Langmuir
Letter
pubs.acs.org/Langmuir

Ionic-Complementary Peptide Matrix for Enzyme Immobilization and Biomolecular Sensing

Hong Yang, Shan-Yu Fung, Mark Pritzker, and P. Chen*

Department of Chemical Engineering, University of Waterloo, 200 University Avenue West, Waterloo, Ontario, Canada N2L 3G1

Received April 23, 2009. Revised Manuscript Received June 4, 2009

A novel electrochemical biosensing platform is described using biocompatible, self-assembled ionic-complementary peptide nanofibers. The compatibility of a graphite electrode modified by these peptide nanofibers with enzymes is demonstrated using a model enzyme glucose oxidase (GOx). A glucose biosensor has been successfully fabricated by incorporating this enzyme into the modified electrode. From measurement of its electrode response and sensitivity, this nanofiber-modified electrode shows promise as an enzyme-based biosensor. The findings presented here demonstrate excellent potential of the use of ionic-complementary peptides to modify electrode surfaces for biomolecular sensing and diagnostics.

Introduction

Biomolecular surface modification is critical to many biological applications. Such an approach can enhance the material surface with regard to biocompatibility, molecular recognition, and bioactivity.[1−3] In particular, the development of biosensors and biofuel cells relies on the effective immobilization of enzymes on a biocompatible template that allows the preservation of their native structure and function.[4−7] Here, we report the use of a new type of nanobiomaterial, ionic-complementary peptides, for an enzyme immobilization application. We show that the nanobiomaterials studied herein can modify an electrode surface to enhance its compatibility with the model enzyme glucose oxidase (GOx). The patterned nanofibers assembled on the surface have outward-facing COOH and NH_2 functional residues, which are ideal sites for enzyme immobilization. These nanofibers are used as the basis for a model GOx biosensor and demonstrate that these peptides are potential biomolecular surface modifiers for use in (bio)molecular sensing.

Various materials have been used as surface modifiers to extend enzyme activity upon immobilization on a surface. For example, surface modification with poly(ethylene glycol) (PEG) and star-shaped poly(ethylene oxide) (PEO) can preserve the native conformation of bovine serum albumin (BSA) and ribonuclease H (RNase H) upon their adsorption on glass substrates.[8,9] In addition, chitosan and poly(vinyl alcohol) (PVA) have been mixed with pig liver esterase and glucose oxidase (GOx), respectively, to form a biocompatible biohybrid film on surfaces that maintains enzyme activity.[10,11]

For the past decade, the use of peptides has gained increasing attention from many materials scientists. This is mainly attributed to the biocompatibility and molecular recognition of peptides with other (bio)molecules or cells, resulting in many novel polymer−peptide hybrid molecules as surface modifiers for implants or tissue engineering applications.[12−15] Recently, self-assembled dipeptide nanotubes have been used to modify a graphite electrode and enhance its electrochemical activity (e.g., electronic conductivity).[16] These peptide nanotubes were further used to modify a gold electrode to fabricate glucose and ethanol biosensors with improved sensitivity.[17] More recently, metallized peptide nanowires have been employed as conduits for electronic signals between a redox enzyme (NADH peroxidase) and a carbon-nanotube electrode to enhance detection sensitivity.[18]

Here, we employ a novel approach using ionic-complementary peptides to modify an electrode and enhance its compatibility with enzymes for biosensor applications. Our previous studies have shown the ability of one ionic-complementary self-assembling peptide, EAK16-II, to modify both hydrophilic and hydrophobic surfaces via surface-assisted assembly by forming distinct patterned nanostructures.[19,20] A simple adjustment of the solution pH can alter the charge state of the peptide, which in turn affects its interaction with the surface and assembly behavior on

*To whom correspondence should be addressed. E-mail: p4chen@cape.uwaterloo.ca.

