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# Affinity Biosensor for Avidin Using a Double Functionalized Dendrimer Monolayer on a Gold Electrode

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**We have developed an affinity biosensor system based on avidin–biotin interaction on a gold electrode. As the building block of an affinity-sensing monolayer, a fourth-generation (G4) poly(amidoamine) dendrimer having partial ferrocenyl-tethered surface groups was prepared and used. The unmodified surface amine groups from dendrimers were functionalized with biotinamidocaproate, and the biotinylated and electroactive dendritic monolayer was constructed on a gold electrode for the affinity-sensing surface interacting with avidin. An electrochemical signal from the affinity biosensor was generated by free glucose oxidase in electrolyte, depending on the degree of coverage of the sensing surface with avidin. The sensor signal decreased correlatively with increasing avidin concentration and approached a minimum level when the sensing surface was fully covered with avidin. The detection limit of avidin was about 4.5 pM, and the sensor signal was linear ranging from 1.5 pM to 10 nM under optimized conditions. From the kinetic analysis using the biotinylated glucose oxidase, an active enzyme coverage of 2.5 × $10^{-12}$ mol/$cm^2$ on the avidin-pretreated surface was registered, which demonstrates the formation of a spatially ordered and compact protein layer on the derivatized electrode surface.** 

***Key Words:*** **affinity biosensor; avidin–biotin; dendrimer; ferrocenyl; glucose oxidase.**

For an affinity-biosensing interface with high sensitivity and selectivity, construction of a molecularly organized sensing surface representing a high density of ligand groups with adequate accessibility, fulfilling efficient affinity reaction and easy signal generation, is required. In this respect, much research effort has been devoted to the development of affinity sensors based on the alkanethiol self-assembled monolayers (SAM)[2] (1, 2), silane-modified layers (3, 4), and polymer-grafted layers (5). The establishment of an effective biosensing interface especially draws a growing quest in diagnostic research fields.

During the past decade, there have been expanding interests in the newly introduced synthetic "dendritic polymer" (dendrimer) (6, 7) and its application to the related areas including drug delivery, energy harvesting, ion sensing, catalysis, and information storage (8). The unique characteristics of dendrimers such as structural homogeneity, integrity, controlled composition, and multiple homogeneous chain ends available for consecutive conjugation reaction expand its use as the material of choice (9). A number of approaches adopting dendrimers as the building block for the nanostructures have been conducted (10–13). Recently, we have shown that the dendrimers can be utilized as the bioconjugating reagents for construction of a multilayered enzyme nanostructure (14).

In this paper, an electrochemical affinity biosensor based on the avidin–biotin interaction (15, 16) was developed. As the affinity-biosensing surface, a monolayer of ferrocenyl-tethered and biotinylated dendrimer was constructed on a gold electrode. The signaling principle of the affinity biosensor is depicted in Scheme 1. A monolayer of double-functionalized dendrimer plays a role as a molecular gate for free diffusing and signaling molecules in electrolyte. Nonlabeled free glucose oxidase (GOx) in electrolyte, as a diffusional tracer, generates an electrochemical signal, depending on the degree of coverage of the sensing surface with avidin. The maximum current is obtained

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[2] Abbreviations used: SAM, self-assembled monolayer; GOx, glucose oxidase; FAD, flavin adenine dinucleotide; DMSO, dimethyl sulfoxide; b-GOx, biotin–amidocaproyl labeled glucose oxidase.

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when the surface is free of avidin, and the signal becomes minimal as the sensing surface is fully covered with avidin. Typical cyclic voltammograms for each case with background are shown in Scheme 1 (bottom panel: A, B). Kinetic analysis using biotinylated glucose oxidase, forming an enzyme adlayer on the avidin-pretreated surface, was also carried out to demonstrate the active coverage and the spatial organization of proteins on the derivatized gold electrode.

