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Stepwise surface regeneration of electrochemical immunosensors working on biocatalyzed precipitation

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A new strategy of stepwise surface regeneration for electrochemical immunosensors, working on a biocatalyzed precipitation reaction, has been developed. The strategy is based on the combination of deposited product thin-film dissolution and bound-protein displacement reactions from the modified sensor surfaces. As a model system, surfaces functionalized with biotin groups and their affinity recognition/displacement reactions with antibiotin antibody molecules were chosen and investigated for affinity-sensing and stepwise regeneration reactions.

Introduction

With the advent of LOC (lab-on-a-chip) or μ-TAS (total analysis system) technology,[1–3] there has been an increasing demand for efficient methodologies for affinity biosensing, registering biospecific binding reactions such as antigen–antibody, ligand–receptor, protein–protein, and nucleic acids interactions. Objectively, research trends mainly follow two directions consisting of the developments of novel transduction techniques, particularly stressing parallel sensing with array-type sensors,[4,5] and the design of affinity surfaces presenting desired characteristics such as useful surface functionality, adequate immobilization density, biocompatibility, resistance to nonspecific binding, *etc*. We have recently developed a signalling strategy for immunosensors that converts the biospecific affinity reactions into electrochemical signals.[6] The affinity reaction was designed so that precipitation and deposition of an insulating film on the sensing surface was induced by the catalytic reaction of enzymes labelled to the bound target molecules.[7–9] The signal registration was performed by electrochemical tracking of the changes in the electrode surface area by voltammetry.

However, it should be noted that the majority of the reported affinity sensing surfaces, including ours, were designed and used as the single-use, disposable type. Developing an efficient method to regenerate the affinity surfaces has long been a subject of research but still remains to be further explored. For the regeneration and reuse, modified sensing surfaces should undergo dissociation steps, including treatments of extreme pH, temperature adjustment, and chaotropic reagents, which often hamper irreversibly the activities of bound target molecules and of sensing surfaces themselves.[10,11]

In this Communication, we present a novel strategy of stepwise surface regeneration for immunosensors, enabling the removal of precipitated thin-films while maintaining the biological activity of bound protein molecules, and the complete renewal of the platform affinity surface by displacement dissociation of bound protein molecules for the next round of functionalization/biosensing. Details are reported herein.

Experimental

Reagents and instrumentation

Amine-terminated poly(amidoamine) dendrimer (G4) is manufactured by Dendritech and was purchased from Aldrich. Ferrocene methanol was purchased from Aldrich and used as received. 3,3-dithiopropionic acid bis-*N*-hydroxysuccinimide ester (DTSP), biotinyl-ε-amidocaproic acid *N*-hydroxysulfosuccinimide ester (sulfo-NHS-biotin), d-biotin, and 4-chloro-1-naphtol (4-CN) were purchased from Sigma and used as received. Antibiotin monoclonal IgG–horseradish peroxidase (HRP) conjugate (BN34 clone) was from Sigma and used without further purification. All other materials used were of the highest quality available and purchased from regular sources.

Voltammetric measurements were carried out with a potentiostat/galvanostat (Model 660A, CHI instruments) connected to a laptop computer. A standard three-electrode configuration with a gold working electrode, a platinum wire auxiliary electrode, and an Ag/AgCl reference electrode was used.

Construction of the sensing surface

As the first step, a DTSP self-assembled monolayer (SAM) was constructed on the freshly evaporated gold surfaces to render the surface amine-reactive. The DTSP SAM was prepared by dipping the gold substrate in 5 mM DTSP in DMSO for 1 h. After monolayer formation and rinsing steps with DMSO and ethanol, the electrode surface was modified with poly(amidoamine) dendrimer. A diluted ethanolic solution of dendrimer (1% w/w) was reacted (2 h) with the succinimidyl ester-activated surfaces. Then, the modified surfaces were rinsed and immersed in bicarbonate buffer (0.1 M, pH 9.5) to equilibrate the surface for ligand functionalization reaction. An aqueous solution of sulfo-NHS-biotin (2 mM) was reacted with the terminal amine groups from the dendrimer monolayer. After reaction for 2 h, the resulting surfaces were rinsed with bicarbonate buffer and distilled water, and were stored in phosphate buffered saline solution (PBS, pH 7.4, 10 mM phosphate, 2.7 mM KCl, and 138 mM NaCl containing 0.05% (v/v) Tween 20) for further biospecific affinity reactions.

