Multilayered Assembly of Dendrimers with Enzymes on Gold: Thickness-Controlled Biosensing Interface

ARTICLE *in* ANALYTICAL CHEMISTRY · APRIL 2000

Impact Factor: 5.64 · DOI: 10.1021/ac991299a · Source: PubMed

2 AUTHORS:

Hyun C Yoon
Ajou University / Korea
87 PUBLICATIONS **1,853** CITATIONS
SEE PROFILE

Hak-Sung Kim
Korea Advanced Institute of Science and Tec…
168 PUBLICATIONS **3,905** CITATIONS
SEE PROFILE

Anal. Chem. **2000,** *72,* 922−926

Multilayered Assembly of Dendrimers with Enzymes on Gold: Thickness-Controlled Biosensing Interface

Hyun C. Yoon and Hak-Sung Kim*

Department of Biological Sciences, Korea Advanced Institute of Science and Technology, 373-1, Kusung-dong, Yusung-ku, Taejon 305-701, Korea

A new approach to construct a multilayered enzyme film on the Au surface for use as a biosensing interface is described. The film was prepared by alternate layer-by-layer depositions of G4 poly(amidoamine) dendrimers and periodate-oxidized glucose oxidase (GOx). The cyclic voltammograms obtained from the Au electrodes modified with the GOx/dendrimer multilayers revealed that bioelectrocatalytic response is directly correlated to the number of deposited bilayers, that is, to the amount of active enzyme immobilized on the Au electrode surface. From the analysis of voltammetric signals, the coverage of active enzyme per GOx/dendrimer bilayer during the multilayer-forming steps was estimated, which demonstrates that the multilayer is constructed in a spatially ordered manner. Also, with the ellipsometric measurements, a linear increment of the film thickness was registered, supporting the formation of the proposed multilayered structure. The E5D5 electrode showed the sensitivity of 14.7 μA·mM^{-1} glucose·cm^{-2} and remained stable over 20 days under day-by-day calibrations. The proposed method is simple and would be applicable to the constructions of thickness- and sensitivity-controllable biosensing interfaces composed of multienzymes as well as a single enzyme.

Driven by interest in designing interfaces at the molecular level, research on the investigation of organized thin films (OTFs) has developed into an important area in analytical chemistry and molecular device technologies.[1,2] Moreover, the inclusion of biomolecules, i.e., enzymes, antigens/antibodies, ion channels, and nucleic acids, has extended its utility to artificial biological membranes[3,4] and biosensors.[5−8]

As a building unit for the organic thin films, highly branched dendritic macromolecules are of great interest.[9,10] They possess a unique surface of multiple chain ends, and the number of surface groups can be precisely controlled as a function of synthetic generations. For example, fourth-generation (G4) poly(amidoamine) (PAMAM) dendrimers have 64 surface amine groups. The high concentration of functional end groups of dendrimers enables synthetic modifications for the molecularly ordered nanostructures. These include deposition of dendritic multilayers via Pt^{2+} complexation,[11] electrostatic interaction,[12] and reaction with grafted copolymer.[13,14]

There have been a number of approaches for constructing catalytic enzyme films on electrodes,[15−20] but the quest for the molecularly organized and stable films continues. The multilayered configuration adopting dendrimers has several advantages from the viewpoint of biosensing application. First, the dendrimers provide multiple conjugation sites, and a densely functionalized and structurally stable architecture can easily be obtained. Second, due to the interior void structure of dendrimers,[9] the resulting multilayer film will represent minimal diffusional restriction for analytes and electron-transferring substances (mediators),[21,22] ensuring desired enzymatic and electrocatalytic reactions over the

* Corresponding author: (phone) 82-42-869-2616; (fax) 82-42-869-2610; (e-mail) hskim@sorak.kaist.ac.kr.