## MATERIALS AND METHODS

### *Chemicals and Reagents*

Amine-terminated G4 poly(amidoamine) dendrimers are manufactured by Dendritech, Inc. (Midland, MI) and were purchased from Aldrich. Ferrocene carboxaldehyde, ferrocene methanol, sodium borohydride, 3,3-dithiopropionic acid bis-*N*-hydroxysuccinimide ester, biotinyl-ε-amidocaproic acid *N*-hydroxysulfosuccinimide ester, and D-(+)-glucose were used as supplied. Avidin from hen egg white (Sigma), immunopure avidin (Pierce), glucose oxidase type VII (EC 1.1.3.4. from *Aspergillus niger*, Sigma), and biotin–amidocaproyl-labeled glucose oxidase (Sigma) were used as received from the manufacturers. All other chemicals were of analytical grade and used without further purification. Doubly distilled and deionized water with specific resistance over 18 MΩ · cm was used throughout this work.

### *Preparation and Characterization of Ferrocenyl-Tethered G4 Poly(amidoamine) Dendrimers*

Surface primary amines of $NH_2$-terminated G4 poly(amidoamine) dendrimers were partially modified with ferrocenyl groups through the imine-forming, amine–aldehyde reaction. Ferrocene carboxaldehyde (7.5 mg) dissolved in 3.75 ml methanol was added dropwise to a 0.25-ml portion of 10% (w/w) G4 dendrimer solution containing hydrochloric acid as a catalyst. The reaction mixture was slowly stirred for 2 h, and 5 mg of sodium borohydride was slowly added and stirred for 1 h to reduce carbon-to-nitrogen double bonds. The reaction product was purified through lipophilic gel-permeation chromatography (Sephadex LH-20, Pharmacia) using methanol as the eluent. Purity and degree of modification were determined by using UV/Vis and $^1$H NMR spectroscopies. Purity of the product was verified with thin-layer chromatography, and degree of ferrocene modification was determined spectrophotometrically at 438 nm using a ferrocene standard curve in methanol. The NMR spectrum was obtained to confirm the conjugation and reduction reactions: $^1$H NMR (500 MHz, $CD_3OD$, TMS): $\delta$ 4.23 ($C_5H_4$ [H-2, H-5]), 4.17–4.13 ($C_5H_4$ [H-3, H-4] and Cp), 3.55 ($CH_2NH$), 3.35–2.25 (dendrimer protons).

### *Construction of Ferrocenyl-Tethered Dendrimer Monolayer on Gold Surface and the Surface Functionalization with Biotin*

The affinity-sensing monolayer was constructed on the evaporated gold surface. 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 affinity-sensing monolayered electrodes. The bottom-up synthetic procedure started with the introduction of amine-reactive SAM on the gold surface. By chemisorption of 5 mM 3,3-dithiopropionic acid bis-*N*-hydroxysuccinimide ester in DMSO for 2 h, an amine-reactive functional monolayer on the gold electrode surface was prepared. After SAM formation and rinsing steps with DMSO and methanol, the electrode surface was incubated with the ferrocenyl-tethered dendrimer solution in methanol. The purified ferrocenyl-tethered dendrimer solution, 22 μM (based on the dendrimer concentration), was reacted with the 3,3-dithiopropionic acid bis-*N*-hydroxysuccinimide ester monolayer on the gold electrode in methanol for 2 h. Immobilization of ferrocenyl-tethered dendrimers on the electrode surface was verified by cyclic voltammetric and coulometric tests of the resulting electrode. After thorough rinsing with methanol and distilled water, the electrode was immersed in bicarbonate buffer (0.1 M, pH 9.5) for further functionalization with biotin. Biotinyl-ε-amidocaproic acid *N*-hydroxysulfosuccinimide ester (biotin–NHS, 2 mg/ml in water) was reacted with the remaining terminal amines of the dendritic monolayer by addition to the electrode-immersed bicarbonate buffer. The biotin–NHS concentration in the final reaction mixture was 2 mM. After reaction for 1 h, the electrode was washed with bicarbonate buffer and water and was stored in phosphate buffer solution (0.1 M, pH 7.2) prior to use.