Immunosensing and regeneration

Before the immunosensing, the prepared electrodes were clamped to Teflon electrode holders. The holder was designed to expose the active electrode area of 0.148 cm^2. After assembly, the biospecific affinity reaction on the biotin-functionalized surfaces was performed with a target protein, antibiotin monoclonal IgG–HRP conjugate. Aliquots of anti-

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body samples (50 μL, 100 μg mL^{-1}) were prepared in PBS and incubated at the electrodes for 30 min at room temperature. After the reaction, the antibody-associated surfaces were subjected to the signal measurement step.

For the signal generation, HRP-catalyzed precipitate formation reaction with 4-CN was adopted in this study. Fifty μL aliquots of 4-CN (50 mM) and 30% hydrogen peroxide were added to 1 mL of PBS solution briefly before the precipitation reaction. Then, the electrode surface was subjected to the signal generation reaction by incubating with the analysis mixture for 10 min. The surfaces were then thoroughly washed and subjected to the electrochemical transduction step.

For the regeneration of sensor surfaces, a combination method consisting of (1) dissolution of the precipitate film with an ethanolic PBS solution and (2) displacement dissociation of bound IgG–HRP conjugates with free biotin was performed. Dissolution of the precipitate thin-film was conducted with the ethanolic PBS solution (1 : 9 EtOH/PBS, v/v) treatment (5 min). The displacement reaction was performed by exchanging the buffer with the solution of biotin (20 mM, in PBS) and incubating for 10 min.

Results and discussion

The construction of modified affinity surfaces, the signal generation reaction, and the routes of sensor surface regenerations are summarized in Scheme 1. The affinity surfaces were fabricated onto the e-beam evaporated gold surfaces *via* SAM technique.[12] A fourth-generation poly(amidoamine) dendrimer monolayer was formed onto an amine-reactive DTSP SAM.[13] As the base template for biofunctionalization, terminal amine groups from dendrimer monolayers were functionalized with sulfo-NHS-biotin, and model affinity reactions were performed with antibiotin antibody samples. Antibiotin monoclonal IgG–HRP conjugate (BN34 clone) was used for the enzyme-catalyzed signal generation. Precipitate thin-film formation was performed with a reagent mixture of 4-CN and hydrogen peroxide.

Fig. 1 shows a signalling result from biotin/antibiotin IgG affinity sensors based on biocatalyzed precipitation. For the freshly prepared affinity sensors, well-developed ferrocene/ferricinium redox waves were registered by cyclic voltammetry in the presence of 0.1 mM ferrocene methanol in electrolytes (Fig. 1(A)). After the biospecific antibiotin IgG–HRP association (30 min) and precipitation (10 min) reactions, immunoelectrodes exhibited typical background voltammograms, showing no distinguishable redox peaks (Fig. 1(B)). From the result, we assumed that the electrode surface was entirely blocked with the insoluble precipitate, benzo-4-chlorocyclohexadienone, which was produced by the biocatalytic reaction of labeled HRP with 4-CN. The colour change in photographs of the exposed biosensor surface upon signaling reaction confirmed the deposition of the precipitate thin-film (Fig. 1, inset).

After signalling reaction with the immunosensor, the affinity surfaces were irreversibly modified with bound protein conjugates and insoluble precipitate thin-films, excluding the possibility of reuse. Thus, we propose strategies of stepwise