(1) Ratner, B. D.; Bryant, S. J. *Annu. Rev. Biomed. Eng.* **2004**, *6*, 41–75.
(2) Morra, M. *Expert Rev. Med. Devices* **2007**, *4*, 361–372.
(3) Vasita, R.; Shanmugam, I. K.; Katt, D. S. *Curr. Top. Med. Chem.* **2008**, *8*, 341–353.
(4) Laurent, N.; Haddoub, R.; Flitsch, S. L. *Trends Biotechnol.* **2008**, *26*, 328–337.
(5) Oh, S. J.; Hong, B. J.; Choi, K. Y.; Park, J. W. *OMICS* **2006**, *10*, 327–343.
(6) Mansur, H. S.; Orefice, R. L.; Vasconcelos, W. L.; Lobato, Z. P.; Machado, L. J. C. *J. Mater. Sci.: Mater. Med.* **2005**, *16*, 333–340.
(7) Tan, Y.; Deng, W.; Ge, B.; Xie, Q.; Huang, J.; Yao, S. *Biosens. Bioelectron.* **2009**, *24*, 2225–2231.
(8) Yang, Z.; Yu, H. *Adv. Mater.* **1997**, *9*, 426–429.
(9) Groll, J.; Amirgoulova, E. V.; Ameringer, T.; Heyes, C. D.; Rocker, C.; Nienhaus, G. U.; Moller, M. *J. Am. Chem. Soc.* **2004**, *126*, 4234–4239.
(10) Gutierrez, M. C.; Jobbagy, M.; Rapun, N.; Ferrer, M. L.; del Monte, F. *Adv. Mater.* **2006**, *18*, 1137–1140.
(11) Wei, X.; Cruz, J.; Gorski, W. *Anal. Chem.* **2002**, *74*, 5039–5046.
(12) Hirano, Y.; Mooney, D. J. *Adv. Mater.* **2004**, *16*, 17–25.
(13) He, W.; McConnell, G. C.; Schneider, T. M.; Bellamkonda, R. V. *Adv. Mater.* **2007**, *19*, 3529–3533.
(14) Raynor, J. E.; Petrie, T. A.; Gracia, A. J.; Collard, D. M. *Adv. Mater.* **2007**, *19*, 1728.
(15) Boateng, S.; Lateef, S. S.; Crot, C.; Motlagh, D.; Desai, T.; Samarel, A. M.; Russell, B.; Hanley, L. *Adv. Mater.* **2002**, *14*, 461–463.
(16) Yemini, M.; Reches, M.; Rishpon, J.; Gazit, E. *Nano Lett.* **2005**, *5*, 183–186.
(17) Yemini, M.; Reches, M.; Gazit, E.; Rishpon, J. *Anal. Chem.* **2005**, *77*, 5155–5159.
(18) Yeh, J. I.; Lazareck, A.; Kim, J. H.; Xu, J.; Du, S. *Biosens. Bioelectron.* **2007**, *23*, 568–574.
(19) Yang, H.; Fung, S.-Y.; Pritzker, M.; Chen, P. *J. Am. Chem. Soc.* **2007**, *129*, 12200–12210.
(20) Yang, H.; Fung, S. Y.; Pritzker, M. D.; Chen, P. *PLoS One* **2007**, *2*, e1325–11.

