Biotechnology and Bioprocess Engineering **2004**, 9: 107-111

Bioelectrocatalyzed Signal Amplification for Affinity Interactions at Chemically Modified Electrodes

Hyun C. Yoon[1]* and Hak-Sung Kim[2]

[1] Department of Biotechnology, Ajou University, Suwon 442-749, Korea
[2] Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon 305-701, Korea

Abstract A comparative study was performed to evaluate the signal amplification strategies in electrochemical affinity sensing, which included the direct electron transfer and diffusible-group mediated electron transfer between label enzymes that were specifically bound to target proteins and chemically modified electrode surfaces. As a platform surface for affinity recognition reactions, a double functionalized poly(amidoamine) dendrimer monolayer that was modified with ferrocene and biotin groups was constructed on a gold surface. With the chemically modified electrode, a model affinity sensing with avidin was investigated. The advantages of adopting the diffusible-group mediated signaling strategy were demonstrated in terms of signal sensitivity and stability.

Keywords: biosensor, immunosensor, electrochemical, avidin, affinity

INTRODUCTION

With the growing interest in the lab-on-a-chip (LOC) or micro-total analysis system (μ-TAS) and their integration with biosensor technology, much effort is being devoted toward parallel analysis with a high density and an acceptable signal sensitivity [1-4]. This relatively new research field of using microelectromechanical systems (MEMS)-based microdevices in biological applications, or bioMEMS, now possesses a major part of the relevant technology. For the above objective, researchers follow two main approaches consisting of the development of efficient signal transducing strategies and the introduction of new sensing materials or interface designs [5,6]. Especially for the implementation of immunoassays into microsystem-based biosensors, registering biospecific recognition reactions of antibody/antigen and ligand/receptor, a signal transducing method that is flexible in operation and whose signal is highly quantifiable is indispensable. The current research trend is shifting from the lateral-flow immunoassay strip [7,8], of which the signal is semi-quantifiable with the colorimetric detection, toward the microsystem-based immunosensing LOC with the readily quantifiable electrochemical signaling [9-11].

We report here a comparative study for the evaluation of signal amplification strategies in electrochemical affinity biosensing. The electrochemical current from the biosensor was generated by the biocatalytic reaction of redox enzymes that were labeled to the target proteins in either the direct electron transfer between the prosthetic group of the label enzyme and the electroactive functionality on the electrode surface or the shuttled electron transfer facilitated by diffusible mediators in the bulk electrolyte. The target protein adopted in the current study was avidin that holds the potential for versatile use in biorecognition reactions.

***Corresponding author**
Tel: +82-31-219-2512 Fax: +82-31-219-2394
e-mail: hcyoon@ajou.ac.kr

MATERIALS AND METHODS

Reagents and Instrumentation

Amine-terminated fourth generation poly(amidoamine) dendrimer was manufactured by Dendritech and was purchased from Aldrich. The syntheses of partial ferrocenyl-tethered dendrimers have previously been described [12], and the dendrimer with ca. 23% surface modification was used in this study. 3,3-Dithiopropionic acid bis-*N*-hydroxysuccinimide ester (DTSP), biotinyl-amidocaproic acid *N*-hydroxysulfosuccinimide ester, and D-(+)-glucose were purchased from Sigma. Ferrocene methanol was purchased from Aldrich and was used as received. Avidin from hen egg white and biotin-amidocaproyl labeled glucose oxidase (b-GOx, from *Aspergillus niger*, minimum 4 moles biotin per mole protein) were from Sigma 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, such as cyclic voltammetry and coulometry, were carried out with an electro-