### *Affinity Sensing of Avidin*

Before the affinity-sensing tests, the electrode was removed from the storage solution, rinsed, air dried, and clamped to a Teflon electrode holder. The holder was designed to expose the defined electrode area of 0.148 $cm^2$ and the reaction well volume was 3 ml. Chronocoulometry was employed for the determination of electrode surface area. After rinsing with phosphate buffer (0.1 M, pH 7.2), avidin sample was added to the affinity-sensing surface, incubated for 30 min at room temperature, and rinsed. The sensor signals were registered from cyclic voltammograms at each electrode by adding glucose oxidase (30 μg/ml) as a diffusional catalytic signal generator in the presence of 10 mM glucose solution under dioxygen-free conditions. The avidin stock solution of 1 mg/ml was prepared with phosphate buffer (0.1 M, pH 7.2), and the avidin–biotin

affinity reaction was performed after proper dilution. The protein concentration in each sample was determined by Bradford's method (17).

### *Kinetic Analysis Using Biotin–Amidocaproyl-Labeled Glucose Oxidase*

To verify the affinity-sensing interface on the gold electrode surface in terms of the active coverage and the spatial organization of protein molecules, a kinetic analysis was performed using the biotin-labeled GOx. The biotin-functionalized electrode was incubated with 0.1 mg/ml of avidin for 30 min, rinsed thoroughly, and reacted with 1 mg/ml of biotin–amidocaproyl-labeled glucose oxidase (b-GOx). The concentration of avidin (0.1 mg/ml) was chosen, because the biotin-functionalized electrode surface was found to be fully covered with avidin at this concentration under standard reaction conditions (see Scheme 1B). After formation of the glucose oxidase adlayer on the avidin layer via the avidin–biotin recognition reaction, the electrode was rinsed, and the cyclovoltammetric response from the electrode was registered using 0.1 mM ferrocene methanol as a diffusional electron-transferring mediator and 10 mM glucose as a substrate in electrolyte. The kinetic analysis is based on the previous work of Savéant's group (18, 19), and the sequence of reactions is shown in Scheme 2.

### *Instrumentation*

Electrochemical measurements were performed with a BAS CV-50W electrochemical analyzer (West Lafayette, IN). A standard three-electrode configuration with a platinum gauze counter electrode and an Ag/AgCl (3 M NaCl, BAS) reference electrode was used. All experiments were performed at room temperature (25 ± 2°C) under argon atmosphere, unless otherwise specified. The glucose stock solutions were prepared with phosphate buffer (0.1 M, pH 7.2) and were allowed to mutarotate overnight before use. The electrolyte solutions were dideoxygenated with argon bubbling for 20 min before each voltammetric run. The $^{1}$H NMR spectra were recorded on a Bruker AMX FT500 NMR spectrometer. The UV/Vis absorption spectra were obtained using a spectrophotometer (Shimadzu UV2100) at 25°C.