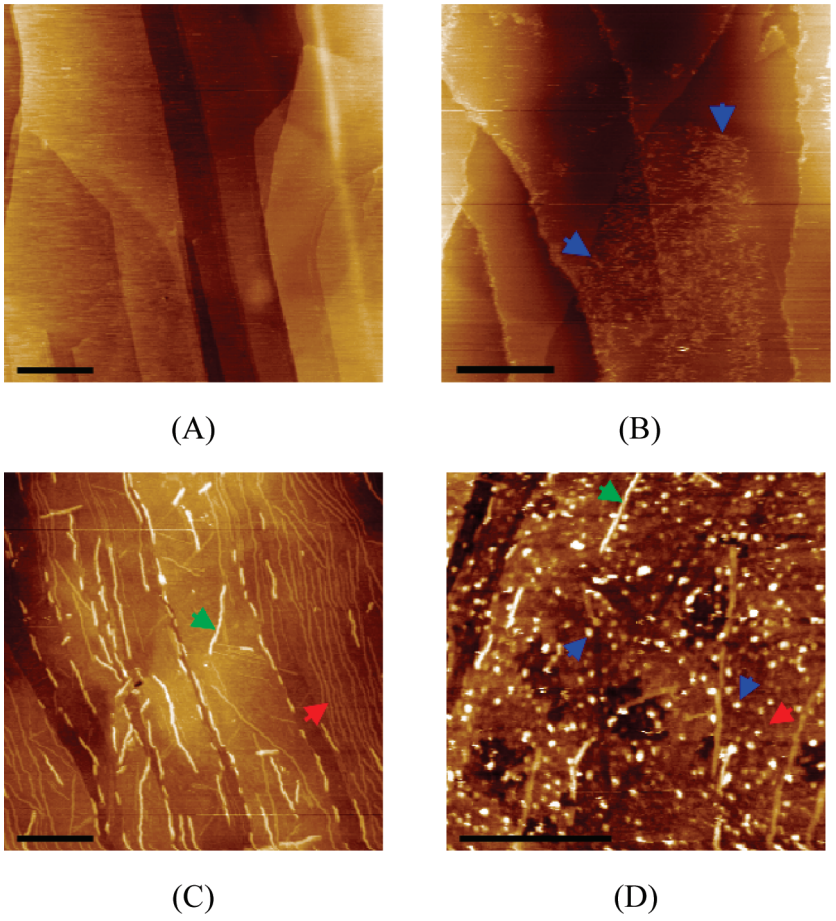

Figure 1. AFM images of (A) bare HOPG, (B) GOx on HOPG, (C) EAK16-II-modified HOPG, and (D) GOx on EAK16-II-modified HOPG electrodes collected in 50 mM phosphate buffer (pH 5.5). Scale bars represent 200 nm.

the surface. Mechanochemical control over such a surface-assisted assembly has further been demonstrated to enable local patterning of peptide nanofibers on surfaces.[21] Moreover, the peptide contains charged residues (e.g., E and K with COOH and NH_2 groups, respectively) that are ideal reaction sites for the immobilization of biomolecules. Based on these findings, we hypothesize that the ionic-complementary peptide nanofibers may serve as biocompatible surface modifiers for enzyme immobilization. A model GOx biosensor has been fabricated to evaluate this hypothesis.

Results and Discussion

For the proof-of-concept, the compatibility of the peptide nanofibers, aimed at modifying a highly ordered pyrolytic graphite (HOPG) electrode, with enzymes is first studied. Glucose oxidase (GOx) is the model enzyme for this study. The compatibility of the surface with an enzyme is partially indicated by the enzyme morphology on the surfaces using *in situ* atomic force microscopy (AFM). It should be noted that the peptide and GOx concentrations and incubation times are maintained lower in the experiments aimed at producing samples for AFM imaging than those used in the subsequent stage when the glucose biosensor is fabricated. Our experience showed that this procedure is necessary to ensure that nanostructure and patterning could be more clearly discerned in the AFM images. As shown in Figure 1A, the AFM image of a bare HOPG surface shows an atomically flat, layer-by-layer structure of graphite. When the GOx solution is deposited on HOPG, amorphous aggregates of GOx molecules with a height of ~1 nm are observed on the HOPG surface within 20 min incubation (Figure 1B). Upon adsorption on HOPG, these amorphous structures of GOx are very different from its native globular shape ($7 \times 5.5 \times 8$ nm^3),[22] indicating that GOx is likely denatured on the HOPG surface. This could be due to the strong hydrophobicity of the bare HOPG that makes the surface far from biocompatible.[23] Evidence has been reported that trypsin denatures irreversibly when adsorbing onto a hydrophobic polystyrene surface.[24]