(1) Zhong, C.-J.; Porter, M. D. *Anal. Chem.* **1995**, *67*, 709A−715A.
(2) Ulman, A. *An Introduction to Ultrathin Organic Films: From Langmuir−Blodgett to Self-Assembly*; Academic Press: Boston, 1991.
(3) Müller, W.; Ringsdorf, H.; Rump, E.; Wildburg, G.; Zhang, X.; Angermaier, L.; Knoll, W.; Liley, M.; Spinke, J. *Science* **1993**, *262*, 1706−1708.
(4) Cornell, B. A.; Braach-Maksvytis, V. L. B.; King, L. G.; Osman, P. D. J.; Raguse, B.; Wieczorek, L.; Pace, R. J. *Nature* **1997**, *387*, 580−583.
(5) Willner, I.; Heleg-Shabtai, V.; Blonder, R.; Katz, E.; Tao, G.; Bückmann, A. F.; Heller, A. *J. Am. Chem. Soc.* **1996**, *118*, 10321−10322.
(6) Motesharei, K.; Ghadiri, M. R. *J. Am. Chem. Soc.* **1997**, *119*, 11306−11312.
(7) Berggren, C.; Johansson, G. *Anal. Chem.* **1997**, *69*, 3651−3657.
(8) Jordan, C. E.; Frutos, A. G.; Thiel, A. J.; Corn, R. M. *Anal. Chem.* **1997**, *69*, 4939−4947.
(9) Tomalia, D. A.; Naylor, A. M.; Goddard, W. A., III *Angew. Chem., Int. Ed. Engl.* **1990**, *29*, 138−175.
(10) Fischer, M.; Vögtle, F. *Angew. Chem., Int. Ed. Engl.* **1999**, *38*, 884−905.
(11) Watanabe, S.; Regen, S. L. *J. Am. Chem. Soc.* **1994**, *116*, 8855−8856.
(12) Tsukruk, V. V.; Rinderspacher, F.; Bliznyuk, V. N. *Langmuir* **1997**, *13*, 2171−2176.
(13) Liu, Y.; Bruening, M. L.; Bergbreiter, D. E.; Crooks, R. M. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 2114−2116.
(14) Liu, Y.; Zhao, M.; Bergbreiter, D. E.; Crooks, R. M. *J. Am. Chem. Soc.* **1997**, *119*, 8720−8721.
(15) Kuwabata, S.; Okamoto, T.; Kajiya, Y.; Yoneyama, H. *Anal. Chem.* **1995**, *67*, 1684−1690.
(16) Riklin, A.; Willner, I. *Anal. Chem.* **1995**, *67*, 4118−4126.
(17) Bourdillon, C.; Demaille, C.; Moiroux, J.; Savéant, J.-M. *Acc. Chem. Res.* **1996**, *29*, 529−535.
(18) Shin, M.-C.; Yoon, H. C.; Kim, H.-S. *Anal. Chim. Acta* **1996**, *329*, 223−230.
(19) Hodak, J.; Etchenique, R.; Calvo, E. J.; Singhal, K.; Bartlett, P. N. *Langmuir* **1997**, *13*, 2708−2716.
(20) Anzai, J.; Takeshita, H.; Kobayashi, Y.; Osa, T.; Hoshi, T. *Anal. Chem.* **1998**, *70*, 811−817.
(21) Zhao, M.; Tokuhisa, H.; Crooks, R. M. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 2596−2598.
(22) Tokuhisa, H.; Zhao, M.; Baker, L. A.; Phan, V. T.; Dermody, D. L.; Garcia, M. E.; Peez, R. F.; Crooks, R. M.; Mayer, T. M. *J. Am. Chem. Soc.* **1998**, *120*, 4492−4501.

10.1021/ac991299a CCC: $19.00
Published on Web 01/27/2000

whole range of multilayers. Third, the remaining reactive groups after film formation are accessible for further modifications with artificial redox mediators for the reagentless biosensing or pendant groups for specific purposes.

Here, we report a new approach to construct a thickness-tunable enzyme multilayer film on the Au electrode surface for use as a biosensing interface. The multilayered enzyme film was prepared by using G4 PAMAM dendrimers and periodate (IO_4^-)-oxidized glucose oxidase (GOx) via alternate layer-by-layer depositions on an aminated Au surface. On the basis of voltammetric and ellipsometric studies, we demonstrate that the enzyme/dendrimer multilayered film is formed in a spatially ordered manner and stable on an Au electrode surface, and the resulting electrode is useful for the biosensing application.