## RESULTS AND DISCUSSION

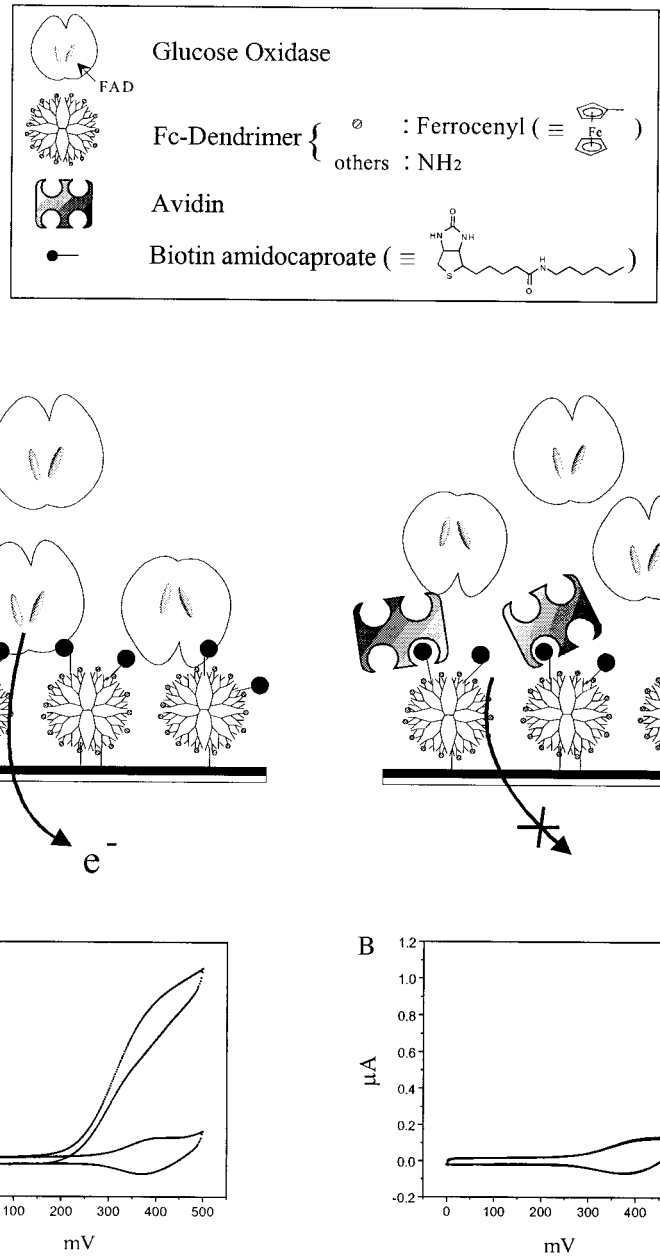
### *Preparation of Ferrocenyl-Tethered Poly(amidoamine) Dendrimers*

According to the signaling principle of the affinity biosensor depicted in Scheme 1, amine groups of dendrimers are used for immobilization on the electrode surface and functionalization with ferrocenyls and biotinyl groups. First, amine groups were partially modified with ferrocene carboxaldehyde, and the resulting Schiff bases were reduced with sodium borohydride after the conjugation reaction. The number of ferrocenyl groups bound per dendrimer molecule was determined spectrophotometrically at 438 nm with ferrocene standard in methanol. As mentioned above, surface amine groups are used for immobilization on the electrode surface and two functionalization reactions to introduce ferrocenyls and biotinyl groups, and we thought that the ferrocenyl-tethered dendrimer with one-third functionalization might be satisfactory for construction of the affinity-sensing monolayer. Thus, we prepared ferrocenyl-tethered dendrimers having about 30% modification by controlling the molar ratio between ferrocene carboxaldehyde and dendritic surface amines. From spectrophotometric analysis, the percentage modification of dendritic surface amines to ferrocenyls was found to be about 32% when the molar ratio of amine to ferrocene carboxaldehyde was 2.5, which indicates that 20 or 21 surface amine functionalities of dendrimers were modified to ferrocenyls.

### *Construction of Biotinylated Ferrocenyl-Dendrimer Monolayer on the Gold Surface*

For construction of an affinity-sensing monolayer on gold electrodes, a bottom-up synthetic procedure was attempted. First, an amine-reactive SAM was prepared by chemisorption of 3,3-dithiopropionic acid bis-*N*-hydroxysuccinimide ester on gold surfaces. Then, the activated succinimidyl ester groups were reacted with the amine groups of ferrocenyl-tethered dendrimers. Finally, an aqueous solution of biotinyl-ε-amidocaproic acid *N*-hydroxysulfo-succinimide ester was added to the electrode for biotinylation of the remaining amine groups of the ferrocenyl-tethered dendritic monolayer.

