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Biocatalytic precipitation induced by an affinity reaction on dendrimer-activated surfaces for the electrochemical signaling from immunosensors

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We have developed a strategy of signal generation for immunosensors that transduces biospecific affinity recognition reactions into electrochemical signals. The cyclic voltammetric method, tracking the precipitation of insoluble products onto the sensing surface and the subsequent decrement in the electrode area, was chosen for signal registration. Precipitation of insolubilities was induced by the catalytic reaction of enzymes, which were labeled to the biospecifically attached protein or antibody molecules. As a model system for affinity recognition, we have investigated the functionalization of biotin groups to the sensing monolayer and their biospecific interactions with anti-biotin antibody molecules. The immunosensing interface was developed onto the dendrimer-activated self-assembled monolayers (SAMs), as the base template for the functionalization of the antigen moiety and signal generation. The advantages of using dendrimer-activated SAMs in comparison to the plain modified thiolate SAMs for the sensing surface were shown in terms of sensing performances, and the analytical characteristics of the resulting immunosensor were examined. Additionally, the sensing system was applied for biotin/(strept)avidin couples, extending the applicability of the developed strategy.

Introduction

There has been an increasing demand for efficient analytical tools for immunoassays in the fields of clinical analysis and biochemical studies. Based on the requirement, a new scientific field in bioassay has been emerging, linking bioanalytical techniques with microelectronics technology. Especially, immuno- or affinity-sensing techniques, registering biospecific interactions such as antigen–antibody, ligand–receptor and protein–protein recognition reactions, are under great demand in terms of miniaturization and automation. In addition, recent completion of the human genome project opened a new research field of proteomics, making the development of immunoassay techniques more important.

There exist many transduction methodologies for immunosensors such as optical (luminescence, fluorescence, surface plasmon resonance), electrochemical (amperometric, conductimetric, potentiometric), and gravimetric (quartz crystal microbalance) techniques.[1,2] For each of the methodologies, technical developments are pursued to accomplish parallel analyses with high throughput/output as well as the invention of novel signaling methodologies. Of the transduction techniques in current use, electrochemical methods are believed to meet the needs of sensor miniaturization and automation due to the relatively direct application of the microelectronics that have been developed during the last decades.[3–5] On the other hand, optical techniques are being developed for clinical laboratory uses in the form of bench-top scale instruments, due to the rather complicated instrumentation and accompanying difficulties in miniaturization.[6,7] Therefore, for the POCT (point-of-care-testing) application, signal transduction strategies based on electrochemical methods are assumed to be the choice. To date, a number of immunosensing formats were reported based on electrochemical techniques, including amperometric signaling from the labeled-enzyme catalysis that was induced by antigen/antibody interactions,[8,9] capacitive sensing with interdigitated electrodes,[10] and impedimetric transduction techniques.[11,12]

From the viewpoint of biosensor construction and its miniaturization for high-throughput analysis, the surface on which biological entities are immobilized and biospecific interactions take place is important. Thus, specially designed and modified surfaces were developed to achieve biospecific interactions with high yields and to maintain the activity/stability of functionalized biological species. In our previous reports, we have shown that poly(amidoamine) dendrimer-modified surfaces could effectively be made onto electrode surfaces and functionalized with chemical and biological ligand groups for the purpose of affinity and catalytic biosensing.[13–15] From these studies, it was shown that dendrimer films could be used as the platform for biofunctionalization on which efficient biospecific interactions take place.

In the present work, a strategy of signal generation for immunosensors that converts the biospecific affinity recognition reactions into electrochemical signals is proposed. The cyclic voltammetric method, electrochemically tracking the decrements in the electrode area resulting from the precipitation of insoluble product, was chosen for signal registration. Precipitation and deposition of an insulating film on the sensing surface was induced by the catalytic reaction of enzymes labeled to the biospecifically associated target molecules.[16,17] Anti-biotin monoclonal antibody and (strept)avidin were used as model recognition targets for biotinyl groups which were functionalized on electrode surfaces. As the template layer for the functionalization and affinity sensing, dendrimer-activated SAMs were employed. Modified-SAM supported layers were also tested and compared with the dendrimer-assisted ones. Details are reported herein.

