

Spatial Confinement Induced Enzyme Stability for Bioelectronic Applications

Gang Wang and Siu-Tung Yau*

Department of Electrical and Computer Engineering, Cleveland State University, Cleveland, Ohio 44115

Received: January 8, 2007; In Final Form: June 8, 2007

The stability of glucose oxidase (GOx) immobilized on a silicon electrode can be enhanced using spatial confinement as described in this paper. We show that when GOx is assembled in semiopen spatially confined structures created on the unmodified surface of a silicon electrode, the enzyme's activity is preserved under destabilizing conditions. GOx-immobilized silicon electrodes were treated with guanidinium chloride and high temperature. Cyclic voltammetry measurement of the treated electrodes showed the proper redox characteristics of GOx and indicated the GOx-catalyzed electrooxidation of glucose. When GOx was immobilized on flat silicon electrodes, voltammetric measurement showed null result presumably caused by denaturation of the enzyme. The effect of spatial confinement on enzyme stability is also revealed by analyzing the characteristic rate constants and the kinetic parameter for the complete catalytic process of glucose.

Introduction

A thrust in the present bioelectronic research is to obtain enhanced electron transfer at the interface between immobilized proteins/enzymes and the electrode of amperometric devices such as biofuel cells and biosensors. Sensors with high sensitivity and biofuel cells with high current density can be realized with high interfacial electron transfer.^{1,2} However, in general, when enzymes are immobilized on inorganic solid surfaces, interfacial interactions and an altered environment may induce changes in the native conformation of enzymes. Drastic changes may produce detrimental effects on enzymes so that they lose their enzymatic activity.³ The problem of enzyme denaturation is, in general, solved by introducing an intervening layer of organic materials between the electrode and proteins/enzymes.^{4–8} However, the thickness of the intervening layer causes additional impedance for electron transfer. Denaturation can also be caused by the heat generated at the enzyme–electrode interface of biofuel cells. Thus, techniques need to be developed for preparing an enzyme–electrode interface, which provides enhanced electron transfer with increased enzyme stability.

Previously, it has been shown experimentally that when proteins are encapsulated in porous materials⁹ or entrapped inside layered materials,¹⁰ they are protected against denaturation with retained bioactivity. A recent theoretical treatment of spatial confinement of proteins has been put forward based on the estimation of the change of the folding free energy of a protein as a function of spatial confinement.^{11,12} For a protein confined in a cubic cage, the free energy difference exhibits a minimum when the size of the cage is just slightly larger than the size of the native protein. This is the condition for the confined protein to acquire maximum stability. Even when the cage size is 6 times that of the native protein, the stabilization due to spatial confinement is still on the order of $k_B T$. However, the high degree of confinement may have implications on the reaction kinetics. For bioelectronic applications, the requirement of sensitivity and current density makes these immobilization techniques unsuitable.

In this paper, we describe an enzyme/protein immobilization technique which, when applied to GOx immobilized on an unmodified semiconductor electrode, renders enhanced stability for the immobilized enzyme against destabilizing conditions. The technique consists of creating grooves on an unmodified semiconductor electrode such as a silicon wafer and assembling an enzyme into these semiopen spatially confined structures. Electrochemical characterization indicates the presence of the enzymatic activity of GOx immobilized on both silicon surfaces containing grooves and the bare silicon surface. The two kinds of enzyme-immobilized electrodes were treated with a denaturing agent and high temperature. It was found that only the spatially confined enzyme preserved its enzymatic activity after the treatments. We analyze different quantities of the catalytic process of glucose using the preserved enzymatic activity of the spatially confined GOx. These quantities including characteristic rate constants and the Michaelis constant provide additional information about the effect of spatial confinement on enzyme stability.

Experimental Section

GOx (EC 1.1.3.4, 15.5 units/mg, from *Aspergillus niger*) and β -D-(+)-glucose were purchased from Sigma-Aldrich and used as received. All other chemicals were of analytical grade and used without further purification. GOx was dissolved in 0.01 M PBS at pH 7 to reach a concentration of 10 mg/mL. Glucose solutions were prepared overnight before use to allow equilibration of anomers. All solutions were prepared with water (18.2 M Ω cm) from a Direct-Q 5 Millipore system.