EXPERIMENTAL SECTION

Reagents and Materials. Amine-terminated G4 PAMAM dendrimers are manufactured by Dendritech, Inc. (Midland, MI) and were purchased from Aldrich. Glucose oxidase type VII (EC 1.1.3.4. from *Aspergillus niger*) was used as received from Sigma. 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.

Carbohydrate groups on the peripheral surface of the GOx molecule were oxidized with periodate to carbaldehydes. For this, a 20 μM GOx solution (5 mL, in 0.1 M phosphate buffer, pH 6.8) was reacted with 30 mg of sodium metaperiodate for 1 h at 4 °C in the dark. The reaction was stopped with 25 mM ethylene glycol (30 min, 25 °C), and the product was purified and concentrated with ultrafiltration (molecular weight cutoff, 30 000, Centricon).

Construction of Multilayered Enzyme Electrode. Gold disk electrodes (electrode area, 0.033 cm^2) were used as substrates for the enzyme/dendrimer multilayer construction. Before layer-forming process, electrodes were polished with 1 and 0.25 μm diamond slurries, subjected to sonication in doubly distilled water, and treated with an etching solution of $HNO_3/HCl/H_2O$ (1:3:4, v/v/v) for 1 min. After rinsing, the electrodes were polished with a 0.05 μm alumina slurry to a mirror finish and thoroughly cleaned. Cyclovoltammetric tests in 0.5 M H_2SO_4 were performed to check the surface contamination.

The layer-forming process started with the introduction of amine functionalities on the Au surface by the chemisorption of aqueous cystamine dihydrochloride (10 mM, 1 h), as shown in Figure 1. The resulting aminated Au surface was cleaned to remove physically adsorbed monomers and then modified with the enzyme monolayer (E1D0) by dipping in 40 μM IO_4^--oxidized GOx solution (pH 6.8, 0.1 M phosphate buffer) for 1 h at room temperature. The enzyme/dendrimer bilayer (E1D1) was formed by immersing the resulting enzyme monolayer in an aqueous G4 PAMAM dendrimer solution (~10 mM, based on the primary amine concentration) for 1 h, followed by rinsing. The Schiff bases formed were reduced by 5 mM $NaBH_3CN$ at 4 °C in the dark during 30 min. In addition, the free carbaldehyde groups on the periphery of GOx were blocked with 10 mM ethanolamine (pH 9.5, titrated with concentrated HCl) for 30 min to avoid self-polymerization. Repetition of the above procedure led to the alternating GOx/dendrimer multilayer network with the desired number of bilayers (EnDn).

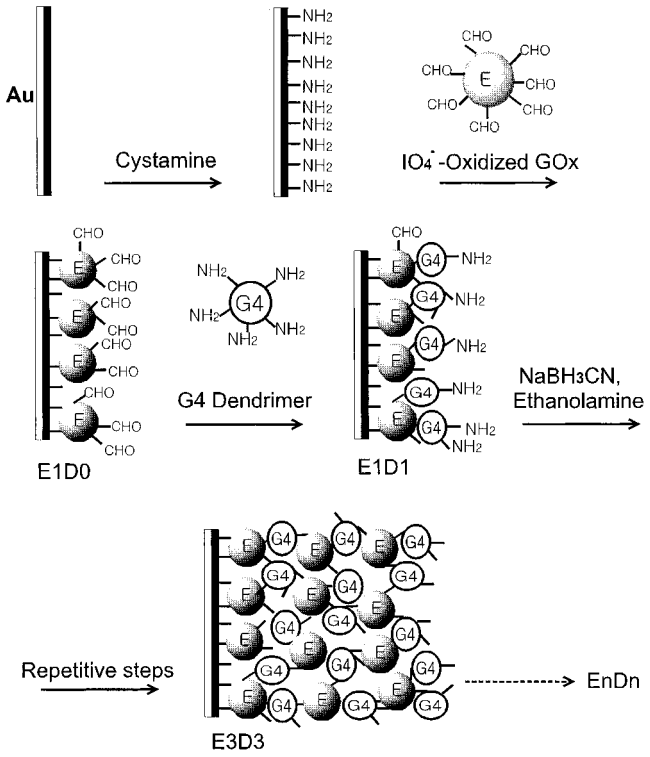

Figure 1. Schematic representation of the multilayered GOx/dendrimer network construction on the Au electrode surface. See Experimental Section for details.