Covalent immobilization of ferrocenyl-tethered dendrimers on the 3,3-dithiopropionic acid bis-*N*-hydroxysuccinimide ester SAM was confirmed by cyclic voltammetry. As shown in Scheme 1 (see also Fig. 1, vide infra), the background and unmediated cyclic voltammograms represent the surface-immobilized electroactive ferrocenyl groups. Cyclic voltammograms of electrode with the ferrocenyl-tethered dendrimer monolayer were also obtained as a function of potential sweep rate. The registered voltammograms were typical for the surface-confined electroactive groups, as evidenced by the small (~20 mV) peak separation ($\Delta E_{p}$), the full width at half maximum ($\Delta E_{fwhh}$) of ca. 100 mV, and the linear proportionality of peak currents with sweep rate (data not shown). In addition, the cyclic voltammograms remained unaltered during repetitive sweep cycles, which indicates that the ferrocenyl-tethered dendrimers were covalently immobi-




**SCHEME 1.** Construction and proposed operational principle for the affinity biosensor based on the avidin–biotin interaction on a gold electrode surface. (Top) Molecular models of the chemicals used for electrode construction and affinity biosensing. (Bottom) Typical cyclic voltammetric signal traces for (A) the avidin-free surface that was not reacted with avidin and (B) the fully blocked surface that was reacted with 10 $\mu$g/ml avidin. See text for details.

lized onto underlying SAM and the resulting affinity-sensing monolayer was maintained stably.

To verify the biosensor's signaling principle, which is based on the steric hindrance from the avidin–biotin interaction at the electrode surface, dependency of the sensor signal on the avidin concentration was investigated. For this, electrodes were incubated with avidin samples of various concentrations followed by rinsing steps, and the cyclic voltammograms were obtained. As shown in Fig. 1, the traces represent typical ferrocenyl-mediated and enzyme-catalyzed voltammograms, reaching a plateau at ca. +400 mV vs Ag/AgCl. The voltammograms showed essentially an inverse proportionality to the avidin concentration. From the avidin concentration of 1 ng/ml (curve B), a gradual decrease in oxidative catalytic current was observed. At the avidin concentration of 10 $\mu$g/ml (curve F), an almost identical voltammogram to the background (curve G)

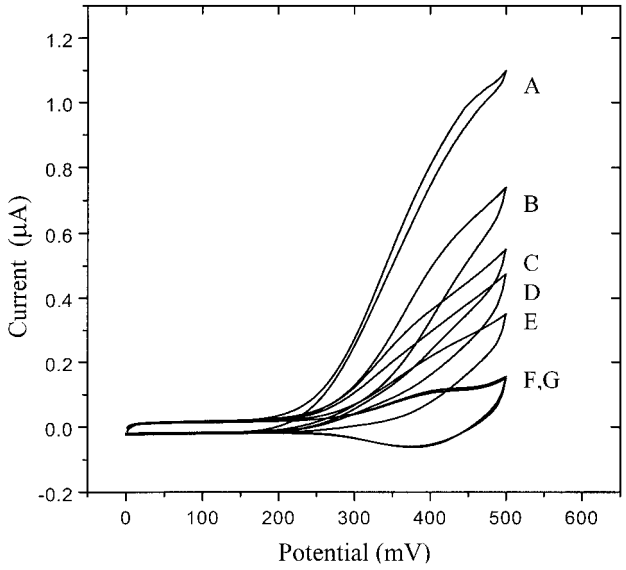



**FIG. 1.** Cyclic voltammograms of the affinity biosensors as a function of reacted avidin concentration: (A) 0, (B) 1 ng/ml, (C) 10 ng/ml, (D) 100 ng/ml, (E) 1 $\mu$g/ml, and (F) 10 $\mu$g/ml. Cyclic voltammograms were obtained in the presence of 30 $\mu$g/ml of GOx as a signal generator and 10 mM glucose as a substrate; (G) background voltammogram in the absence of enzyme and substrate. All curves were registered in a dideoxygenated 0.1 M phosphate buffer (pH 7.2) solution under argon atmosphere. Potential scan rate was 5 mV/s.

was registered, suggesting that the affinity-sensing surface was fully covered with avidin.