Heavily doped ($\rho < 0.005 \Omega$ cm) n-type silicon wafers with (111) orientation were used in this experiment. The grooves were made by one scratching action on the surface of a silicon wafer using a sewing needle.¹³ The needle was held with a pair of pliers that was held manually by hand. A moderate pressure was applied to the needle while the surface of a 1 cm \times 1 cm silicon wafer was scratched. The size of the scratched region was about 3 mm \times 0.5 mm. The scratched wafer was cleaned in acetone, 2-propanol, and deionized water. Adhesive tape was used to mask the wafer to form a 2 mm \times 2 mm square about the scratched region. To deposit GOx on both nonscratched and

* To whom correspondence should be addressed. E-mail: s.yau@csuohio.edu. Fax: (216) 687-5405. Phone: (216) 875-9783.

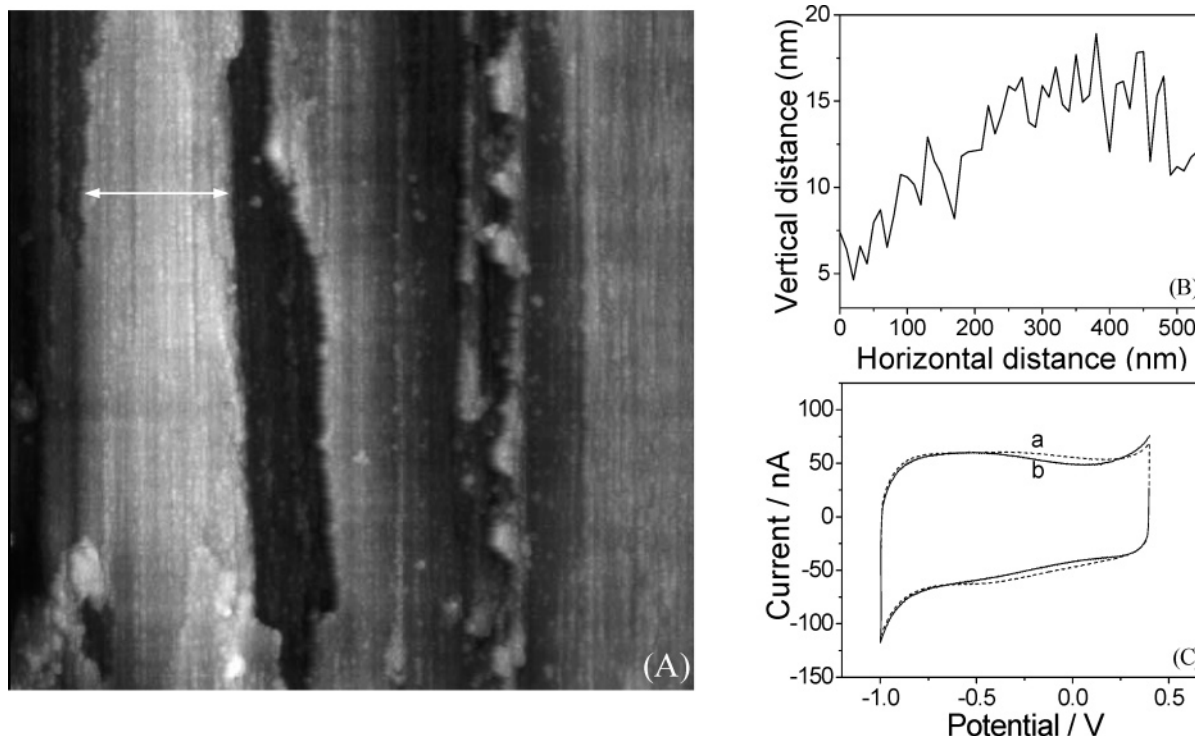


Figure 1. (A) AFM image ($3\ \mu\text{m} \times 3\ \mu\text{m}$) of a scratched silicon wafer. The span of the gray scale is 400 nm. (B) Line profile of the location as indicated by the arrows in (A). (C) CVs of the scratched silicon (curve a) and flat silicon (curve b) electrodes in PBS at pH 7.0.