Instrumentation. Electrochemical measurements were performed with a BAS CV-50W electrochemical analyzer (W. 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 8.0) and were allowed to mutarotate overnight before use. Ferrocenemethanol (0.1 mM, final concentration) was used as a diffusional electron-transferring mediator. The electrolyte solutions were deoxygenated with argon bubbling for 20 min before each voltammetric run.

For the ellipsometry, freshly evaporated Au 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 enzyme/dendrimer multilayer deposition. Ellipsometric thicknesses were determined on a Jobin Yvon spectroscopic ellipsometer with dried mono- and multilayers in air with a 70° angle of incidence at 632.8 nm wavelength. Film thicknesses were based on a refractive index of 1.465.[23,24] At least five different locations on each sample were measured and the average was then calculated. Those ellipsometric thicknesses had an uncertainty level of ±2 Å.

RESULTS AND DISCUSSION

Construction of the GOx/Dendrimer Multilayered Electrodes and Analysis of the Bioelectrocatalytic Response. First,

(23) Lestelius, M.; Liedberg, B.; Tengvall, P. *Langmuir* **1997**, *13*, 5900−5908.
(24) The thickness of a G4 PAMAM dendrimer monolayer prepared via multiple amine/Au interactions without intervening adhesive layer was 26 ± 2 Å, which agreed with Tokuhisa et al.,[22] suggesting substantial distortion of dendrimers on the flat Au surface.

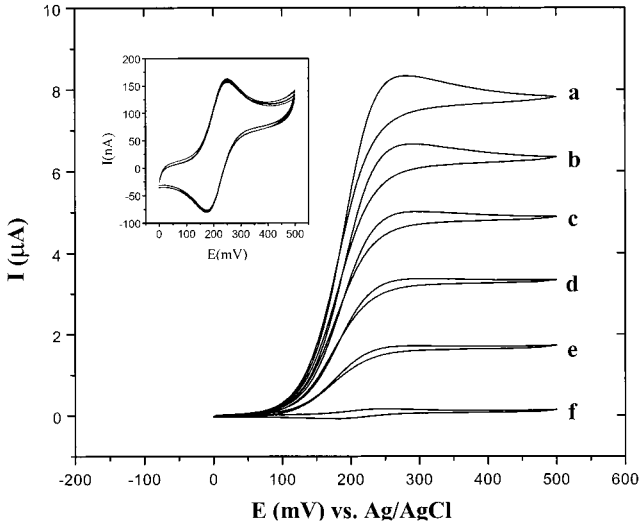

Figure 2. Cyclic voltammograms of the GOx/dendrimer multilayered electrodes in the presence of 0.1 mM ferrocenemethanol as a diffusional electron-transferring mediator: (a) E5D5, (b) E4D4, (c) E3D3, (d) E2D2, and (e) E1D1 in the presence of 20 mM glucose; (f) E1D1 in the absence of glucose, unmediated. The inset shows the cyclic voltammograms for each layer numbers in the absence of glucose in solution. All curves were registered in 0.1 M phosphate buffer (pH 8.0) under Ar. Potential scan rate was 5 mV·s^{-1}.

we focused on the exploitation of a simple process leading to the formation of a multilayered glucose oxidase/dendrimer assembly on the Au electrode surface. The key to this process is the formation of Schiff bases between amine groups of dendrimers and carbaldehyde groups on the peripheral surface of IO_4^--oxidized GOx. Through alternate depositions of IO_4^--oxidized GOx and amine-terminated PAMAM dendrimers, multilayers with the desired thickness can be prepared. The idealized schematic representation of the multilayered enzyme/dendrimer assembly, based on the voltammetric and ellipsometric studies, is as shown in Figure 1.