On the contrary, the EAK16-II-modified HOPG surface indicates good compatibility with GOx. Upon 20 min immersion of HOPG in 0.2 mg mL^{-1} EAK16-II solution, nanofiber matrices are formed on the surface (Figure 1C). Two types of nanofibers are observed. One is a mature fiber with a height of 2.7 ± 0.3 nm (denoted by a green arrow) that is believed to form in the solution and then adhere to the surface.[19,25] The other is a small fibril with a height of ~1 nm (red arrow). These small fibrils are formed by peptide assembly on the surface following a nucleation and growth mechanism.[19,21] The resultant nanofiber matrix may provide a compatible environment for the enzyme immobilization. As shown in Figure 1D, the adsorbed GOx has a globular

(21) Yang, H.; Fung, S.-Y.; Pritzker, M.; Chen, P. *Angew. Chem., Int. Ed.* **2008**, *47*, 4397–4400.
(22) Hecht, H. J.; Kalisz, H. M.; Hendle, J.; Schmid, R. D.; Schomburg, D. *J. Mol. Biol.* **1993**, *229*, 153–172.
(23) Davis, J. J.; Hill, A. O. *Chem. Commun.* **2002**, 393–401.
(24) Koutsopoulos, S.; Patzsch, K.; Bosker, W. T. E.; Norde, W. *Langmuir* **2007**, *23*, 2000–2006.
(25) Fung, S. Y.; Keyes, C.; Duhamel, J.; Chen, P. *Biophys. J.* **2003**, *85*, 537–548.

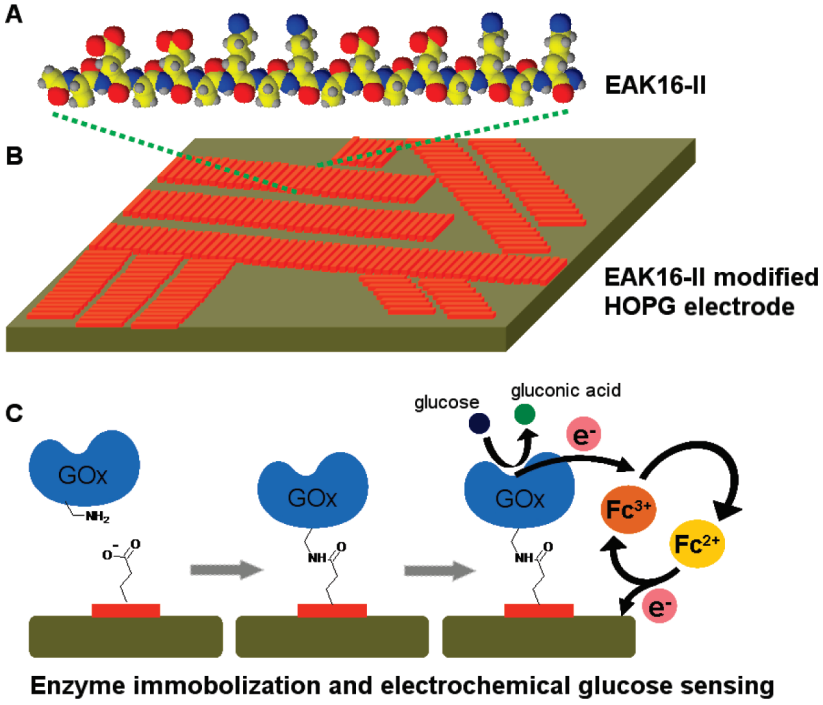

Figure 2. Schematic diagram of an ionic-complementary peptide modified HOPG electrode for GOx immobilization and glucose sensing. (A) EAK16-II molecular structure, (B) EAK16-II nanofiber coatings on HOPG electrode, and (C) GOx immobilization and glucose sensing mechanism.

shape (blue arrows) with a height of ~5 nm on the peptide nanofiber-modified HOPG surface. The observation of this morphology suggests that GOx retains its native structure on the peptide nanofiber matrix, in contrast to the denatured one on the bare HOPG surface. It is worth noting that the uncovered area on HOPG in Figure 1D may also induce denaturation of GOx; however, it is difficult to directly observe the amorphous structure of denatured GOx in Figure 1D due to the loss of AFM resolution arising from the dramatic difference in height of the denatured (~1 nm) and native GOx (~5 nm).