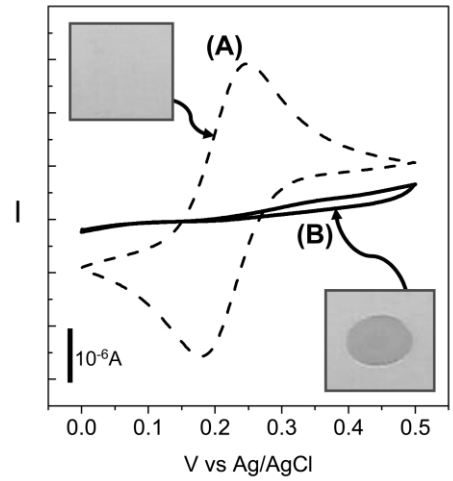
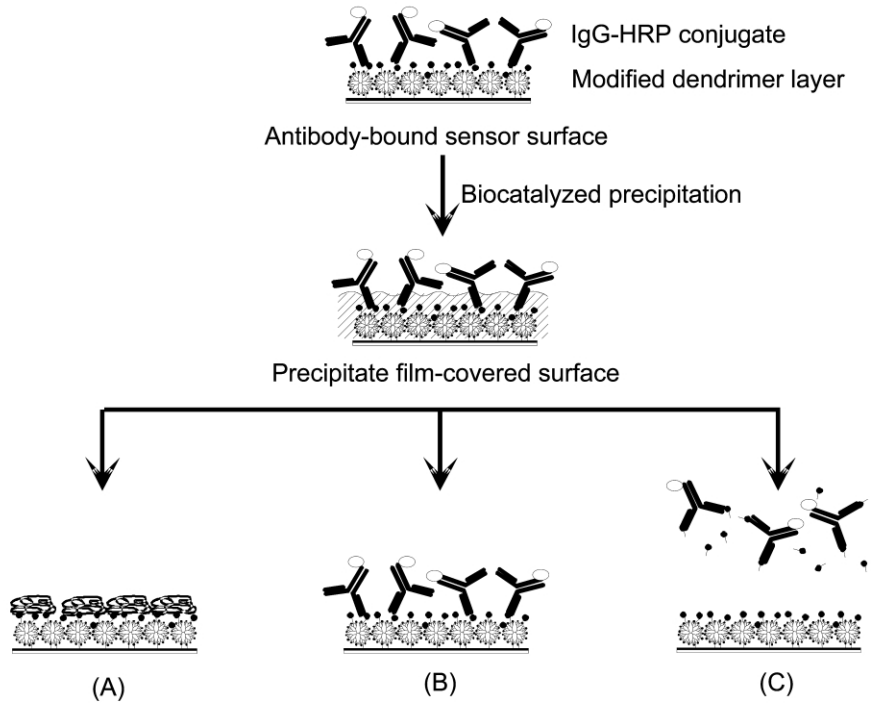


Fig. 1 Voltammetric traces for affinity sensor signalling: a biotin-functionalized surface before (A) and after (B) target protein (antibiotin IgG–HRP) association and precipitation reaction steps. Voltammetric measurements were performed in 0.1 M phosphate buffer (pH 7.0), containing 0.1 mM ferrocene methanol as a signal tracer. Inset: CCD camera images of a sensor surface upon signalling reactions.



Scheme 1 Schematic illustration of affinity biosensor construction, biocatalyzed precipitation, and the stepwise surface regeneration reactions. The dimensions of the components are not drawn to scale for simplicity.

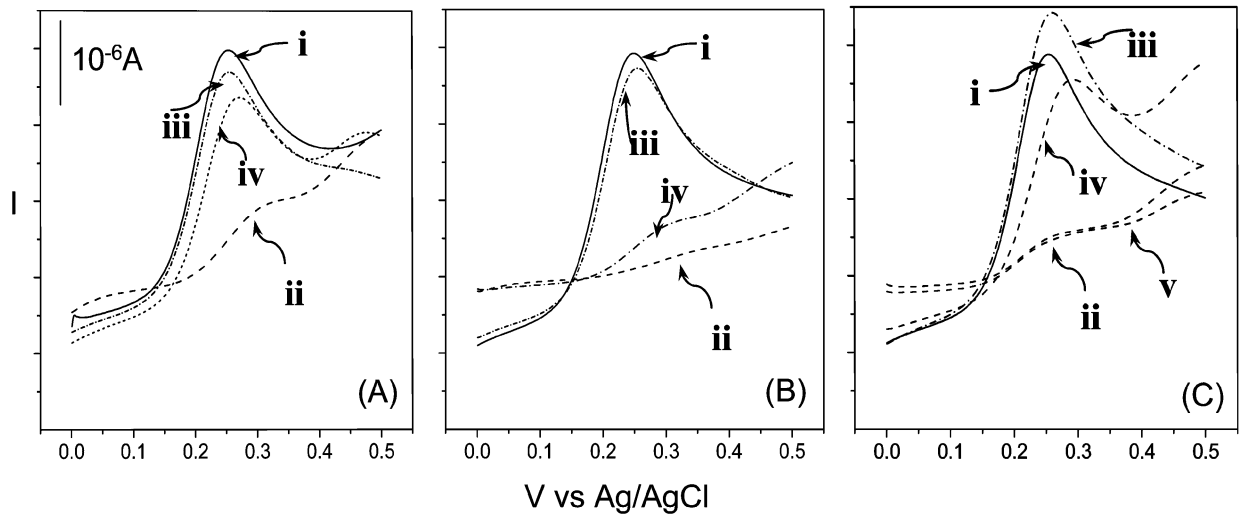


Fig. 2 Voltammetric traces for affinity sensor signalling upon different surface regeneration strategies: (A) organic solvent treatment, (B) ethanolic PBS solution treatment, (C) combination of ethanolic PBS solution treatment and displacement dissociation reactions. See text for details.