To evaluate the usefulness of this strategy, we have prepared the mono- and multilayered assemblies and investigated their bioelectrocatalytic characteristics. Figure 2 shows the cyclic voltammograms obtained from the Au electrodes modified with the GOx/dendrimer bilayers when 0.1 mM ferrocenemethanol was used as a diffusional electron-transferring mediator. Cyclic voltammograms a−e represent the bioelectrocatalytic signals of five, four, three, two, and one GOx/dendrimer bilayers associated Au electrodes, respectively, in the presence of 20 mM glucose. Anodic responses were remarkably increased for the multilayered electrodes due to the increased amount of enzyme deposited on the Au electrode surfaces. However, the electrodes fabricated with native GOx, instead of IO_4^--oxidized enzyme, showed no detectable signal, which suggests that the multilayer growth proceeds through the proposed reaction mechanism, the formation of Schiff bases between IO_4^--oxidized GOx and amine groups from NH_2-terminated poly(amidoamine) dendrimers and subsequent reduction with $NaBH_3CN$. As can be seen from the cyclic voltammograms for the multilayered electrodes, the anodic plateau current showed an almost linear relationship with the number of deposited enzyme/dendrimer bilayers. Thus, it is thought that each bilayer contains the same amount of active enzyme, and the enzyme/dendrimer multilayer is constructed in a spatially ordered manner.

Scheme 1

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

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

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

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

Fc and Fc$^+$: reduced and oxidized forms of 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

To substantiate the above claims, bioelectrocatalytic responses from the GOx/dendrimer mono- and multilayer-associated electrodes were kinetically analyzed. This is based on the previous work of Savéant's group on the GOx multilayered assemblies using antigen/antibody-conjugated[17,25] and avidin/biotin-conjugated enzyme couples.[26]

The bioelectrocatalytic reaction consists of the sequence of reactions depicted in Scheme 1. In the absence of mass transport limitations, plateau current from the ferrocenemethanol-mediated cyclic voltammograms, I_p, is expected to obey the following equation,[26,27]

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

where F is the Faraday's constant, S the electrode area, Γ_E the surface concentration of enzyme, [Fc] the mediator concentration, [G] the glucose concentration in solution, and $k_{red} = k_1k_2/(k_{-1} + k_2)$. On the basis of the equation and the known rate constant values[26,27] ($k_2 = 700$ s^{-1}, $k_3 = 1.2 \times 10^7$ M^{-1}·s^{-1}, $k_{red} = 1.1 \times 10^4$ M^{-1}·s^{-1}), the surface concentration of GOx deposited in the enzyme/dendrimer film was determined. Chronocoulometry was employed for the determination of electrode surface area, and 0.033 cm^2 was registered from an Anson plot.[28]

As shown in Table 1, the coverage of active GOx per bilayer, Γ_E, was estimated to be 1.8×10^{-12} mol·cm^{-2} with good precision, and total coverage ($\Gamma_{E,\,total}$) was linearly proportional to the number of deposited bilayers. The increment in the active GOx coverage per bilayer was retained during the multilayer-forming process. From these results, it follows that each enzyme/dendrimer bilayer has a satisfactory level of active enzyme coverage and that the multilayered structure is formed in a spatially ordered manner. In other words, precise control over the amount of active enzyme immobilization is possible through layer-by-layer depositions of enzyme and dendrimer, and thereby, the sensitivity of the GOx/dendrimer multilayered electrode can be tuned to the desired level by adjusting the number of deposited bilayers.

(25) Bourdillon, C.; Demaille, C.; Moiroux, J.; Savéant, J.-M. *J. Am. Chem. Soc.* **1995**, *117*, 11499−11506.
(26) Anicet, N.; Bourdillon, C.; Moiroux, J.; Savéant, J.-M. *J. Phys. Chem. B* **1998**, *102*, 9844−9849.
(27) Bourdillon, C.; Demaille, C.; Gueris, J.; Moiroux, J.; Savéant, J.-M. *J. Am. Chem. Soc.* **1993**, *115*, 12264−12269.
(28) Adams, R. N. *Electrochemistry at Solid Electrodes;* Marcel Dekker: New York, 1969.