Next, we show the potential use of such a peptide-modified, enzyme compatible surface for molecular biosensing. The procedure of enzymatic electrode fabrication and its sensing mechanism are illustrated in Figure 2. First, the HOPG electrode is modified by EAK16-II nanofibers. As noted in the Experimental Section, the EAK16-II nanofibers on the HOPG surface are then activated by contacting the surface with a phosphate buffer containing EDC and NHS before covalent immobilization of GOx. AFM images of the surface after activation and before GOx immobilization show that the nanofiber morphology is unchanged by the contact with EDC and NHS (data not shown). In the next step, the GOx is covalently immobilized on the peptide nanofiber matrix through amide bond formation between the amine groups on GOx and the carboxylate groups of glutamic acid residues (E) on the peptide. After the GOx is immobilized, its catalytic activity for glucose oxidation is measured using Fc^{3+} (ferrocenecarboxylic acid, FCA) as a mediator for electron transduction.[26]

The enzymatic activity of the immobilized GOx on the electrode is characterized using cyclic voltammetry. Figure 3A shows cyclic voltammograms of the GOx-immobilized EAK16-II-modified HOPG electrode in 0.1 M potassium phosphate buffer (pH 7.0) containing 0.2 mM FCA in the absence and presence of 5, 10, and 20 mM glucose. In the absence of glucose, the characteristic and well-behaved voltammogram of FCA is observed (Figure 3A, black line). In the presence of glucose, we observe an electrocatalytic anodic current, which increases with rising glucose concentration, indicating that GOx molecules are effectively immobilized on HOPG and remain active.[27] It is worth noting that GOx physically adsorbed on bare HOPG without EAK16-II also exhibited an increase in the electrocatalytic anodic current, but to a much less degree (Figure 3A inset). With the presence of 20 mM glucose, the current was $<20\ \mu$A, which was even smaller than that (~30 μA) of GOx-immobilized EAK16-II-modified HOPG electrode with 5 mM glucose. This indicates that the surface modification of HOPG by EAK16-II can enhance GOx catalytic activity, probably due to the preservation of native GOx on the peptide modified HOPG.

A calibration curve correlating the electrochemical response with glucose concentration is further established based on chronoamperometric measurements. $I-t$ curves for the enzyme electrode shown in Figure 3B are acquired at a constant potential of 0.45 V in the presence of various concentrations of glucose (0−20 mM). The background-subtracted steady-state currents (obtained within 2 min) at various glucose concentrations are used to construct the calibration curve shown in Figure 3C. It can be seen that anodic currents rise with increasing glucose concentration until they reach a plateau at high glucose concentrations (>12 mM); the shape of this curve is consistent with that expected for enzyme catalysis obeying typical Michaelis−Menten kinetics.[27] The response is linear up to 10 mM glucose (Figure 3C, inset), which covers the range of 3.5−6.5 mM required for clinical applications.[28] In addition, the sensitivity is found to be 26 nA mM^{-1} mm^{-2}, which is more than 6 times

(26) Chaubey, A.; Malhotra, B. D. *Biosens. Bioelectron.* **2002**, *17*, 441–456.

(27) Zhang, S.; Wang, N.; Yu, H.; Niu, Y.; Sun, C. *Bioelectrochemistry* **2005**, *67*, 15–22.

(28) Heider, G. H.; Sasso, S. V.; Huang, K. M.; Yacynych, A. M.; Wieck, H. J. *Anal. Chem.* **1990**, *62*, 1106–1110.