scratched silicon, a drop of the GOx solution was placed on the masked surface, and the sample was sealed in a container for incubation. A wet cotton swab was used to wipe the unscratched region within the square to remove the GOx. The GOx-groove electrode was finally rinsed with PBS and transferred with the sample covered in PBS to an electrochemical cell for measurement. Chemical treatment of the samples was carried out by incubating them in 4 M guanidinium chloride (GdmCl) for 4 h. Heat treatment was carried out by immersing the samples in water at $80\ ^\circ\text{C}$ for 60 min. The treated electrodes were rinsed with PBS.

The electrochemical measurement system consisted of a conventional three-electrode cell and a potentiostat (Princeton Applied Research, model 283). GOx-groove electrodes were used as the working electrode with an active area of $0.04\ \text{cm}^2$. A commercial (Microelectrode, Inc.) Ag/AgCl (3 M KCl, saturated with AgCl) electrode was used as the reference electrode. A platinum wire was used as the counter electrode. All the experiments were carried out at room temperature ($22 \pm 1\ ^\circ\text{C}$) in 0.01 M PBS as the supporting electrolyte. Deoxygenated PBS was obtained by purging the PBS using highly pure nitrogen for 15 min and maintained under a nitrogen atmosphere during the measurements. Atomic force microscopy (AFM) of the samples was performed using the tapping mode.

Results and Discussion

Semiopen spatially confined structures have been created by scratching the surface of a heavily doped n-type silicon wafer that contained its native oxide. As shown previously, scratching the surface of a silicon wafer results in parallel grooves with different widths and depths on the nanometer scale.¹³ Figure 1A is an AFM image of a scratched silicon wafer surface, showing parallel grooves and scattered “debris” just outside some grooves due to the scratching. A line profile of a region on the surface as indicated by the white line in Figure 1A is shown in Figure 1B. The line profile shows that the grooves

have a typical cross-sectional size of $25\ \text{nm} \times 6\ \text{nm}$ (width \times depth). Note that the actual size of the grooves should be smaller than the measured size due to the finite size of the AFM tip. Given that the dimension of the GOx molecule is $60\ \text{\AA} \times 52\ \text{\AA} \times 77\ \text{\AA}$,¹⁴ it is estimated that about two or three GOx molecules can be accommodated per cross-section of the groove. Figure 1C shows the cyclic voltammograms (CVs) of the scratched silicon wafer (curve a) and the flat silicon wafer (curve b). Since the scratched silicon was cleaned using deionized water and left under ambient conditions for about 30 min before being used in the voltammetric measurement, an oxide layer is expected to have formed in the scratched region. Figure 1C shows that the two CVs are almost identical.

Previously, we have shown that, when immobilized on the native oxide of silicon, GOx exhibits its proper redox characteristics with preserved enzymatic activity.¹⁵ The AFM image of Figure 2A shows that the same region as shown in Figure 1A is covered with a near monolayer of spherical structures. The smallest structure has a diameter of about 21 nm. It is known that, in AFM imaging, the tip-induced convolution effect usually makes nanometer-sized objects appear to be up to several times larger. We assign the 21 nm spherical structures to be a GOx molecule. Within the near monolayer, there are also larger elongated structures of about $30\ \text{nm} \times 50\ \text{nm}$, which are likely due to the aggregation of two to three GOx molecules. An inspection of Figures 1A and 2A indicates that all of the surface features other than those indicative of the grooves appearing in Figure 1A are visible in Figure 2A. Thus, a conclusion can be made that the GOx molecules that were immobilized in the grooved area were partly confined by the grooves.