Table 1. Coverage of Active GOx for Enzyme/Dendrimer Multilayers Determined from the Cyclic Voltammetric Data[a]

layer no./EnDn	$\Gamma_{E,\,total}$[b] (10^{-12} mol·cm^{-2})	Γ_E[c] (10^{-12} mol·cm^{-2})
E1D1	1.7 ± 0.1	1.7
E2D2	3.3 ± 0.2	1.6
E3D3	5.2 ± 0.1	1.9
E4D4	7.0 ± 0.1	1.8
E5D5	8.8 ± 0.1	1.8

[a] Plateau currents were measured from the background-corrected voltammograms resulting from subtraction of the glucose-free voltammograms from the mediated (20 mM glucose in solution) voltammograms. [b] Values are the average of triplicated determinations. [c] Active enzyme coverage of each bilayer was calculated from the subtraction of $\Gamma_{E,\,total}$ of the $(n-1)$th layer from $\Gamma_{E,\,total}$ of the nth layer.

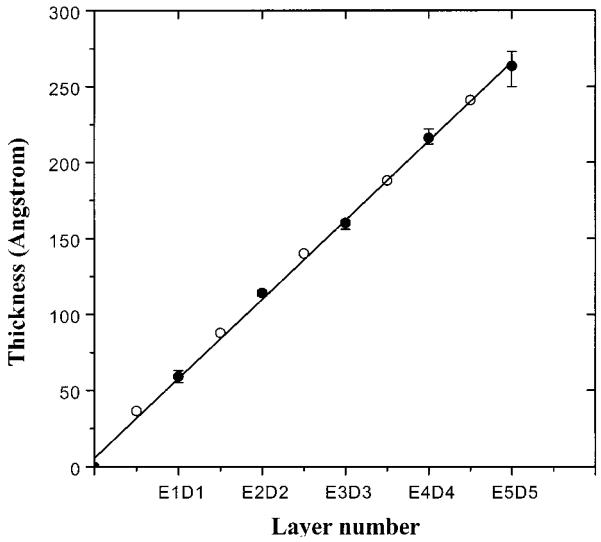

Figure 3. Ellipsometric thickness of the GOx/dendrimer assemblies on Au surfaces vs the number of deposited layers. Open circles represent the thickness of the GOx-terminated multilayers (En + 1Dn).

Additionally, it should be pointed out that the unmediated voltammograms of ferrocenemethanol remained unaltered even though the number of GOx/dendrimer bilayers increased from one to five (Figure 2, inset). This observation supports that the multilayer network has a highly permeable structure, which originates from the interior void architecture of G4 dendrimer itself.[9] It is likely that the structural characteristic of GOx/dendrimer multilayer leads to facile diffusion of glucose and ferrocenyl mediator over the entire assembly, minimizing the transport barrier, which also contributes to the signal enhancement.

Ellipsometric Characterization of the GOx/Dendrimer Multilayered Assembly. For the direct tracing of multilayer growth in detail, ellipsometric measurements were performed with the mono- and multilayers prepared on freshly evaporated Au surfaces. Figure 3 shows the change in film thickness as a function of the GOx/dendrimer layer growth cycle. Film thickness was found to increase in linear proportion to the number of deposited layers, and the increment in thickness per bilayer deposition was ~52 Å. When either dendrimer or GOx was absent during the multilayer-forming process, as a control, film growth was not

