody interactions at immunoelectrodes with bioelectrocatalyzed enzymatic signal amplification[11]. The unique point of the method is that the signal is generated by the back-filled enzymes onto the immunosensor surface, circumventing the use of enzyme-tagged antibody and alleviating the signal instability from low affinity binding. The signaling strategy is schematically summarized in Figure 1. As a model biorecognition reaction, the dinitrophenyl (DNP) antigen-functionalized electrode was fabricated and the anti-DNP antibody was used as the target analyte. Glucose oxidase (GOX) was chosen to amplify the electrochemical signal by enzymatic catalysis. During the immunosensing process, 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 underlying dendrimer layer by an imine formation reaction (Box, Figure 1). From the bioelectrocatalyzed signal registration with the immobilized GOX, the surface density of biospecifically-bound antibody molecules could be estimated. Using this method, the electrochemical signal could be stably maintained because the signal generating enzyme was separated from the analyte antibody and covalently attached to the surface even though the analyte itself was dissociated from the surface by its limited and low affinity to surface-bound antigen.

Tracking of the Electrochemical Immunosensor Signals before and after Antibody Dissociation Reactions

Based on this idea, we evaluated the signal stability

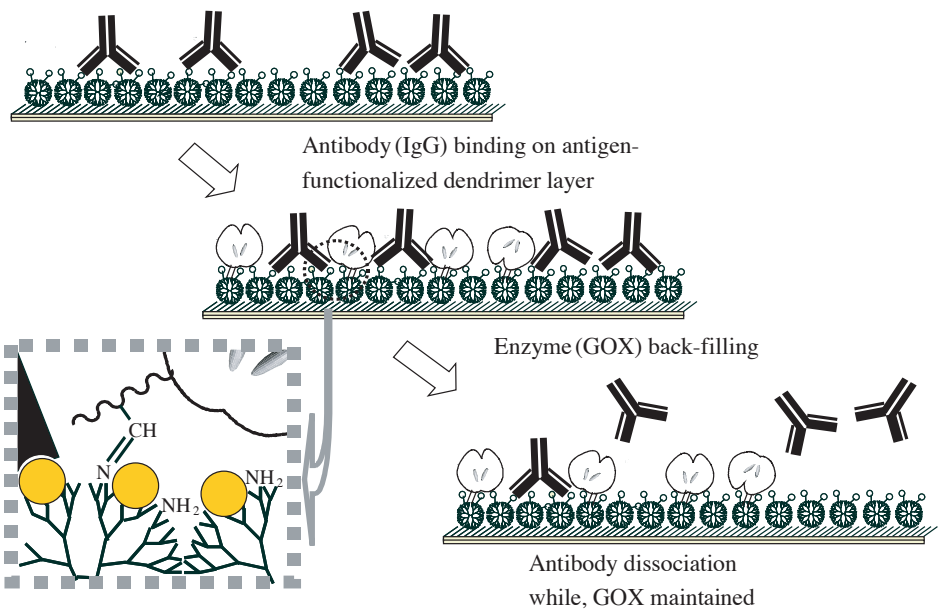


Figure 1. Schematic illustration of the enzymatic back-filling signaling method.

from the back-filling immunoassay with a number of surface treatments that would induce changes in biospecific intereactions at sensor surfaces. Two methods of surface treatment that dissociate biospecifically-bound antibodies from the sensor surface, including molecular displacement using dinitrophenylacetic acid (free antigen) and elution with a pH 2.8 buffer, were employed. In addition, treatment with a chaotrophic reagent such as SDS that deactivates both the biospecifically-bound antibodies and the covalently attached GOX was performed. These artificial conditions mimic the surface changes during immunosensor signaling under harsh environments.