The CV of this GOx-groove sample is shown in Figure 2B. The CV shows the redox peaks of the immobilized GOx with a formal potential of $-0.32\ \text{V}$ vs Ag/AgCl. This sample was then immersed in 4 M GdmCl, a denaturant, for 4 h. It was shown that if GOx is treated with 4–6 M GdmCl for 2 h, extensive unfolding of the enzyme takes place due to the strong

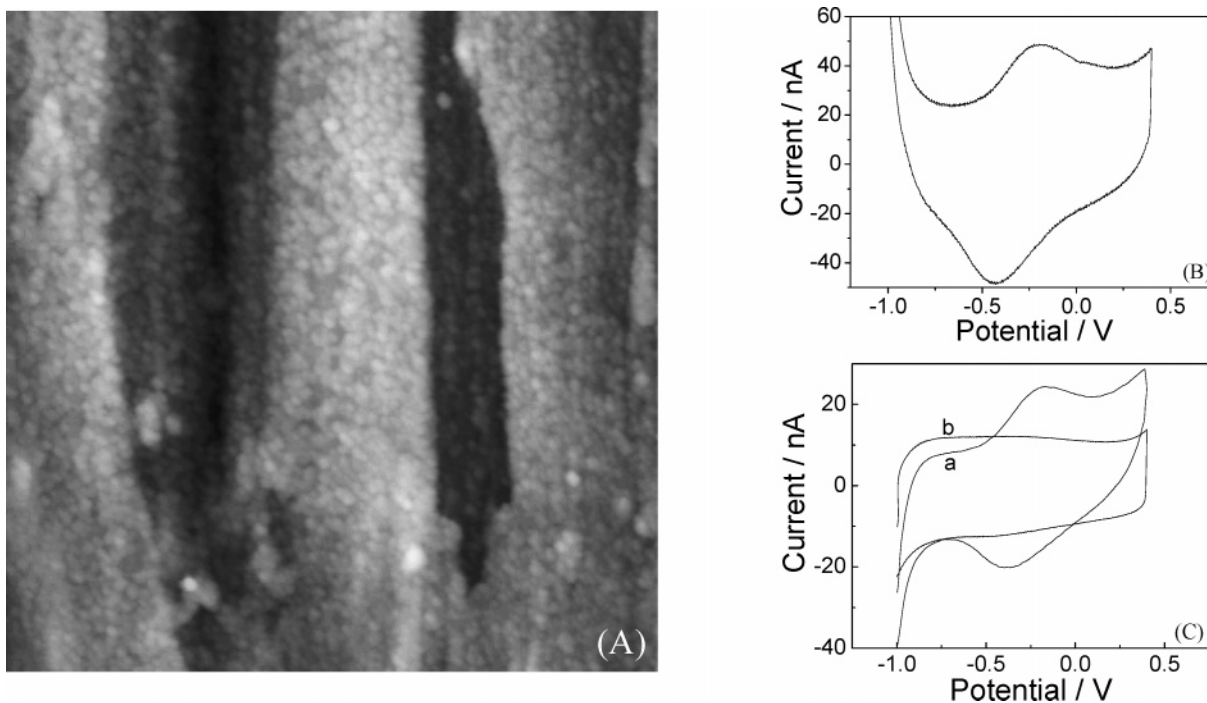


Figure 2. (A) AFM image ($2 \mu\text{m} \times 2 \mu\text{m}$) of the same region as in Figure 1A. The span of the gray scale is 400 nm. (B) CV of the GOx-groove sample as in (A) before treatment. (C) CV (curve a) of the GOx-groove sample after a 4 h GdmCl treatment. Curve b is the CV of a GOx-immobilized flat silicon sample. The background signal due to the silicon without GOx as shown in Figure 1C has been subtracted for all the CVs.

destabilization character of GdmCl.^{16,17} Figure 2C shows the CV (curve a) of the GdmCl-treated GOx-groove sample. The presence of the redox peaks on the CV indicates that the treated GOx was able to retain its redox reaction with the silicon. The formal potential of this redox reaction is about -0.31 V. Figure 2C also shows the CV (curve b) of a GOx-immobilized flat silicon that was treated with GdmCl under identical conditions. The CV is featureless, indicating the detrimental effect the treatment produced on the redox process of the immobilized enzyme. AFM imaging (not shown) showed that there was still a near monolayer of GOx on the flat electrode surface after the treatment. The absence of redox peaks is likely due to denaturation. However, the GOx immobilized on the grooved surface has survived the treatment.