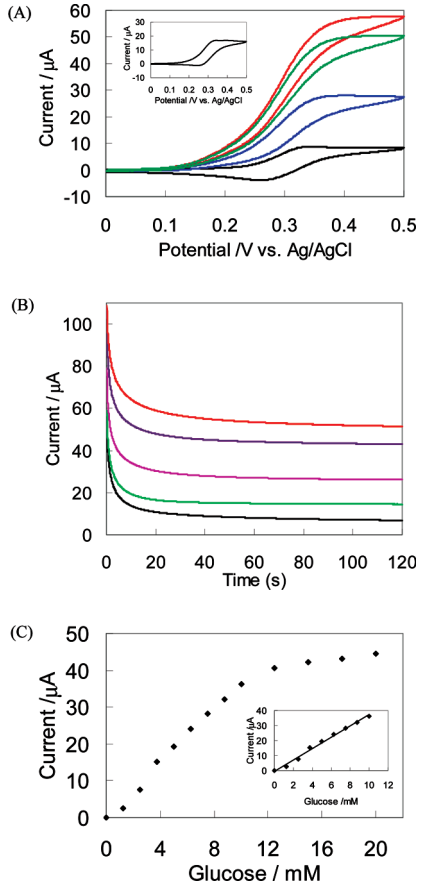

Figure 3. (A) Cyclic voltammograms of the GOx-immobilized EAK16-II-modified HOPG electrode obtained in 100 mM potassium phosphate buffer (pH 7) containing 0.2 mM FCA and 0 mM (black line), 5 mM (blue line), 10 mM (green line), and 20 mM glucose (red line). Inset shows CV of unmodified HOPG electrode with GOx physically adsorbed in 20 mM glucose. (B) Amperometric responses of the GOx-immobilized EAK16-II-modified HOPG electrode in 100 mM potassium phosphate buffer (pH 7.0) containing 0.2 mM FCA and various concentrations of glucose (0, 2.5, 5, 10, and 20 mM from bottom to top). (C) Calibration curve for the GOx-immobilized EAK16-II- modified HOPG electrode; the inset shows the linear range of the calibration curve, $r^2 = 0.97$.

higher than that obtained with GOx on thiol-modified gold nanotubes and gold electrodes (~4 nA mM^{-1} mm^{-2}).[29,30]

In summary, we have shown that an ionic-complementary peptide can modify an enzyme electrode for (bio)molecular sensing. The presence of ionic-complementary peptide nanofiber assemblies on an electrode surface not only provides functional groups to immobilize enzymes but also improves the surface compatibility with enzymes. The activity and stability of the immobilized enzymes also appear to be enhanced. The information obtained in this study provides evidence for the potential use of self-assembling peptides such as EAK16-II to fabricate biocompatible electrode surfaces for immobilization of other enzymes and their use in (bio)molecular sensing.

Experimental Section

Materials. The ionic-complementary peptide EAK16-II ($C_{70}H_{121}N_{21}O_{25}$, molecular weight 1657 g mol^{-1}) has the sequence AEAEAKAKAEAEAKAK, where A corresponds to alanine, E to glutamic acid, and K to lysine. This peptide was purchased from CanPeptide Inc. (Quebec, Canada) with a purity > 95% (purified by reverse-phase high-performance liquid chromatography). The N-terminus and C-terminus of the peptide were protected by acetyl and amino groups, respectively.

Highly ordered pyrolytic graphite (HOPG, ZYB grade) was obtained from SPI Supplies (West Chester, PA). The dimensions of HOPG for AFM imaging and electrochemical measurements are 2 cm × 2 cm and 1.2 cm × 1.2 cm, respectively. Glucose oxidase (GOx, EC 1.1.3.4 type X-S, from *Aspergillus niger*), glucose, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), *N*-hydroxysulfosuccinimide (NHS), ferrocenecarboxylic acid (FCA), and potassium ferricyanide were all purchased from Sigma-Aldrich (Oakville, ON, Canada).

Atomic Force Microscopy. AFM Imaging of the GOx on HOPG and EAK16-II-modified HOPG surface was performed using a PicoScan AFM (Molecular Imaging, Phoenix, AZ) in 500 μL of 50 mM potassium phosphate buffer (pH5.5) in an environmentally controlled chamber at room temperature to minimize evaporation of the solution. The EAK16-II-modified HOPG was obtained with 200 μL of 0.2 mg mL^{-1} EAK16-II immobilized on HOPG for 20 min. This modified surface was then rinsed with pure water to remove the loosely attached peptide prior to liquid AFM imaging. To collect AFM images of GOx on bare HOPG and EAK16-II-modified HOPG, 30 μL of 0.1 mg mL^{-1} GOx was injected into a liquid cell with 500 μL of 50 mM potassium phosphate buffer (pH 5.5) inside. The AFM imaging was then performed after ~30 min.