The electrochemical enzymatic back-filling assays were performed with the DNP-functionalized surfaces by the method described in *Section 3.1*. The surfaces were treated sequentially with PBS containing anti-DNP antibody and periodate-activated GOX. After these steps, bioelectrocatalyzed signals from each electrode were registered by cyclic voltammetry. The three surface treatments were then conducted, followed by a signaling with another cyclic voltammetry process. Figure 2 shows each result based on antibody dissociation with free DNP displacement (A), dissociation by a low pH elution (B), and protein denaturation with the addition of SDS (C). Anodically-amplified voltammograms for bioelectrocatalysis with surface-immobilized enzyme, GOX, before and after the treatment, as well as the background voltammogram, are shown. The voltammetric traces from the immunosensors exhibited a typical pattern for the ferrocene-mediated bioelectrocatalysis of an anodically-elevated sigmoidal curve, and the peak current reached approximately +350 mV vs. Ag/AgCl reference electrode. However, because most of the resulting voltammograms exhibited stable current around +400 mV, differences in signals at +400 mV between the GOX-amplified and background voltammograms were collected and registered as the sensor signals (Table 1).

Figure 2A shows the voltammetric result for the DNP displacement treatment after the completion of antibody dissociation. From Figure 2A and Table 1, we observed that the signals were maintained at 96.4 ±3.2% of their initial levels, although the antibody was dissociated through a displacement procedure with free antigen. This is due to the strong covalent binding of GOX, the real signal generator, to the amine functionality of underlying dendrimer monolayer. The back-filling assay, therefore, presented stable signals despite the fact that the analytes have low affinities with their immobilized ligand couples. A schematic model for the occurrence of association and dissociation of antibodies at the surface is depicted as an inset of Figure 2A. In addition, the antibody elution method using a buffer exchange with a low

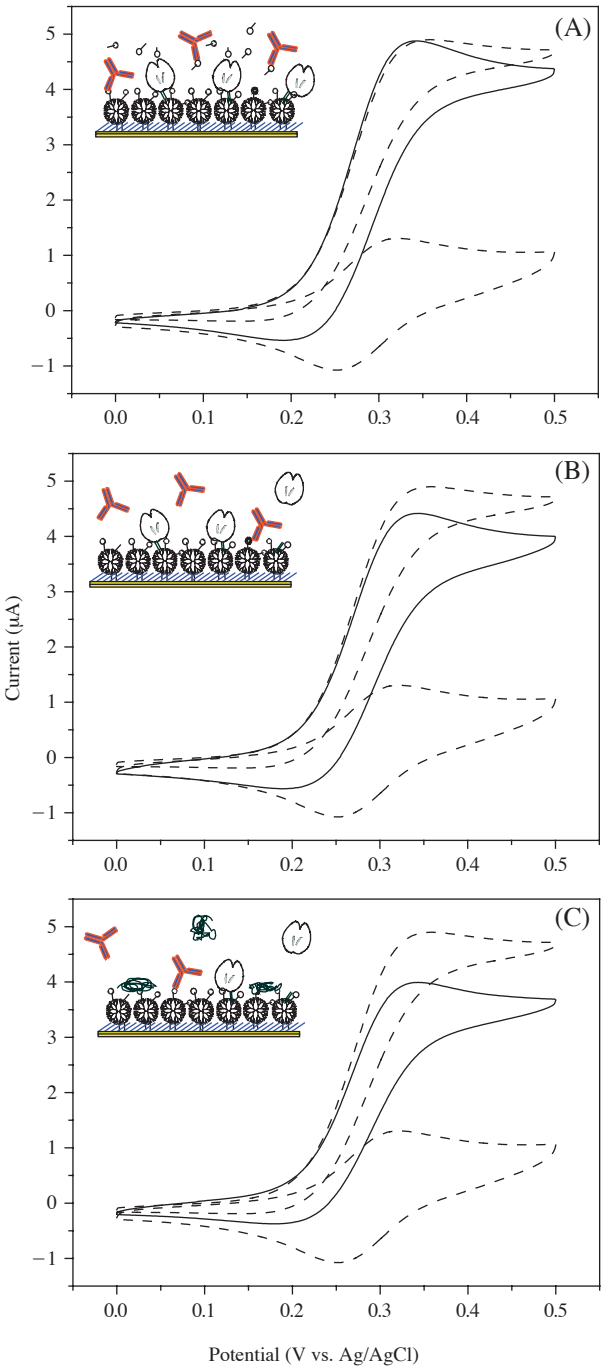


Figure 2. Registered cyclic voltammograms from the 'back-filling' immunoassay before and after antibody dissociation reactions. (A) Displacementive dissociation with the addition of free-DNP on the electrode surface, (B) elution with a low pH buffer solution, and (C) denaturation of bound proteins by the SDS treatment.