To study the effect of heat, a GOx-groove sample was subjected to high temperature. It is known that an elevated temperature induces instability of enzymes, causing denaturation to occur. A recent study shows that thermally induced denaturation of GOx occurs at 55.8 ± 1.2 °C with the dissociation of the flavin cofactor.¹⁸ The GOx-groove sample was exposed to 80 °C for 60 min. Figure 3 shows the CV (curve a) of the heat-treated sample. The redox peaks of GOx are present with a formal potential of -0.31 V. On the contrary, GOx immobilized on flat silicon electrodes show no peaks (curve b) presumably due to the dissociation of the flavin cofactor.

The redox properties, i.e., positions of redox peaks and formal potential, of the two kinds of treated GOx-groove samples and of the untreated GOx-groove sample are listed in Table 1. The presence of the redox peaks on the CV indicates the presence of the redox process between the enzyme and the electrode. The retained redox process of GOx after the destabilizing treatment can be expressed more quantitatively. Since a near monolayer of GOx was present on the electrode, the surface density Γ of active GOx, which gave rise to the redox process, can be estimated using the redox peak current, constants, and experimental parameters.¹⁹ The value of Γ for the GdmCl-treated sample is calculated to be 2.93×10^{-12} mol/cm², while that

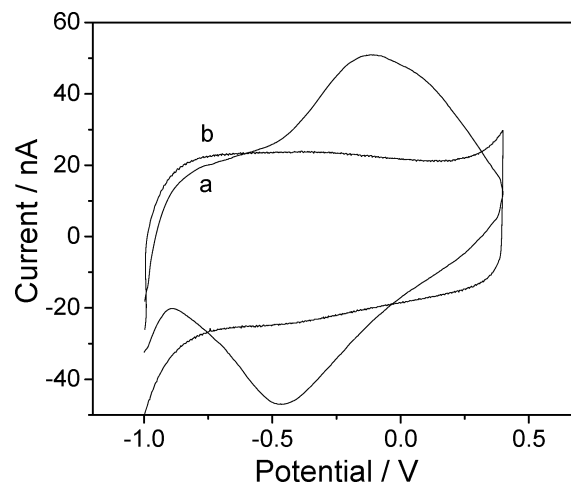


Figure 3. CV (curve a) of a GOx-groove sample and CV (curve b) of a GOx-immobilized flat silicon sample. Both samples have been exposed to 80 °C for 60 min. The background signal due to the silicon without GOx as shown in Figure 1C has been subtracted for both curves.

TABLE 1: Redox Properties of Treated and Untreated GOx-Groove Samples^a

sample	E_{Red} (V)	E_{Ox} (V)	$E_{1/2}$ (V)
GdmCl	-0.43	-0.19	-0.31
high temp	-0.48	-0.14	-0.31
untreated	-0.43	-0.19	-0.32

^a E_{Red} , E_{Ox} and $E_{1/2}$ are the positions of the reduction peak and oxidation peak, and formal potential, respectively.

for the same sample before treatment is 3.73×10^{-12} mol/cm². The corresponding values for the high-temperature-treated sample are 3.24×10^{-12} and 5.18×10^{-12} mol/cm². In both cases, the value of Γ for the treated sample is very close to that for the untreated sample. Thus, the detrimental effect of the treatment on the redox properties of GOx is insignificant.

The retained redox process of GOx, however, does not necessarily imply that the enzyme's activity was preserved. A