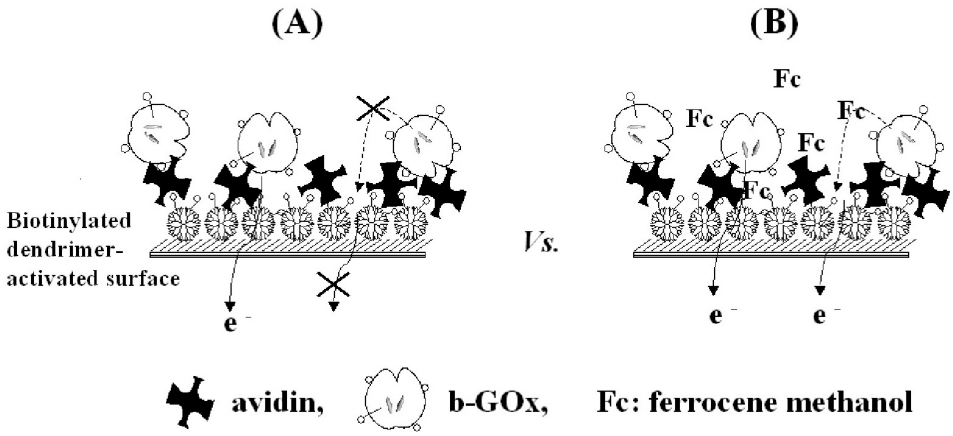

Fig. 1. Schematic representation of the affinity sensing surface and the signal generation modes: (A) the direct electron transfer mode and (B) the diffusible-group mediated electron transfer mode. Assumed paths of the electrons are shown. For simplicity, the dimensions of the components are not drawn to scale.

chemical analyzer (BAS CV-50W) 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 were used. All experiments were performed at room temperature under ambient conditions.

Procedure for the Affinity Biosensor Construction

The affinity biosensing interface was made on an evaporated gold surface, which was prepared through resistive evaporation of 200 nm of gold (99.999%) onto a titanium-primed (20 nm) silicon [100] wafer. Freshly prepared surfaces were used as base substrates for the affinity-sensing monolayer construction. Prior to the bottom-up layering processes, the electrode surfaces were cleaned by immersing them in piranha solution (1:4 by volume of 30% H_2O_2 and concentrated H_2SO_4, 5 min). Piranha solution reacts violently with most organic materials and must be handled with extreme care.

As the first step of affinity-sensing monolayer formation, a DTSP self-assembled monolayer (SAM) was constructed on the cleaned gold surfaces to render the surface amine-reactive for dendrimer functionalization. The DTSP SAM was prepared by dipping the gold substrate in a 5 mM DTSP in DMSO for an hour. After the SAM formation and rinsing steps with DMSO and ethanol, the electrode surface was modified with ferrocenyl-tethered dendrimer. A diluted ethanolic solution of dendrimer (1% w/w) was allowed to react for an hour with the DTSP-SAM activated surfaces. Then, the dendrimer-modified surfaces further underwent the biotin functionalization step. The modified surfaces were rinsed and immersed in bicarbonate buffer (0.1 M, pH 9.5) in order to change the environment from an organic to an aqueous solvent. An aqueous solution of biotinyl-amidocaproic acid *N*-hydroxysulfosuccinimide ester (2 mM) reacted (2 h) with the remaining terminal amine groups from the dendrimer monolayer. Other functional groups have been employed for ferrocene functionalization and attachment to the activated electrode surface. The resulting surfaces were rinsed and stored in phosphate buffered saline solution for affinity biosensing.

Affinity Biosensing Procedure

Prior to affinity sensing, electrodes 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 avidin, the natural recognition couple to the surface functionalized biotin. Aliquots of avidin samples (50 μL) of predetermined concentrations were prepared in the phosphate buffered saline solution and were incubated for 30 min at the biosensor surface. After rinsing, the avidin-associated surfaces were subjected to the enzyme-labeling step with b-GOx (1 mg/mL) for 30 min.

For the signal generation, electrochemical tests were conducted to register the current from bioelectrocatalysis at the affinity sensing electrodes. The anodically generated current from the redox reaction of b-GOx, which was mediated by either the surface confined ferrocenyls groups or the freely diffusible ferrocene mediators, was registered by cyclic voltammetry. For the direct signaling mode, voltammetric measurements were conducted in a 0.1 M phosphate buffer (pH 7.0) in the presence of 10 mM glucose as the enzyme substrate. In the mediated signal amplification mode, signal currents were registered by cyclic voltammetry in the presence of ferrocene methanol (0.1 mM) as a free-diffusing electron transferring mediator and glucose substrate (10 mM).