Experimental

Materials

Amine-terminated fourth generation poly(amidoamine) dendrimer is manufactured by Dendritech and was purchased from

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Aldrich. Ferrocene methanol and 2,2-(ethylenedioxy)-bis(ethylamine) (DADOO) were 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), and 4-chloro-1-naphthol (4-CN) were purchased from Sigma and used as received. Anti-biotin monoclonal antibody–HRP conjugate (BN34 clone) and HRP labeled avidin (HRP–avidin) were from Sigma and used without further purification. Poly-peroxidase labeled streptavidin (polyHRP–SA) was obtained from Endogen and used after proper treatment as recommended by the manufacturer. 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 work.

Construction of the affinity sensing surface

Base substrates for the affinity sensing monolayers were thin film gold surfaces, which were prepared by e-beam evaporation of 200 nm of gold onto titanum-primed (40 nm) silicon wafers (Si[100]). As the first step, a DTSP SAM was constructed on the freshly prepared gold surfaces to render the surface amine-reactive. Prior to the chemisorption of DTSP and SAM formation, the gold surfaces were cleaned by immersing them in piranha solution for 5 min. (**Caution:** piranha solution will react violently with most organic materials and must be handled with extreme care.) After cleaning, the DTSP SAM was prepared by dipping (1 h) the gold substrate in a 5 mM DTSP in DMSO. After monolayer formation and rinsing steps with DMSO and ethanol, the electrode surface was incubated with G4 poly-(amidoamine) dendrimer solution. A diluted ethanolic solution of dendrimer (1% w/w) was reacted (2 h) with the prepared succinimidyl ester-activated surfaces. As a comparative test, an ethanolic DADOO solution (20 mM) was used for the surface modification of the DTSP SAM in place of the dendrimer.

After thorough rinsing of the surface to remove the weakly adsorbed dendrimers, the modified surfaces were immersed in bicarbonate buffer (0.1 M, pH 9.5) to equilibrate the surface for further biotinylation reaction. Biotinylation of the dendrimer-modified layers was performed with sulfo-NHS-biotin, an activated ester of biotin with an extended amidocaproate arm. An aqueous solution of sulfo-NHS-biotin (2 mg mL^{-1}, in water) was reacted with the terminal amine groups from the dendrimer monolayer by addition to the electrodes immersed in bicarbonate buffer. The sulfo-NHS-biotin concentration in the final reaction mixture was adjusted to 2 mM. After reacting for 2 h, the resulting surfaces were rinsed with bicarbonate buffer and distilled water, and were stored in PBST for further biospecific affinity reactions with (strept)avidin or antibody samples.

Affinity sensing procedures

Before the affinity-sensing tests, the prepared electrodes were rinsed and clamped to Teflon electrode holders. The holder was designed to expose the active electrode area (0.148 cm^2) and the reaction volume (3 mL). Biospecific protein binding on the constructed affinity sensing surfaces was performed with model analyte samples including anti-biotin monoclonal antibody, avidin, and streptavidin. All three protein samples are in the form of horseradish peroxidase (HRP) conjugates for the enzyme-catalyzed signal generation reaction. Especially for the purpose of signal amplification, poly-peroxidase labeled streptavidin (polyHRP–SA) was used.

After assembly, biospecific affinity reactions were performed with target protein samples. For the antibiotin IgG–HRP, aliquots of antibody sample (100 µL) of predetermined concentrations ranging from 1 ng mL^{-1} to 1 mg mL^{-1} were prepared in PBST and were incubated at the electrode for 30 min at room temperature. For the HRP–avidin and polyHRP–SA, lyophilisate samples were dissolved into PBST to the concentration of 20 µg protein mL^{-1} and were incubated at the electrode. After incubation reaction, the antibody- or (strept)avidin-associated surfaces were subjected to the signal generation/measurement step after thorough rinsing to detach the weakly adsorbed protein molecules from the surface.

For the signal generation, an enzyme-catalyzed precipitate formation reaction was adopted and the reaction couple of HRP with 4-CN was used in this study. Ethanolic 4-CN was initially prepared to a concentration of 50 mM. Fifty µL aliquots of prepared 4-CN 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 it with the analysis mixture for a determined time (*vide infra*). The surfaces were then thoroughly washed and subjected to the electrochemical transduction step.