As mentioned above, the signaling principle of the affinity biosensor is based on the concentration-dependent coverage of the sensing surface with avidin and consequent steric hindrance preventing free GOx in the electrolyte from accessing the electrode surface. Therefore, an efficient electron transfer from GOx to the immobilized ferrocenyls is critical for the highly sensitive signal generation. In the bioelectrocatalytic reaction of enzymes that have an FAD prosthetic group, such as GOx, and employ ferrocene as an electron-transferring mediator, the oxidative current is generated via three main steps: encounter of ferrocene with enzyme, adequate positioning of ferrocene at the reaction pocket which connects the FAD to the enzyme surface, and electron transfer from the prosthetic group to ferrocene. Of these, the second step has been known to be a rate-determining step (20), which is also the case in this work, even though ferrocenyls are immobilized on the electrode surface. In this context, the high density of ferrocenyls immobilized on the electrode surface through tethering onto the multiple functional groups of dendrimers would facilitate proper positioning, leading to efficient electron transfer from prosthetic groups to ferrocenyls, interferrocenyls, and the electrode in this system.

From the observation that the affinity biosensor exhibited a negligible signal change when the ferrocenyl-tethered dendrimer was not biotinylated, it is evident that the derived catalytic current with dependency on avidin concentration is attributed to the specific avidin–biotin recognition rather than nonspecific protein adsorption to the electrode surface. When the nonbiotinylated electrode was used as a control, dependency of the anodic current on the avidin concentration disappeared, and only small signal retardation was observed, which might be due to the nonspecific binding of avidin. Additionally, we prepared a set of affinity-sensing surfaces that were approximately half-active by lowering the concentration of biotinylating reagent and/or shortening the reaction time. As a result, we obtained half-active affinity biosensors showing only half signal retardation even under fully blocked conditions (>10 $\mu$g/ml avidin), which also supports that nonspecific protein adsorption on the electrode surface is negligible.

### *Characterization of the Affinity-Sensing Surface by Kinetic Analysis*

To confirm the proposed signaling principle and to characterize the interaction at the affinity-sensing surface in more detail, bioelectrocatalytic signals from the affinity biosensor were kinetically analyzed. For this, biotin-functionalized electrodes were fully covered with avidin by incubating with 0.1 mg/ml avidin for 30 min. The resulting electrodes were then treated with 1 mg/ml of b-GOx and rinsed, and the bioelectrocatalytic current was registered by cyclic voltammetry in the presence of ferrocenyl methanol as an electron-transferring mediator and glucose (Fig. 2). From the derived current magnitude, the surface coverage of active enzyme can be calculated. The bioelectrocatalytic reaction between GOx and the ferrocene mediator is depicted in Scheme 2. In the absence of diffusion limitation, the plateau current from the mediated cyclic voltammograms, $I_p$, is expected to obey the equation

$$\frac{1}{I_p} = \frac{1}{2FS\Gamma_E}\left(\frac{1}{k_3[\mathrm{Fc}]} + \frac{1}{k_2} + \frac{1}{k_{red}[G]}\right),$$

where $F$ is Faraday's constant, $S$ is electrode area, $\Gamma_E$ is surface concentration of enzyme, [Fc] is mediator concentration, $[G]$ is 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 ($k_2 = 700\ s^{-1}$, $k_3 = 1.2 \times 10^7\ M^{-1}\ s^{-1}$, $k_{red} = 1.1 \times 10^4\ M^{-1}\ s^{-1}$) (18, 19), we determined the density of b-GOx associated on the avidin-pretreated electrode surface. Chronocoulometry was employed for the determination of electrode surface area, and 0.148 $cm^2$ was registered from an Anson plot (21). From the voltammograms of Fig. 2