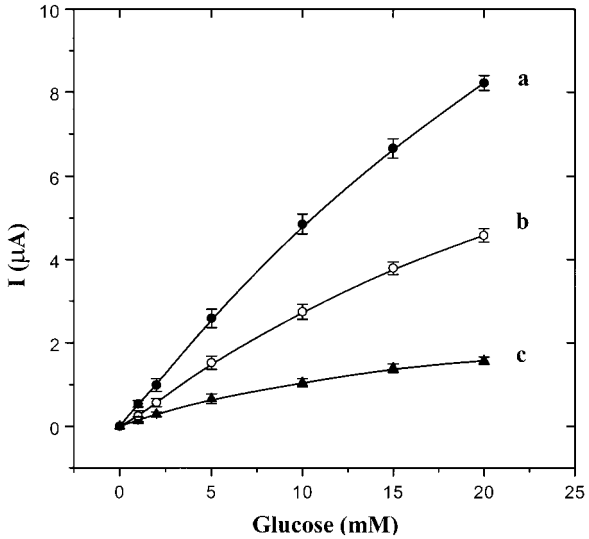

Figure 4. Calibrations for the GOx/dendrimer multilayered electrodes as a function of glucose concentration. Ferrocenemethanol (0.1 mM) was used as an electron-transferring mediator: (a) E5D5, (b) E3D3, and (c) E1D1. Bioelectrocatalytic signal values were registered from the respective cyclic voltammograms at +275 mV vs Ag/AgCl. Other conditions were the same as in Figure 2.

observed. The linear increase in film thickness during the process indicates that each depositing layer grows in an orderly manner. Considering these observations and the known bulk dimensions of assembling G4 PAMAM dendrimer (45 Å)[29] and native GOx (60 × 52 × 77 Å),[30] each layer seems to act as a submonolayer platform on which layer-forming counterparts are linked alternately, and as schematically shown in Figure 1.

Analytical Performance of the GOx/Dendrimer Electrode. To address the analytic usefulness of multilayered enzyme electrodes, calibration curves were obtained for the electrodes associated with one, three, and five GOx/dendrimer bilayers (Figure 4). The oxidative currents were measured for each electrode as a function of glucose concentration in solution. As shown in Figure 4, the anodic signals were significantly amplified for the multilayered electrodes, and this also indicates that the amount of enzyme loaded on Au increases correlative to the number of deposited bilayers. The sensitivity calculated from the linear region of calibration curve was 3.2 μA·mM^{-1} glucose·cm^{-2} for the E1D1 electrode and increased to 8.3 and 14.7 μA·mM^{-1} glucose·cm^{-2} for the E3D3 and E5D5 electrodes, respectively. This result clearly shows that the sensitivity of the EnDn electrode can be adjusted by modulating the deposition number of enzyme/dendrimer bilayers. The ability to control the sensitivity of the electrode should be useful, especially for the electrode containing multiple enzymes with significantly different activities.

The stability of the multilayered enzyme/dendrimer film was evaluated by tracing the sensitivity of the electrode response during extended uses. An E5D5 electrode was stored in phosphate buffer (0.1 M, pH 7.0) at room temperature and subjected to the day-by-day calibrations at room temperature under ambient air conditions. As a result, the sensitivity of the electrode response

(29) Determined by size exclusion chromatography in a citric acid buffer (pH 2.7). Technical data supplied by Dendritech, Inc., Midland, MI.

(30) Hecht, H. J.; Kalisz, H. M.; Hendle, J.; Schmid, R. D.; Schomburg, D. *J. Mol. Biol.* **1993**, *229*, 153−172.

was found to be maintained over 80% of the initial value over 20 days.

In summary, we have demonstrated that dendrimers can be effectively used as building units for the construction of thickness-controllable and spatially ordered biocomposite films. The proposed method is simple and can be directly applied to the bioelectrocatalytic electrodes. The response of the resulting enzyme/dendrimer electrode was significantly enhanced with multilayer growth, which indicates that the sensitivity is tunable by controlling the film thickness. Currently, miniaturized enzyme electrodes in conjunction with microchip technology attract much attention, and high sensitivity is a prerequisite. In this regard, the method developed here should make a significant contribution to the fabrication of microbiosensors. Furthermore, the synthetic manipulation of surface groups of dendrimers and/or conjugating biomolecules is expected to find a wide application in the areas of biomimetic membranes and surface biofunctionalization as well as bio- and chemical sensors.

ACKNOWLEDGMENT

We are grateful to Professor Chul-Hi Han and Mr. Joon-Ho Kim (Department of Electrical Engineering, KAIST) for valuable technical assistance.

Received for review November 11, 1999. Accepted December 1, 1999.

AC991299A