Instrumentation

Cyclic 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 Au thin-film working electrode, a Pt wire auxiliary electrode, and an Ag/AgCl (3 M NaCl, BAS) reference electrode was used throughout the study. Cyclic voltammetric measurements were performed either in 0.1 M phosphate buffer, pH 7.0, as the background electrolyte or in a buffer solution containing 0.1 mM ferrocene methanol for signal registration. All experiments were performed at room temperature under ambient conditions.

Inspections of the sensor surfaces for precipitate formation and color change were performed with a color CCD camera-installed optical microscope system.

Results and discussion

Major concerns for the development of affinity biosensors analyzing biospecific recognition reactions at sensor surfaces can be summarized as follows. Biomolecular interactions in solution should be reproduced on the sensor chip surfaces, where only limited surface concentrations of the functional groups are available. Therefore, effective biofunctionalization of the surfaces with desirable density as well as conserved activity of the immobilized biomolecules is important. Another important issue is the invention of an effective transduction strategy to provide correct signals rapidly from the biosensors. In comparison to the cases of typical test-tube and microwell assays, the surface concentrations of the immobilized biomolecules at the affinity biosensor are relatively low, of the order of 10^{-12} mol cm^{-2}.[13,15,18] Thus, a powerful signaling methodology that enables the effective transduction of biomolecular interactions into a scalable signal is indispensable.

To accomplish this objective, we devised a specially designed sensing interface that is modified with a dendrimer-assisted SAM, which is functionalizable with biological ligand groups for affinity sensing. And an electrochemical method for the biosensor signaling, which is based on the formation of an enzyme-catalyzed insoluble precipitate on the sensor surface and the tracking of changes in the electrode surface area, was developed.

A schematic representation of the chemically modified and biofunctionalized electrode surface and the signal generation reaction are summarized in Fig. 1. The affinity sensing layers were fabricated onto the e-beam evaporated gold surfaces *via*

the SAM technique. As an adhesive layer for the dendrimer monolayer formation, an amine-reactive DTSP SAM was firstly prepared on a gold electrode surface. Succinimidyl ester groups from the DTSP SAM readily react with terminal amine functional groups from the dendrimers yielding covalent amide linkages. It is known that amine-terminated dendrimer molecules form SAM-like structures on gold surfaces through multiple amine/Au interactions.[19] However, the resulting layer was not found sufficiently stable to endure the following successive modification and reaction steps. We used, therefore, a DTSP adhesive layer to ensure the stability of the affinity sensing interface. After the DTSP SAM formation and rinsing steps, the dendrimer solution was reacted to yield a covalently-modified dendrimer monolayer. The dendrimer-modified portion of the electrode surface worked as the base platform for further ligand functionalization and biospecific interactions with target proteins. In this study, we chose the biotinyl ligand group because several types of model proteins are readily available for biospecific recognition reactions including anti-biotin antibody and (strept)avidin, and there also exist further applicational capabilities incorporating biotinylated biomolecules such as nucleic acids, lectins, and biopolymers.

The analyte samples, containing the target proteins, antibiotin IgG–HRP or (strept)avidin–HRP conjugates, were then incubated with the biotinyl group functionalized surfaces. After biospecific affinity reaction and a proper washing step, the signal generation reaction was performed with the target-protein-associated electrodes.

For the precipitate formation reaction, a mixture of H_2O_2 and 4-CN was prepared and used. Peroxidase (HRP)-mediated conversion of 4-CN to benzo-4-chlorocyclohexadienone with H_2O_2 yielded insoluble precipitates on the sensor surface in which the biocatalyzed reaction took place. From the production and gravitational deposition of the insoluble precipitates, a film with a distinct color change was formed on the exposed and protein-associated region of the electrode surface. The result was discernible with naked eyes, and the photographs of resulting surfaces were shown in Fig. 1. The distinct change in color at the exposed region of the affinity-sensor surface (*positive*) confirmed that the biocatalyzed reaction occurred efficiently. However, for rapid registration of the easily quantifiable signals, an electrochemical transduction strategy was developed in this study.

The enzyme label used in this study was HRP. Other enzyme candidates also exist including alkaline phosphatase (ALP) and glucose oxidase (GOx), but HRP was chosen for its superiority in enzymatic sensitivity, endogenous activity, and stability. There are also several substrates of labelled enzymes that produce insoluble precipitates, developed for use in ELISA assays. These include 3,3′-diaminobenzidine, 3-amino-9-ethylcarbazole, tetramethylbenzidine and 4-CN. Of them, 4-CN was chosen because the discrimination of resulting color changes and electrochemical signal quantification were easier in comparison to other compounds.