RESULTS AND DISCUSSION

As a platform surface for affinity biosensors, the electroactive dendrimer modified SAM/gold electrode was developed (Fig. 1). This configuration of the biochip platform surface is believed to be adequate for registering

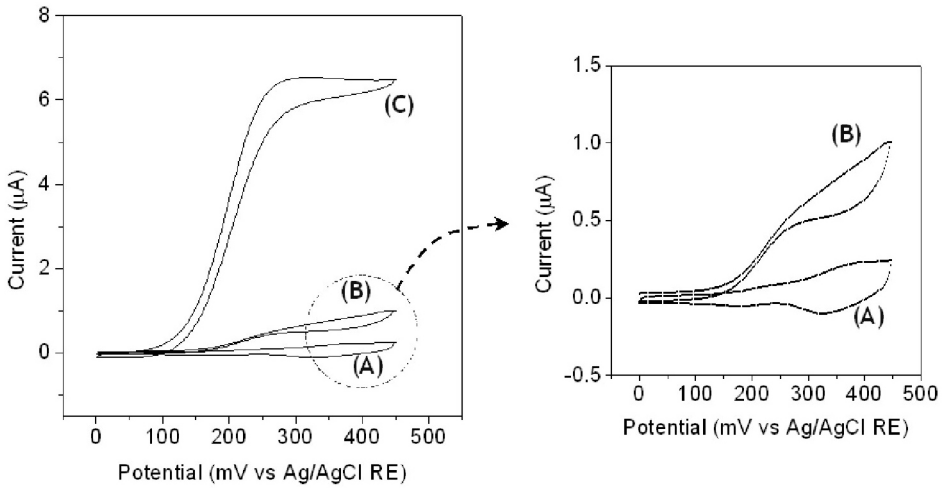

Fig. 2. Voltammetric traces for the affinity biosensor signaling: an avidin-associated surface before (A) and after (B) the b-GOx treatment and bioelectrocatalysis. Trace (C) was registered after the addition of a 0.1 mM ferrocene methanol mediator in the electrolyte. Measurements were performed in a 0.1 M phosphate buffer (pH 7.0) under the potential sweep rate of 5 mV/s. A magnified view of the voltammograms (A) and (B) is presented for clarity on the right.

bioelectrocatalytic signals because the intervening ferrocenyls-functionalized dendrimer monolayer excludes direct contact of enzymes and target proteins to the gold surface and their irreversible adsorption, while maintaining initial electroactivity as the role of the electron transfer path. We have shown that the dendrimer-modified surfaces can be made onto solid substrates (*e.g.,* gold and glass) and can be functionalized with chemical and biological ligands for the purposes of biosensors [12,13] and bio-micropatterning [14].

As a model system for affinity recognition reactions, we investigated the functionalization of biotin groups to the surface monolayer and also their subsequent biospecific interactions with avidin molecules and signal-generating enzyme labels. The electrochemical signal from the affinity biosensor was generated by the biocatalytic reaction of biospecifically bound biotin-amidocaproyl modified glucose oxidase (b-GOx) that was associated onto the preadsorbed avidin layer in either (i) the direct electron transfer between the prosthetic group of b-GOx (FAD: flavin adenine dinucleotide) and the electroactive ferrocenyl functionality on the dendrimer monolayer or (ii) the mediated electron transfer facilitated by diffusible ferrocenyls (ferrocene methanol) that were added in the bulk electrolyte. Fig. 1 shows the contrasting signaling modes schematically.

Cyclic voltammetry was employed to investigate the signal amplifications with affinity biosensors. Fig. 2 represents the signaling results from the direct electron transfer (trace B) and the shuttled electron transfer by diffusible mediators (trace C). As a result, both modes of signaling exhibited anodically amplified voltammograms that are typical for the enzyme-catalyzed and ferrocene-mediated reactions. However, the mode of direct electron transfer (trace B) yielded only a small signal, which is considered to be due to the limited chance of direct electrical communication between the FAD and the surface-functionalized ferrocene group and to the base electrode by the long-range electron hopping mechanism [15,16]. The importance of adequate orientation and positioning of electron transferring constituents in biological systems cannot be overemphasized [17,18]. On the other hand, efficient signal amplification was accomplished in the electron transfer mode that was facilitated by diffusible mediators (trace C, Fig. 2). Fully developed cyclic voltammograms, whose signal magnitudes directly correlated to the associated avidin concentrations, were registered from the experiment. It was also noted that the voltammogram (C) in Fig. 2 showed no further increase in anodic current from the hydrogen peroxide oxidation around +450 mV under ambient conditions, which was found significant in the voltammogram (B), however, suggested the efficient electron transfer from the FAD prosthetic group to the electrode by the diffusible mediator-facilitated electron transfer. Hence, the mediator-facilitated electron transfer mode (Fig. 1B) was employed for avidin affinity sensing, and the merits in terms of signal sensitivity after amplification as well as stability could be expected.