surface regeneration, the combination of dissolution of the precipitate thin-film and displacement dissociation of immobilized proteins.[14,15] First, surface regeneration by the solvent dissolution of the precipitate thin-film was tested as in Scheme 1(A). Voltammetric traces for each steps of the regeneration and reuse of immunosensors are shown in Fig. 2(A). Traces (i) and (ii) represent voltammograms for the newly prepared electrodes and after first protein association and precipitate formation, respectively. The construction of the biosensor surface, the biospecific affinity reaction, and signalling steps were under the same conditions as Fig. 1. After affinity sensing, the thin-film adsorbed surface was treated with an organic solvent to dissolve the water insoluble precipitates. Ethanol drops were applied to the sensor surface and reacted for 5 min. After surface regeneration with ethanol treatment, the electrode exhibited a well-developed oxidation curve as (iii), suggesting that the precipitate thin-film was successfully removed. But after another precipitation reaction with the regenerated surface, the biosensor did not respond (curve (iv)). The result suggests that the ethanol treatment successfully dissolves the insoluble precipitate thin-film from the surface, but the biological activities of biospecifically associated antibodies and labelled-HRP are significantly hampered, as schematically shown in Scheme 1(A). Thus, the solvent treatment method manifests its limitation for the regeneration of the surface and its reuse.

Fig. 2(B) shows the regeneration results from the treatment with a mixture of solvent and aqueous buffer solution for the dissolution of the precipitate film while maintaining the activity of immobilized biomolecules. After surface preparation (i) and signalling reaction (ii), mild surface regeneration was conducted with an ethanolic PBS solution (Scheme 1(B)), containing minimum amount of ethanol for the dissolution of water insoluble precipitates (1 : 9 EtOH/PBS, v/v). After regeneration reaction for 5 min, the electrode exhibited a fully recovered ferrocene oxidation wave (curve (iii)), showing the perfect regeneration and renewal of the surface. After another precipitation reaction with 4-CN and hydrogen peroxide, the electrode responded satisfactorily again (curve (iv)). From the result, mild dissolution treatment did not hamper the activity of immobilized HRP, beneficial for consecutive sensing reactions and further removal of immobilized biomolecules from the surface. It is assumed that about 80% of the initial activity of labelled-HRP is maintained after the regeneration step.

Next, for the complete renewal of the surface for another round of functionalizaton/immunosensing, a combination method consisting of dissolution of the precipitate film with an ethanolic PBS solution and displacement of bound IgG–HRP conjugates with biotin (vitamin H) was performed. After dissolution with the aforementioned ethanolic PBS (5 min) and displacement reaction with 20 mM biotin (10 min), the electrode exhibited voltammograms of fully recovered ferrocene oxidation (Fig. 2(C), curve (iii)). Then, the resulting biosensor was subjected to another precipitation reaction, yielding a deformed voltammogram (iv) but with essentially similar wave shape and peak height to curves (i) and (iii). The deformation of the resulting voltammogram was assumed to be due to the limited displacement yield. After this step, the electrode was made viable for another round of functionalization and immunosensing. Finally, the regenerated surface was used for another biospecific IgG–HRP association and signalling step. The electrode responded correctly (curve (v)), confirming that the sensor surface was regenerated in its initial condition as shown in Scheme 1(C) and is feasible for reuse.

Additionally, the developed sensor surface as well as the regeneration method could be directly applicable to biotin/(strept)avidin reaction couples. (Strept)avidin is a unique tetrameric protein of symmetric dimers, having identical binding sites for biotin to which other biotinylated proteins, ligands, and nucleic acids can be conjugated and used for the construction of platform affinity surfaces.[16–18] The developed stepwise regeneration method based on the combination of product thin-film dissolution and bound-protein displacement reactions would find applications in biosensors, biochips, and LOCs.

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