Table 1. Comparison of the registered electrochemical signals before and after immunosensor treatments. Sampled currents at +400 mV vs. Ag/AgCl reference electrode were collected from each experiment. a The % signal changes indicate the percentage of the signal after the treatment compared to the initial signal.

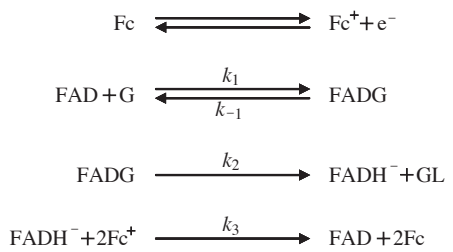
	Registered current before treatment (μA)	Registered current after treatment (μA)	Signal change[a] (%)
Displacementive dissociation	4.83±0.09	4.65±0.21	96.4±3.2
Elution with pH change	4.83±0.09	4.17±0.18	87.0±3.2
SDS denaturation	4.83±0.09	3.81±0.17	79.4±2.2

pH resulted in a moderate decrease in the signal of around 87.0±3.2% (Figure 2B and Table 1). We assumed that the decrease came from the inactivation of GOX by the lowered pH of the electrolyte or the cleavage of imine linkages (Schiff's bases) between GOX and amine groups of the dendrimer layer. Moreover, the molecular denaturation with the addition of SDS exhibited a more significant signal decrease (79.4±2.2%) because SDS, as a chaotropic reagent, would denature or molecularly unfold GOX as well as antibody on surfaces (Figure 2C). From the test, we found that the surface-bound GOX enzyme is stable for chemical changes that would result in the breakdown of biospecifically-bound antibody molecules, and generates stable signals. Therefore, compared with the typical immunosensing methods of signal generation correlating with bound analyte and using the enzyme-tagged secondary antibodies, the enzymatic back-filling method offers better signal stability regardless of the dissociation of analytes from the sensing surface, and is appropriate for the sensing of low-affinity proteins and ligands.

In addition, to compare the surface reactions in a quantitative format, bioelectrocatalytic signals from the voltammetric tests were kinetically analyzed. From the current magnitude derived by GOX electrocatalysis, the surface coverage of active enzyme could be calculated based on a reaction model[12]. The bioelectrocatalytic reaction model between GOX and the employed ferrocene mediator is shown in Figure 3. In the absence of a diffusion limitation as an assumption for simplified analysis, the plateau current from the mediated cyclic voltammograms, I_p, is expected to follow the equation:

$$\frac{1}{I_p}=\frac{1}{2FS\Gamma_E}\cdot\frac{1}{k_3[Fc]}+\frac{1}{k_2}+\frac{1}{k_{red}[G]},$$

where F is Faraday's constant, S is the electrode area, Γ_E is the surface concentration of enzyme, [Fc] is the mediator concentration, [G] is the glucose concentration in solution, and $k_{red}=k_1k_2/(k_{-1}+k_2)$. Based on the above equation and the known rate constant values ($k_2=700\ s^{-1}$, $k_3=1.2\times10^7\ M^{-1}\ s^{-1}$, $k_{red}=1.1\times10^4\ M^{-1}\ s^{-1}$), we determined the density of active GOX associated on the electrode surface[12,13].