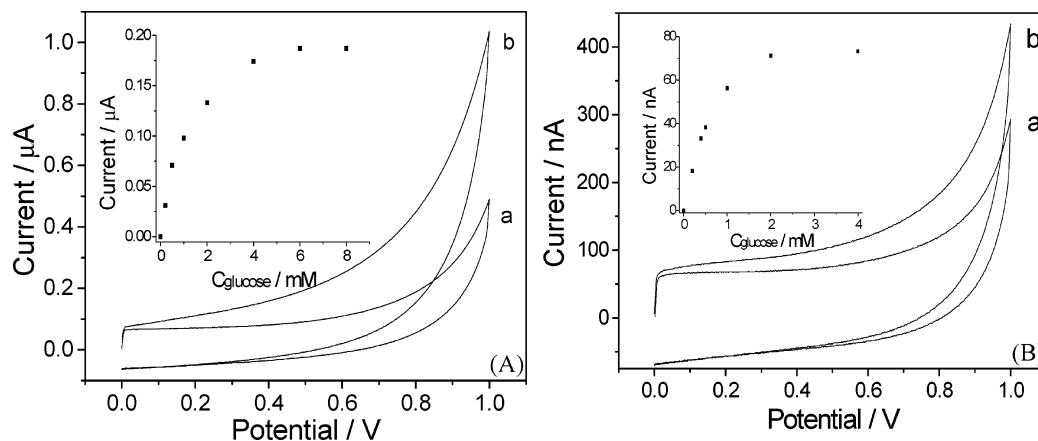


Figure 4. (A) CV of the GdmCl-treated GOx-groove sample in the positive potential range. Curve a was obtained in PBS only. Curve b was obtained with 4 mM glucose added to the PBS. The inset is the calibration of the sample obtained at 0.7 V. (B) CV of the GOx-groove sample exposed to 80 °C in the positive potential range. Curve a was obtained in PBS only. Curve b was obtained with 1 mM glucose added to the PBS. The inset is the calibration of the sample obtained at 0.7 V.

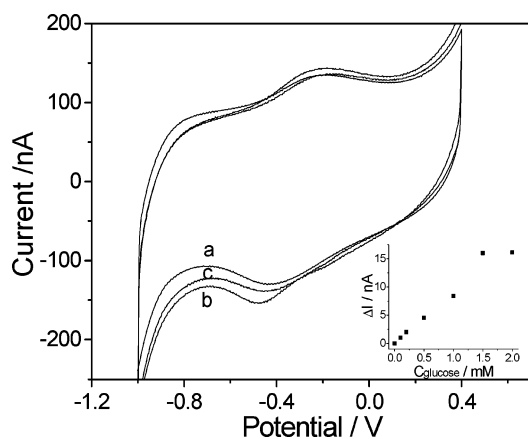


Figure 5. CVs of the GdmCl-treated sample in the negative polarity of the potential. The inset is the calibration curve, where ΔI is the signal current.

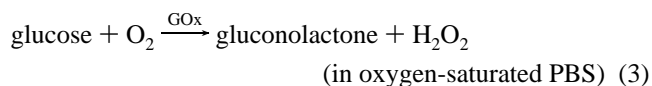
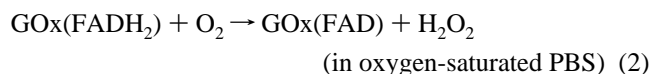
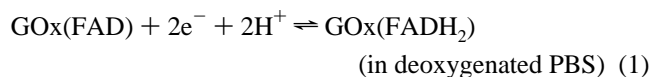
previous study shows that, even when GOx is unfolded so that it loses its catalytic property for the oxidation of glucose, the denatured enzyme can still produce redox peaks on its CV.²⁰ The formal potential of an enzyme is usually used as an indication of changes in the conformation and therefore possible denaturation of a protein.²¹ Table 1 shows that the formal potentials, $E_{1/2}$, of the treated GOx are very close to that of the untreated sample. Although this may imply that the conformation of the immobilized GOx remained unchanged after the treatments, the difference between the $E_{1/2}$ values for the present work and that for the native GOx, namely, -0.34 V vs Ag/AgCl, at pH 7,²² makes a prediction of the preservation of the enzymatic activity of the treated GOx difficult.

To elucidate the enzymatic activity of the treated GOx of the GOx-groove electrode, we characterized the electrodes' response to glucose using two independent electrochemical schemes.