For biosensor signal registration, an electrochemical method was used. A cyclic voltammetric signaling method, tracking the changes in the active electrode area from the precipitation of the insolubilities onto the electrode surfaces, was developed. (Fig. 2) With the freshly prepared, ligand-functionalized (biotinylated) surfaces, cyclic voltammetric tests were conducted in the presence of 0.1 mM ferrocene methanol as an electroactive signal tracer in the electrolyte. (Fig. 2A) As shown in the figure, the electrode showed a typical cyclic voltammogram for the ferrocene/ferricinium redox couple. This cyclic voltammogram was taken as the background signal prior to the biospecific affinity and signaling reactions. After proper washing, the electrode was subjected to consecutive steps: biospecific protein association (30 min) with antibiotin IgG–HRP and precipitation reaction (10 min) with 4-CN. The resulting electrode surface, on which the insoluble precipitate was deposited, was then transferred to buffer solution for rinsing to remove the nonspecifically adsorbed protein molecules and impurities. The electrode was subjected to cyclic voltammetry under the same conditions as Fig. 2A. As a result, in case of the affinity surface that was modified with biospecifically associated protein molecules, the registered voltammogram was typical for the background one, showing no distinguishable redox waves (Fig. 2B). Therefore, we assumed that the electrode surface was entirely blocked by the deposition of insoluble benzo-4-chlorocyclohexadienone that was converted from 4-CN through the biocatalytic reaction of labeled HRP. The authors also found cyclic voltammograms that showed ferrocene/ferricinium redox waves of various peak heights, showing the possibility of correlating the changes in surface area (changes in waveform and peak heights from voltammograms) with the extent of the biocatalytic reaction. The cyclic voltammogram presented in Fig. 2B was from a test after the optimization experiments for the affinity-surface fabrication and signaling reaction had completed. From this result, it was proposed that the insoluble precipitates produced by the labeled enzyme would form an impermeable film on the electrode surface, and the decrements in the active electrode area could be interpreted as the extent of biocatalyzed reaction. Under the controlled reaction conditions, including fixed reaction time

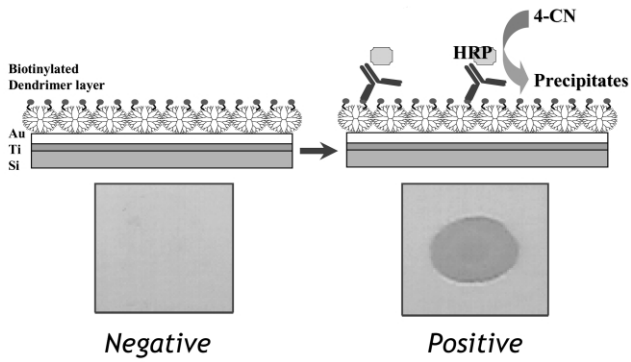

Fig. 1 Schematic representation of the affinity biosensor construction and the proposed operational principle (top), and the CCD camera images of a representative surface upon signaling reaction (bottom). The dimensions of the components are not drawn to scale for simplicity.

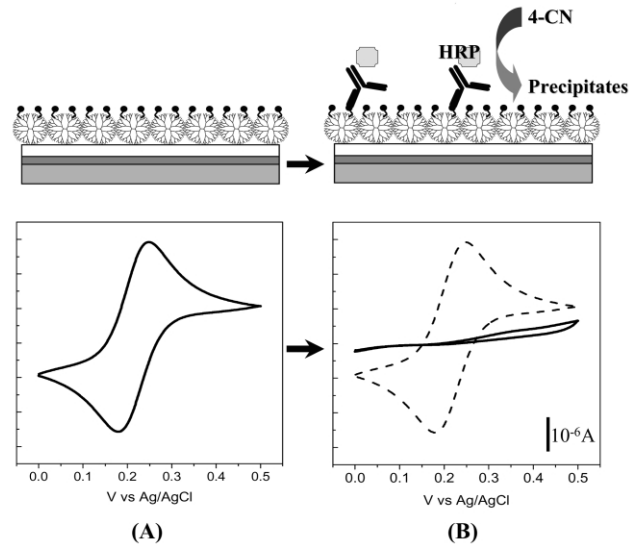

Fig. 2 Cyclic voltammetric traces for sensor signaling at the dendrimer-assisted SAM surfaces: a freshly prepared and biotin-functionalized surface before (A) and after target protein association and precipitation reaction steps (B). Curves were registered in a 0.1 M phosphate buffer (pH 7) containing ferrocene methanol (0.1 mM) as a signal tracer. Potential scan rate was 50 mV s^{-1}.

and concentrations of reagents being used, changes in the electrochemical signals could be correlated to the units of surface-bound HRP and to the number of biospecifically associated antibiotin IgG molecules.