Fc and Fc^+ : reduced and oxidized forms of ferrocenyl mediator
FAD and $FADH^-$: oxidized and reduced forms of the flavin-adenine dinucleotide at the active site in GOX
FADG : enzyme-substrate complex
G and GL : glucose and glucono-lactone

Figure 3. Sequence of reactions for the bioelectrocatalytic oxidation of glucose catalyzed by GOX and electro-mediated by ferrocenyls at sensing interfaces.

From the registered signal values from tests, changes in the coverage of active GOX, Γ_E, were estimated as summarized in Table 2. A surface density of 5.9×10^{-13} mol/cm^2 was registered prior to surface treatment. Changes in Γ_E following surface treatment were calculated as 5.5×10^{-13} mol/cm^2 for displacement by free-diffusing antigen, 4.5×10^{-13} for pH lowering, and 3.8×10^{-13} for the addition of SDS. Taking into account the occupied area on the surface of a GOX molecule, 100 nm^2 per enzyme[14], the ideal density for the compact enzyme monolayer corresponds to 1.7×10^{-12} mol/cm^2. Therefore, we presumed that approximately 35% of the surface area was covered by back-filled enzyme, and about 65% was covered by antibody molecules *via* biospecific recognition. Thus, the registered reduction in GOX density from SDS treatment is equivalent to a deactivation of 36% on the basis of immobilized GOX, which is significant compared to the change (~7%) from displacement with free antigen.

Comparison of the Signal Stability between the Anti-DNP Back-filling Immunoassay and Biotin-avidin Affinity Sensing

As a model for antibody sensing, DNP/anti-DNP

Table 2. Kinetic analysis of changes in surface coverage of active enzyme. Percentile calculation data with ideal coverage as a basis are in parenthesis.

	I_p (μA)	Active GOX coverage, Γ_E (10^{-13} mol cm^{-2})	% Active coverage
Before treatment	4.83	5.9	100 (35)
Displacementive dissociation	4.65	5.5	93 (32)
Elution with pH change	4.17	4.5	76 (27)
SDS denaturation	3.81	3.8	64 (22)
Ideal full coverage	–	17	–(100)

antibody affinity couple was employed and a back-filling immunoassay with GOX electrocatalysis was conducted in this study. To compare the sensor stability, a well-known reaction couple in the sandwich immunoassay format of biotin/avidin/biotin-GOX, exhibiting the most stable biospecific binding, was examined. Using GOX-mediated bioelectrocatalytic signaling, sensor stabilities of both models were compared. The signal stability from each method was registered by using cyclic voltammetry and compared as shown in Figure 4.

In the case of the biotin-avidin interaction, the biotin was functionalized on the dendrimer layer on the gold electrode. On this electrode surface, avidin and biotinylated-GOX were sequentially reacted to fabricate the biotin/avidin/biotin-GOX laminated layer. The stability of the avidin sensor was evaluated by tracking the sensitivity of the electrode response during extended use with a single immunosensor. Sensor signals from cyclic voltammetry were taken every 30 minutes for 3 hours with the same electrode. As a result, the electrode response was found to be gradually decreased, and was maintained at around 52% of the initial value after 3 hrs. For the anti-DNP immunoassay using the back-filling method, a gradual decrease in sensor sensitivity was also observed for extended and repeated use with a single sensor. The registered stability was found to be slightly lower than that of the biotin-avidin sensor, exhibiting about 45% of the initial signal after 3 hrs of usage. Considering the well-known highest stability of the biotin-avidin couple, we believe that the result supports the suggestion that good stability that can be obtained by the enzyme back-filling signaling method.

In conclusion, we have demonstrated the advantage of employing back-filling immobilization of GOX and bioelectrocatalysis for electrochemical immunosensing. By using a few methods of antibody-bound surface regeneration such as the displacement reaction with free antigen and the low pH elution or the chaotropic surface treatment with the SDS addition, we were able to compare the changes in immunosensor sensitivity and the molecular surface. The immunosensor stability with extended timescale usage was also compared with the fabricated immunosensors by the back-filling method and the avidin-biotin sensor of the conventional enzyme-tagging format, and supported the finding of good stability from the enzyme back-filling strategy.