In the first scheme, glucose was introduced to the electrochemical cell that contained a nitrogen-purged phosphate buffer solution (PBS). The potential was then scanned in the positive polarity range. The GOx-induced oxidation of glucose resulted in transporting electrons from glucose via GOx to the silicon. Figure 4A shows the CVs obtained with the GdmCl-treated electrode described above. Curve a was obtained without the presence of glucose, while curve b was obtained with 4 mM glucose in the PBS. The inset is the electrode's calibration curve for glucose. The corresponding CVs of the high-temperature-

treated electrode are shown in Figure 4B. The direction in which the current increases with respect to that of the scanning of the potential as shown in the CVs of Figure 4 indicates that glucose is oxidized so that electrons flow into the electrode. The result shown in Figure 4 shows that the treated GOx is still capable of performing the catalytic process (oxidation of glucose). In other words, the enzymatic activity of the treated enzyme is preserved. Note that no such effect has been observed using electrode immobilized with free FAD or denatured GOx in the literature.^{20,23}

In the second scheme, the potential is scanned in the negative polarity range of the potential. Working in the negative potential range allows one to observe simultaneously the GOx-silicon electron transfer during the catalytic process. In the experiment, the GOx-immobilized silicon electrode was used as the working electrode in cyclic voltammetry measurements, subjected to the following conditions:



The three reactions are a protocol for detecting glucose by monitoring the change in the reduction current of reaction 1, which is the redox reaction of the immobilized GOx. These conditions correspond to the redox reaction of the immobilized GOx as described by reaction 1, coupled respectively to the oxidation of FADH₂ (reaction 2) and to the oxidation of glucose (reaction 3). Figure 5 shows three CVs of the GdmCl-treated sample obtained under the three conditions. Curve a in Figure 5 indicates the presence of the redox reaction of GOx immobilized on the silicon electrode measured in a deoxygenated PBS (reaction 1). When the PBS is saturated with molecular oxygen, the voltammogram changes dramatically with an increase of the reduction peak current and decrease of the oxidation peak current as shown in curve b. The increased reduction peak current indicates that the reduced form of GOx in reaction 1 is oxidized by dissolved O₂ via reaction 2 so that reaction 1 favors more reduction of GOx. Upon adding β-D-(+)-glucose to the O₂-saturated PBS, the reduction peak current

TABLE 2: Characteristic Rate Constants and the Apparent Michaelis Constant K_m of the Catalytic Process of Glucose for Treated and Untreated GOx-Groove Samples

sample	electron-transfer rate constant k_{et} (s^{-1})	turnover rate constant k_{to} (s^{-1})	rate constant of the biocatalytic process k_{red} ($M s^{-1}$)	K_m (mM)
GdmCl	0.35	203	4.69×10^3	1.28
high temp	0.028	75	2.83×10^3	1.08
untreated	0.46	300	5.01×10^3	1.13(GdmCl), 1.47(high temp)

decreases as shown in curve c. Being the substrate of GOx, β -D-(+)-glucose gives rise to a GOx-catalyzed reaction, which also consumes dissolved O_2 as indicated by reaction 3. Therefore, because the two competitive reactions (reactions 2 and 3) consume dissolved O_2 , reaction 2 and hence the reduction process of reaction 1 are slowed. Thus, the reduction peak current decreases with increasing β -D-(+)-glucose concentration, and the amount of glucose is detected by monitoring the decrease in the reduction peak current of the GOx electrode. The inset shows the electrode's calibration curve for glucose.

To gain insight into the effect of spatial confinement on the stability of the immobilized GOx, characteristic rate constants for the complete catalytic process of glucose have been calculated using the results of Figures 2–4 as listed in Table 2. The electron-transfer rate constant k_{et} , a redox quantity and calculated using the Laviron method,²⁴ describes how fast electron transfer takes place at the enzyme–electrode interface. The turnover rate constant k_{to} is a measure of the number of electrons generated by one GOx molecule per second as a result of the catalytic process,^{25–27} while k_{red} is the rate constant describing the entire substance conversion process.^{28,29} The k_{et} values of the treated samples are all less than that of the untreated sample, indicating a changed state or conformation of some of the immobilized GOx caused by the treatments. In fact, the slight discrepancy between the formal potentials of the treated samples and that of the untreated sample shown in Table 1 forecasts this change. Nevertheless, the corresponding k_{to} and the k_{red} for the treated samples are nonzero, and in fact, these rate constants are not drastically different from those for the nontreated sample, indicating preserved enzymatic activity of GOx. Moreover, a comparison between the corresponding k_{to} and k_{red} of the two treated samples indicates that these rate constants reflect a diminished degree of catalytic process echoing, the same degree of conformational change as indicated by k_{et} . Thus, although denaturation conditions may have produced some changes in the conformation of the immobilized GOx, spatial confinement has stabilized GOx against complete denaturation so that the enzyme is still able to bring about the catalytic process of glucose.