Next, to confirm the proposed signaling principle and to evaluate the merits of constructing the immunosensing surface by using dendrimer-assisted SAMs, electrochemical tests with a number of control sets were conducted. Fig. 3 shows the results from the electrochemical tests for dendrimer-assisted SAMs (A), DADOO-assisted SAMs (B), and two control sensor surfaces (C, D) for antibiotin IgG affinity sensing. Each panel presents two cyclic voltammograms that were taken before (dashed lines) and after the antibody–HRP association and precipitation reactions (solid lines). Voltammograms from the same electrode are presented in each panel. Fig. 3A shows the results from a dendrimer-assisted SAM immunosensor, revealing an efficient surface blockage after the biospecific association and precipitation reactions. The formation of an insoluble film by precipitate deposition and accompanying color change were observed after the electrochemical test. For comparison, an immunosensing surface based on the functionalized SAM without an intervening dendrimer layer was employed. A bifunctional amine-terminated oligo(ethylene glycol) linker molecule, DADOO, was used in place of the dendrimers for the platform surface formation onto the DTSP SAM and biotinylation. The oligo(ethylene glycol) incorporated surface is useful for the known resistance of the functional group to nonspecific protein adsorption.[20,21] Under the same experimental conditions as in panel (A), the DADOO-SAM assisted immunosensor exhibited a different voltammetric pattern after antibody association and precipitation reactions. As can be seen in Fig. 3B, the voltammogram after precipitation reaction still exhibited redox waves of ferrocenyls, and did not reach the background one, as in Fig. 3A. The distorted and poorly developed voltammogram revealed that a complete surface blockage was hard to achieve with the plain modified SAM-assisted electrodes. However, it was evident that the proposed signaling principle worked with this surface format because an entirely shielded surface was also attainable when the reaction time for precipitate formation was extended to over 45 min (data not shown). Therefore, it was assumed that the limited number (units) of biospecifically bound biomolecules on the SAM-assisted affinity surface is the reason for the reaction inefficiency. With this limited reaction yield, only a narrow working range was obtainable for the electrochemical affinity-sensor. Also, the required time extension for the signaling reaction limits the usage of this format in comparison to the dendrimer-assisted one.

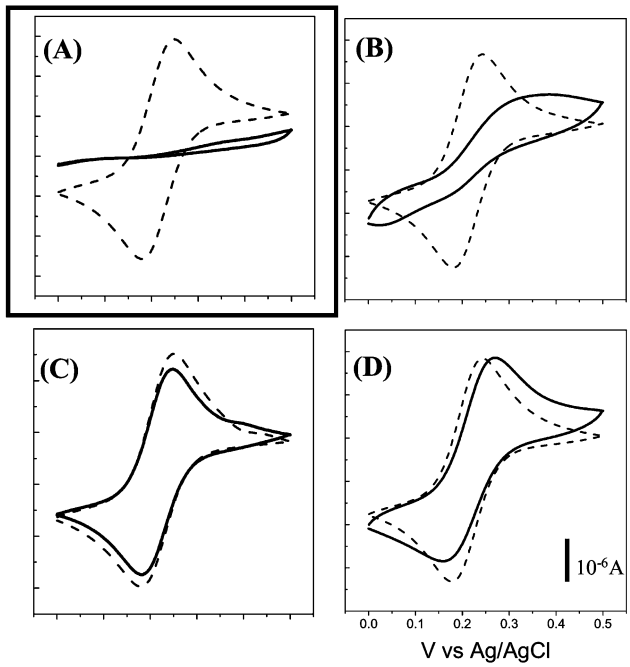

Fig. 3 Cyclic voltammograms of biotin/antibiotin IgG affinity biosensors at different surface construction and signaling conditions: (A) dendrimer-assisted SAM, (B) DADOO-assisted SAM, (C) HRP negative, and (D) ligand negative. Each panel contains voltammograms registered before (dashed curves) and after the signaling step (solid curves). See text for details.