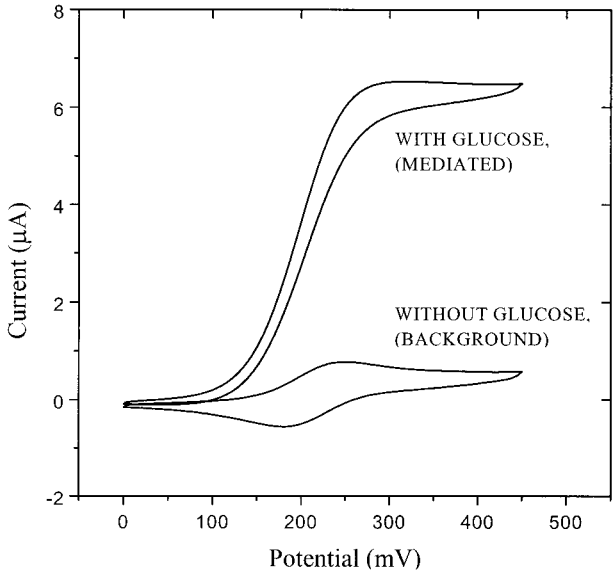



**FIG. 2.** Cyclic voltammetric test for the estimation of active biotinylated glucose oxidase (b-GOx) coverage on the affinity-sensing surface which had been pretreated with avidin. Cyclic voltammograms for the b-GOx immobilized electrode were registered with 10 mM glucose (anodically amplified voltammogram, mediated) and without glucose (background voltammogram). All curves were registered in the presence of 0.1 mM ferrocene methanol as a diffusional electron-transferring mediator. Other conditions were the same as in Fig. 1.

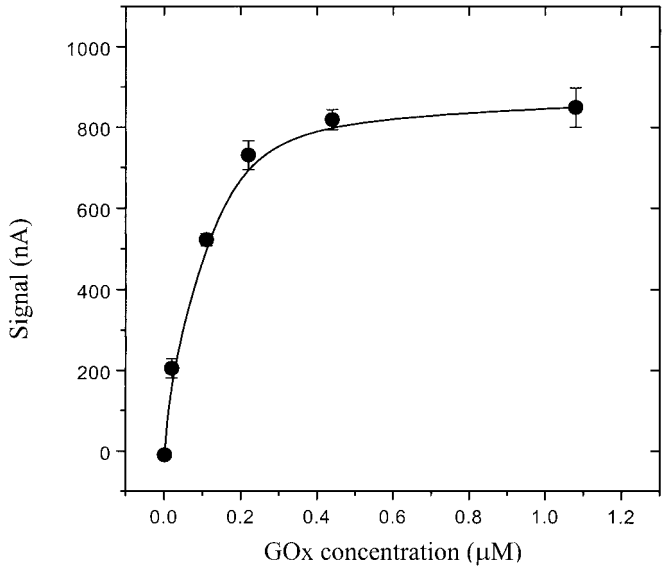



**FIG. 3.** Signal dependency of the affinity biosensor on GOx concentration in electrolyte. Anodic current levels were registered at +400 mV vs Ag/AgCl from the background-subtracted cyclic voltammograms for respective enzyme concentrations. Electrodes that were not reacted with avidin, avidin-free surfaces, were used in this experiment. Conditions for the cyclovoltammetric tests were the same as in Fig. 1.

and repetitive tests, the coverage of active b-GOx, $\Gamma_E$, was estimated to be $2.5 \times 10^{-12}$ mol/cm$^2$, which suggests that a spatially organized enzyme monolayer was formed on the electrode surface. When b-GOx is assumed to be about 60% active in comparison with native enzyme (this is usually the case), an estimated density of $4.2 \times 10^{-12}$ mol/cm$^2$ is obtained, which is comparable to the ideal monolayer coverage of $4.7 \times 10^{-12}$ mol/cm$^2$ (22) based on the known molecular dimension of native GOx. In addition, the mass density of surface-adsorbed strept(avidin) has been reported to be 1.9~2.4 ng/mm$^2$ by optical and acoustic methods (23, 24). This value can be converted to molar density ranging from 2.8 to $3.6 \times 10^{-12}$ mol/cm$^2$, which corresponds to registered active b-GOx coverage. Considering this observation and the known dimensions of GOx ($60 \times 52 \times 77$ Å) (25) and avidin ($55 \times 55 \times 41$ Å) (23), it seems that avidin specifically interacts with biotinylated enzyme and a compact protein layer is formed on the electrode surface.