Based on the above considerations, the signal dependence on the concentration of the applied target protein, avidin, was examined by cyclic voltammetry. As shown in Fig. 3, registered voltammograms exhibited a direct correlation with the applied avidin concentration (voltammetric traces C, D, E). The signal was obtained in the form of bioelectrocatalytic activity of surface bound b-GOx that was reacted biospecifically to the surface-associated avidin. Therefore, the sensitivity and the dynamic range of detection could be controlled by modifying the reaction and signaling conditions such as time duration for the target protein reaction, label enzyme reaction, and concentrations of reagents. In addition, the voltammetric result exhibited the influence of the nonspecific binding of proteins on the affinity surface to the bio-

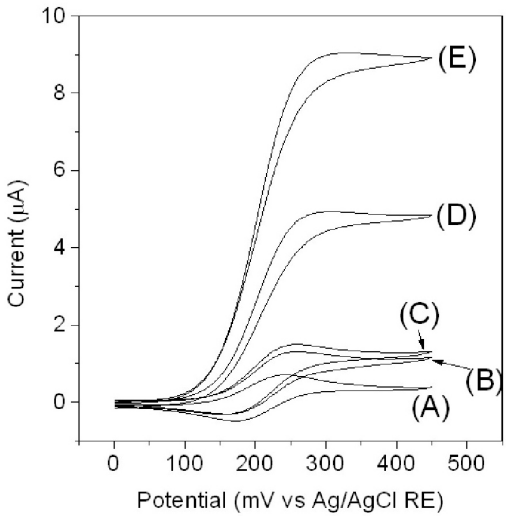

Fig. 3. Cyclic voltammograms of affinity biosensors for avidin by the signaling mode of the diffusible mediator-facilitated electron transfer: (A) background, (B) 0 μg/mL avidin, (C) 100 g/mL, (D) 1 mg/mL, and (E) 2 mg/mL. Conditions for the electrochemical tests were the same as Fig. 2.

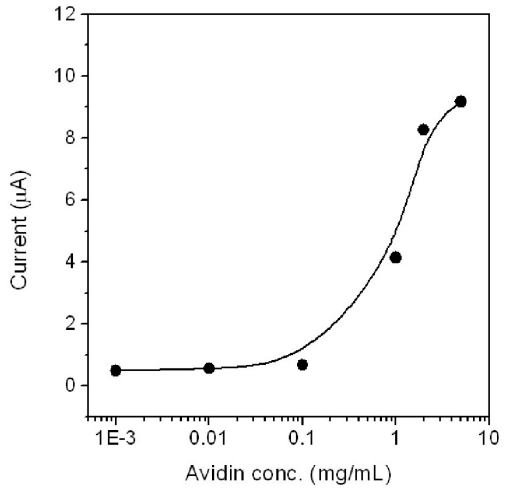

Fig. 4. The calibration curve from the resulting affinity biosensors for avidin by the signaling mode of the diffusible mediator-facilitated electron transfer.

sensor signal. The voltammetric trace (B) in Fig. 3 was obtained when the affinity biosensor was subjected to the signaling step without the avidin treatment. Therefore, the anodic current difference between voltammetric traces (A) and (B) shows the extent of the nonspecific binding of b-GOx to the sensing interface. From the kinetic analysis of the voltammetric data [12,13,19], the surface concentration of the nonspecifically bound enzyme was estimated to be around 3.8×10^{-13} mol/cm^2, which is equivalent to the surface coverage of *ca*. 8 ± 2% (The estimation of the b-GOx density from the kinetic analysis is dependent on the activity of the modified enzyme. When b-GOx is assumed to be *ca*. 60% active in comparison with the native GOx, which is usually the case, the calculated density of 4.7×10^{-12} mol/cm^2 corresponds to the full coverage). Fig. 4 shows the calibration result from the affinity biosensor for avidin. Under the specified reaction conditions in the experimental section, the biosensors exhibited a sigmoidal signal correlation to the logarithmic concentration of the applied avidin samples, having a dynamic detection range from 100 μg/mL to 5 mg/mL.

In conclusion, we have developed a bioelectrocatalyzed signal amplification method for affinity interactions at chemically modified electrodes. The results suggest that the proposed surface configuration would be useful for affinity recognition reactions, and the selected signal amplification strategy could be readily applicable to the sandwich-type electrochemical immunosensing systems. Further studies are under progress to implement the current method into microsystem-based immunoassays.

Acknowledgement H.C.Y. is grateful to Ajou University for financial support through the Research Initiation Program, 2003.

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[Received January 27, 2004; accepted April 6, 2004]