In general, the affinities of antigen-antibody interactions are quite different and the reaction yield of enzyme tagging to antibody is difficult to control. This limits the application of biorecognition couples for multiplexed assays. However, by using GOX as a signal generator that is covalently attached to the sen-

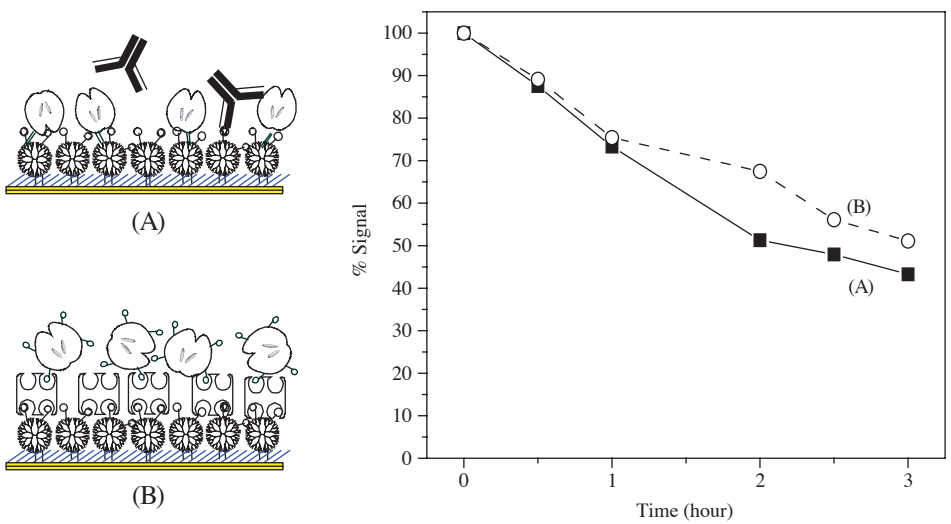


Figure 4. Comparison of signal stability between the 'back-filling' method with anti-DNP testing and a typical affinity sensing with biotin-avidin interaction. The % SIGNAL is the percentage of the registered signal to the initial signal during intermittent tests with one immunosensor.

sing interface and separated from the target antibody, a stable and quantifiable immunoassay would be possible. The application of this signaling strategy to a multiplexed immunosensing format is currently underway in our group.

Methods

Chemicals and Apparatus

3-3′-Dithio-bis (propionic acid N-hydroxysuccinimide ester) (DTSP), glucose oxidase (GOX, EC 1.1.3.4., type VII-S, from *Aspergillus niger*), sulfo-NHS-biotin, avidin (from hen egg white), and β-D-glucose were purchased from Sigma. Poly (amidoamine) generation 4 dendrimer, ferrocenemethanol, and sodium periodate were obtained from Aldrich. 6-(2, 4-dinitrophenyl)aminohexanoic acid succinimidyl ester (DNP-X) as a ligand and anti-dinitrophenyl-KLH (rabbit IgG fraction) as a target antibody were supplied from Molecular Probes and used without further purification. ImmunoPure® IgG elution buffer from Pierce and sodium dodecyl sulfate (SDS) from Junsei were used for the antibody dissociation. All other chemicals used were of the highest quality available and purchased from regular sources. For solutions, double-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.

Electrode Fabrication

The immunosensing surfaces were constructed on evaporated thin-film gold surfaces. 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. Prior to the bottom up layer formation process, the evaporated gold surfaces were cleaned by immersing them in "piranha" solution for 5 min (*The* "*piranha*" *solution reacts violently with most organic chemicals and must be handled with extreme care*.). An amine-reactive self-assembled monolayer (SAM) was formed by dipping the surfaces into a 5 mM DTSP solution in DMSO for 2 h. After washing with DMSO and ethanol, the electrodes were transferred to 0.5% (w/w) poly (amidoamine) dendrimer solution in ethanol for 30 min. After thorough rinsing with ethanol, the electrodes were immersed in a 0.03 mg/mL DNP-X solution in DMSO overnight. After all steps, the electrodes were dipped in PBS (0.1 M, pH 7.2) and stored until the immunosensing step.