$$Fc \rightleftharpoons Fc^+ + e^-$$

$$FAD + G \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} FADG$$

$$FADG \xrightarrow{k_2} FADH^- + GL$$

$$FADH^- + 2\,Fc^+ \xrightarrow{k_3} FAD + 2\,Fc$$

**SCHEME 2.** Sequence of reactions for the bioelectrocatalytic oxidation of glucose catalyzed by glucose oxidase (GOx) and mediated by ferrocenyls at the electrode surface. Fc and Fc$^+$, reduced and oxidized forms of the ferrocenyl mediator; FAD and FADH$^-$, oxidized and reduced forms of the flavin adenine dinucleotide; FADG, enzyme–substrate complex; G and GL, glucose and glucono-lactone.

From these results, it follows that a satisfactory level of active b-GOx coverage was achieved and that the avidin-associated underlying monolayer, which consisted of ferrocenyl-tethered and biotinylated dendrimer on SAM, was constructed in a spatially ordered manner.

### *Affinity Sensing of Avidin*

Signal from the affinity biosensor is affected by the amount of free GOx present in electrolyte. As shown in Fig. 3, the catalytic signal increased almost linearly in correlation to GOx concentration up to 0.2 $\mu$M and reached a plateau. The glucose concentration in electrolyte was fixed at 10 mM and the solution was dideoxygenated with argon. To guarantee the satisfac-

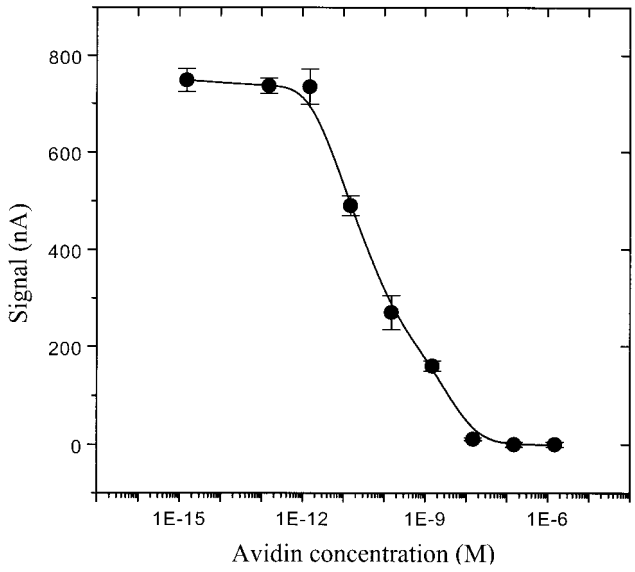



**FIG. 4.** Calibration curve of the affinity biosensor for avidin. Anodic current levels were registered at +400 mV vs Ag/AgCl from the background-subtracted cyclic voltammograms for respective avidin concentrations.

tory signal level from the electrode and to reduce the possibility of nonspecific adsorption of GOx on the sensing surface, the lowest GOx concentration exhibiting a saturated signal level, 0.22 $\mu$M, was chosen in this work.

To evaluate the analytical performance of the affinity biosensor for avidin, calibration experiments were performed (Fig. 4). The electrochemical signals were registered at different avidin concentrations from the respective background-subtracted cyclic voltammograms. Under the specified conditions of adsorption reaction and measurement, the biosensor exhibited a diminishing sigmoidal signal trend to the avidin concentration. The calibration curve was linear ranging from 1.5 pM to 10 nM avidin, and the detection limit was about 4.5 pM. The observed detection limit can be attributed to the unique measurement mode of preimmobilization of avidin, consequent signal generation by free GOx, and signal amplification by the immobilized electron-transferring mediators.

In conclusion, we have developed an affinity biosensor for the sensitive determination of avidin based on the specific interaction of avidin with biotin on a gold electrode. Introduction of a dendrimer as a building unit for the biosensing monolayer and its functionalization is expected to find wide application in the development of affinity biosensors for other analytes.

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