For the biospecific recognition interactions that are derived at the sensor chip surfaces, three major concerns arise. First, the target proteins must be captured biospecifically at the affinity-sensing surfaces within a satisfactory density range. For the reactions at the chip surfaces, the number of available active sites is restricted, contrary to the reactions in test-tubes. From the results of previous reports, the estimated surface density of biomolecules resides in the range $1–5 \times 10^{-12}$ mol cm^{-2}.[15,18] This is attributable to the limited yields of the chemical reactions and the steric limitations from biomolecular interactions, both at chip surfaces. Second, the stability of the associated proteins, target antibody molecules and labeled peroxidases in this study, should be maintained. Third, the nonspecific binding (NSB) of the biomolecules at the affinity-sensing surfaces should be minimized. This last concern can be addressed by the incorporation of NSB-resistant functional groups in the sensing layer or by changing the surface properties, such as surface charge, hydrophobicity, *etc*.

Although there were technological advances for the biosensing interfaces including self-assembled thiol and silane-based monolayers, conventional polymer-supported surfaces are continuously used as platforms for biofunctionalization and biosensing purposes. This is due to the limitation of the SAM technology in that the yields of surface 'bio'-functionalization reactions are restricted and are dependent on experimental variables. To address this issue in view of the above concerns, approaches based on a mixed-SAM with controlled component ratio and grafting or adlayer formation to gain special functions for the sensing surfaces have been attempted.[21–23]

Dendrimers possess unique characteristics such as their exact molecular weight, size, and number of functional groups, which means they are regarded as discrete chemicals in comparison to linear or branched polymers exhibiting polydispersity.[24] Therefore, the use of dendrimers as the building units for the interfacing layer would encompass the advantages of using polymer and SAM-based biosensing surfaces. Based on the experimental results and the considerations described above, a dendrimer-assisted biosensing interface was selected as representing the merits of an organized monolayer and active biofunctionalization with high density.

Additionally, we prepared a set of affinity sensing surfaces for control experiments. When the dendrimer-assisted sensing surface was treated with a non-labeled antibiotin IgG sample (HRP negative, Fig. 3C), changes in the cyclic voltammograms and the color of the sensing surface were negligible after the precipitation reaction, showing that the signaling reaction is catalyzed by the biospecifically associated enzymes. However, a small retardation in the peak current was observed, which might be due to the nonspecific binding of the immunoglobulin molecules to the surface. As another control test, the affinity biosensor exhibited a negligible signal change when the dendrimer-assisted surface was not biotinylated (Ligand negative, Fig. 3D), proving that the derived signal is attributed to biospecific biotin–antibiotin IgG recognition and HRP-mediated precipitation rather than nonspecific protein adsorption to the electrode surface.

For the evaluation of the analytical performance of the developed affinity sensor, calibration experiments for the antibiotin antibody target analyte were conducted. As shown in Fig. 4, the electrochemical signals were registered at different

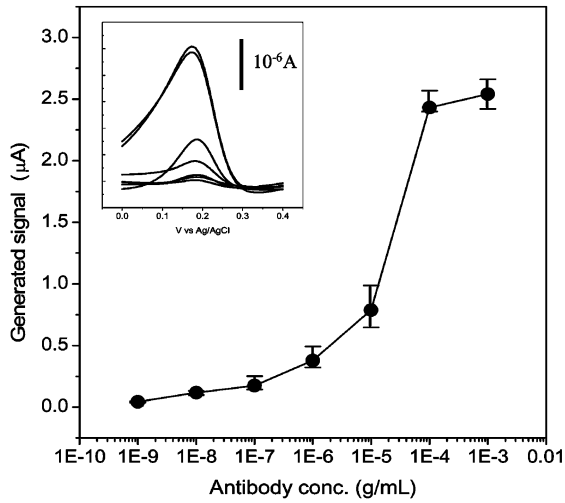

Fig. 4 Calibration curve for the biotin/antibiotin IgG affinity biosensor as a function of target protein concentration. Signal current levels were registered at cathodic peaks from the background-subtracted voltammograms (inset) for respective analyte concentrations.