Electrochemical Immunosensing with the 'Back-filling' Method

Just before the immunosensing tests, the DNP-modified gold electrode was removed from the storage solution, rinsed, air-dried, and clamped to a homemade electrode holder. The apparatus was designed to expose a defined surface area of ca. 0.15 cm^2, and the reaction volume was 2 mL. The biospecific affinity reaction was performed with anti-DNP antibody samples prepared in PBS. Aliquots of anti-DNP antibody samples were prepared and applied at the immunosensor surface for 20 min. After rinsing, the antibody-associated surfaces were subjected to the back-filling step with an activated GOX solution (0.2 mg/mL, in PBS, pH 7.2) for 20 min. After another washing, electrochemical signaling was conducted to register current from the GOX bioelectrocatalysis at the 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 mM ferrocenemethanol as an electron transferring mediator and 10 mM 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, differences in anodic currents at +400 mV between the GOX-amplified and background voltammograms were collected and registered as the sensor signals.

GOX Activation

To achieve covalent immobilization of GOX on the antibody-reacted and dendrimer-modified surface, the enzyme was rendered to be amine-reactive by the oxidation of surface carbohydrates with sodium periodate. GOX and sodium meta-periodate were dissolved in PBS at 2 : 3 (w/w) and reacted at 4°C for an hour. After the induction of carbaldehyde functionality, the activated GOX was purified and concentrated by ultrafiltration with the Centricone, with a molecular weight cut-off of 30,000 daltons. After purification, the activated GOX was used immediately or frozen to avoid protein self-aggregation.

Tracking Electrochemical Signals before and after Antibody Dissociation

The protein solution containing anti-DNP as an

analyte and periodate-oxidized GOX as a signal generator was applied on the DNP-functionalized gold immunosensor surface. After a 20 min binding reaction, various antibody dissociation conditions were applied, including a) a change of pH to 2.8 using ImmunoPure® IgG elution buffer, b) an addition of chaotropic reagent such as sodium dodecyl sulfate (SDS), and c) the biospecific displacement reaction using dinitrophenylacetic acid (10 mM) in the electrolyte. The change in the electrochemical signal between pre- and post-antibody dissociation and/or protein denaturation was investigated with cyclic voltammetry in PBS (0.1 M, pH 7.2) containing 0.1 mM ferrocenemethanol and 10 mM glucose at a scan rate of 5 mV/s.

Comparison of the Signal Stability with the Biotin-avidin Interaction

As a well-known model for highly stable bioaffinity interaction, the biotin-avidin couple was employed and compared. To produce a biotin-modified surface on the electrode, sulfo-NHS-biotin (1 mg/mL in PBS) was set to react on the surface of the dendrimer-modified electrode for 3 hrs. After the completion of this reaction, the electrode was washed and stored in PBS for the next procedure. Avidin solution (0.5 mg/mL in PBS) was applied to the surface of the electrode for 30 minutes to form another layer of avidin on the electrode. Then, the electrode was washed and stored in PBS. Once the avidin layer had been produced, the biotinylated-GOX, which has biotin as a functional group on the lysine group of GOX, was used to create the final layer of GOX on the electrode. The biotinylated-GOX was synthesized by mixing 1 mole of GOX to 12 moles of sulfo-NHS-biotin and leaving it overnight to react at 4°C. The product was then purified and diluted until the concentration was 1 mg/mL in PBS. The concentration was based on the amount of GOX in the original solution. To prepare the GOX adlayer, a rediluted biotinylated-GOX solution (200 μg/mL) was applied on the electrode for 1 h. The electrode was washed in PBS, and electrochemical signaling was conducted. Anodically-generated current from the redox reaction of GOX that is mediated by ferrocene mediators was registered by cyclic voltammetry. Voltammetric measurements were conducted under the same condition as the back-filling method.

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