A scanner with a maximum scan area of 6 × 6 μm^2 was used. Silicon nitride cantilevers with a nominal spring constant of 0.58 N m^{-1} (DNP-S, Digital Instruments, Santa Barbara, CA) and a tip radius of 10 nm were used for tapping mode operation. For the best imaging quality, the tapping frequency was typically set between 16 and 18 kHz and the scan rate was controlled at 0.857 lines s^{-1}. AFM images were obtained at a resolution of 256 × 256 pixels.

Preparation of Enzyme Immobilized Electrode. A total of 200 μL of 0.5 mg mL^{-1} EAK16-II stock solution was injected onto the freshly cleaved surface of HOPG electrodes (1.2 cm × 1.2 cm) and incubated overnight in order to fabricate the EAK16-II-modified electrodes. The modification condition used here was to ensure that a higher peptide coverage percentage on HOPG can be reached based on our previous studies.[20] The EAK16-II-modified electrode surfaces were washed three times with pure water to remove loosely attached EAK16-II. The carboxylic acid groups of EAK16-II on the electrode were then activated with 200 μL of 50 mM potassium phosphate buffer (pH 5.5) containing 2 mM EDC and 5 mM NHS for 1 h at room temperature. This step converts the carboxylate group into its NHS ester, which is susceptible to attack by amines. Consequently, amide bonds form between the enzyme molecules and the glutamic acid residues of the peptide. After the activation, the electrodes were rinsed with a potassium phosphate buffer (pH 5.5) three times and immediately covered with 200 μL of 50 mM phosphate buffer (pH 5.5) containing 1 mg mL^{-1} GOx and incubated overnight at 4 °C. The enzyme electrode was then rinsed with a potassium phosphate buffer (pH 7.0) and used immediately.

Electrochemical Measurements. All electrochemical measurements were conducted with a CHI650A potentiostat (CH Instruments, Inc., Austin, TX). Cyclic voltammetry was carried out in a conventional three-electrode configuration, with the GOx-immobilized EAK16-II-modified HOPG electrode (GOx/EAK16-II/HOPG, geometric area of 1.44 cm^2) as the working electrode, a platinum wire as the auxiliary electrode, and Ag/AgCl (3 M NaCl, Bioanalytical Systems, West Lafayette, IN) as the reference electrode. All potentials reported herein correspond to the Ag/AgCl scale. The scans measured on the GOx-immobilized-EAK16-II-modified HOPG electrode proceeded from 0.0 to 0.5 V and back to 0.0 V at a scan rate of 2 mV s^{-1}. The electrolyte was a 100 mM potassium phosphate buffer containing 0.2 mM FCA

(29) Delvaux, M.; Demoustier-Champagne, S. *Biosens. Bioelectron.* **2003**, *18*, 943–951.
(30) Gooding, J. J.; Pugliano, L.; Hibbert, D. B.; Erokhin, P. *Electrochem. Commun.* **2000**, *2*, 217–221.

and various concentrations of glucose ranging from 0 to 20 mM (diluted from a stock solution prepared at least 24 h prior to use to allow mutarotation to occur). The activity of the immobilized GOx was tested by measuring the current response during cyclic voltammetry experiments in the presence of various concentrations of glucose.

Acknowledgment. We would like to thank Professor Susan Mikkelsen for use of electrochemical equipment and providing very helpful discussions. We also thank Dr. Wei Sun for training with regard to glucose biosensor fabrication and useful discussion. We thank Tony Qian for his help on some electrochemical experiments. Support for this research has been provided by the Natural Sciences and Engineering Research Council of Canada (NSERC), Canadian Foundation for Innovation (CFI), the Canada Research Chairs (CRC) Program for one of the coauthors (P.C.), and the Canada Graduate Scholarships (CGS) Program for another coauthor (S.-Y.F.).