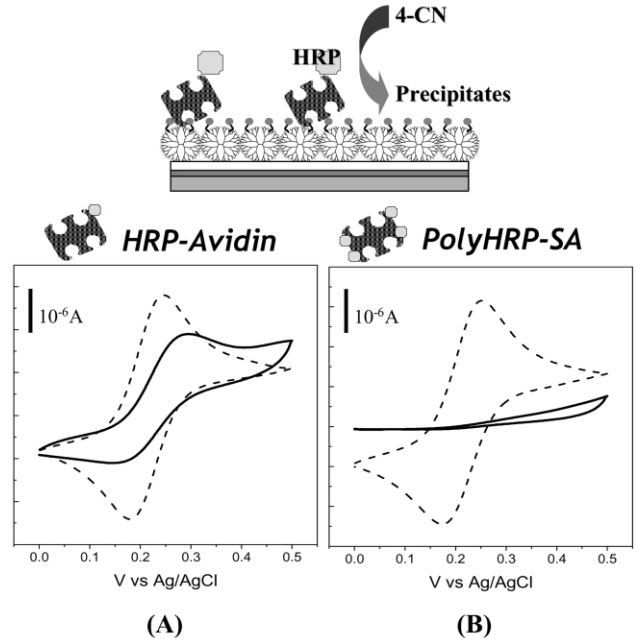

Fig. 5 Top: Schematic representation of biotin/(strept)avidin affinity sensing at the dendrimer-assisted SAM surface. Bottom: Cyclic voltammetric traces for HRP–avidin (A) and polyHRP–SA (B) affinity sensing. Each panel contains voltammograms registered before (dashed curves) and after the signaling step (solid curves). Conditions for the electrochemical tests were the same as Fig. 2.

antibiotin IgG concentrations from the respective background-subtracted cyclic voltammograms. Signal current levels were measured at the cathodic peaks for the ferricinium reduction curves (inset, Fig. 4). Under the specified conditions of the biospecific antibiotin IgG association reaction, the HRP-mediated precipitation reaction, and the measurement step, the immunosensor exhibited a rising sigmoidal calibration curve for the target antibody concentration. From the calibration plot on a semi-logarithmic scale, a dynamic detection range of from 0.1 to 100 μg mL^{-1} antibody concentration was obtained. The dynamic sensing range of an immunosensor is generally affected by physico/chemical variables such as reaction time, temperature, and the concentrations of biochemicals used. Thus, the detection range would have to be modified for further applications.

As an extension to the study, we applied the system to the biotin/(strept)avidin reaction couple, because of its wide usage in the bioanalytical sciences. We investigated the uses of HRP-conjugated avidin (HRP–avidin) and polyHRP-functionalized streptavidin (polyHRP–SA) as target proteins for the dendrimer-assisted and biotinylated sensor surfaces. The idealized schematic illustration of the reaction is included in Fig. 5. As can be seen in Fig. 5, the electrodes treated with HRP–avidin (A) or polyHRP–SA (B) showed responsive changes in their voltammograms after the precipitation reaction. But, the magnitudes of the signal decrements were markedly different and were dependent on the units of enzyme molecules labeled to the target proteins. For polyHRP–SA, a streptavidin molecule was conjugated with a polymer of HRP (polymerization range ~40),[25] an enhanced rate of catalytic precipitation and a faster signaling time (*ca*. 2 min) were obtained (Fig. 5B). Under the same reaction conditions, the HRP–avidin treated immunosensor showed an incomplete surface blockage (Fig. 5A). However, when the reaction time was extended to 10 min, as in the case with the antibiotin IgG–HRP conjugate, a voltammogram representing complete surface shielding was obtained. In addition, a control experiment in which biotin-pretreated HRP–avidin was used for the affinity association and signaling reactions exhibited no response, revealing that the signal observed in Fig. 5 was from a biospecific biotin–avidin interaction and the effect of nonspecific protein adsorption was not significant.

From this study, we have developed a signaling strategy for immunosensors based on a biocatalyzed precipitation reaction and electrochemical transduction. We demonstrated the versatility of the system to (strept)avidin as well as antibodies, and showed the merits of using dendrimer-assisted layers for the functionalization of the biospecific sensing interfaces. From the results, it is expected that dendrimers will be employed as materials for platform formation for protein microarrays and biomolecular micropatterns.

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