As mentioned above, the general approach to elimination of enzyme/protein denaturation occurring in immobilization is to introduce a layer of organic material between the enzyme/protein and the electrode. A k_{et} of $1 \times 10^{-5} s^{-1}$ was obtained for GOx immobilized on a lipid bilayer film modified graphite electrode,³⁰ and k_{et} was found to be $0.026 s^{-1}$ for GOx immobilized on a gold electrode modified with 3,3'-dithiobissulfocinnimidylpropionate.³¹ In the present study, k_{et} for the untreated samples was found to be $0.46 s^{-1}$, which is significantly larger than those obtained with an intervening layer. The enhanced k_{et} is a consequence that the enzyme makes direct contact with the bare electrode. Table 2 shows that this situation occurs even for the treated samples.

Thermally induced denaturation of GOx was shown to proceed from the native state through an intermediate state to a final denatured homodimer structure, from which the flavin cofactors are dissociated.¹⁸ The second step is an irreversible

process of denaturation and has an activation energy of 280 kJ/mol. The fact that not all (about 60%) of the heat-treated GOx has preserved the enzyme's activity (see the discussion in Figure 3) suggests that the minimum spatial confinement resulting in effective protection is one that corresponds to an increased enzyme stability of 280 kJ/mol.

An inspection of the calibration curves of Figure 4 shows that the curves have a fast-rising region at low glucose concentrations C_g followed by a saturation region at higher C_g , indicating the Michaelis–Menten kinetic process. The apparent Michaelis constant K_m provides kinetic information about the catalytic reaction of the glucose–GOx system as measured by cyclic voltammetry.³² K_m is numerically equal to the concentration of the analyte required for the signal current to reach half its maximum value. Therefore, K_m indicates how effective a particular catalytic reaction is. Table 2 indicates that the values of K_m for the treated samples are not very different from that of the untreated sample. The GdmCl-treated sample has a K_m value of 1.28 mM, while that for the same sample before treatment is 1.13 mM. The corresponding K_m values for the high-temperature-treated sample are 1.08 and 1.47 mM. Note that K_m for the native GOx dissolved in a solution is 33 mM,³³ and immobilization of the enzyme may drastically decrease its K_m to single-digit values on the millimolar level.³⁴ Therefore, the difference in K_m between the treated and untreated samples could be a result of experimental error. Since the difference is not significant, the kinetics of the catalytic process seems to be not significantly affected by the treatments.

Conclusion

In this paper we report that when GOx is assembled in grooves made on unmodified silicon wafers, the activity of the enzyme is preserved. The activity persists even when the immobilized enzyme is subjected to destabilizing conditions, which generally induce denaturation. Our results show that the spatial confinement provided by the grooves enhances the stability of GOx against denaturing conditions, since GOx immobilized on a flat silicon wafer loses its redox ability after denaturing treatments. This phenomenon is further elucidated by examining the estimated values of various physical quantities related to the biocatalytic process of GOx. The technique demonstrated here can be used as a general approach for stabilizing enzymes immobilized on an unmodified electrode for bioelectronic applications.

References and Notes

- Scheller, F. W.; Schubert, F.; Fedrowitz, J. *Frontiers in biosensorics*; Birkhauser Verlag: Basel, Boston, Berlin, 1997.
- Barton, S. C.; Gallaway, J.; Atanassov, P. *Chem. Rev.* **2004**, *104*, 4867.
- Norde, W. *Adv. Colloid Interface Sci.* **1986**, *25*, 267.
- Bowden, E. F. *Electrochem. Soc. Interface* **1997**, *40*.
- El, Kasmí, A.; Wallace, J. M.; Bowden, E. F.; Binet, S. M.; Linderman, R. J. *J. Am. Chem. Soc.* **1998**, *120*, 225.
- Leopold, M. C.; Bowden, E. F. *Langmuir* **2002**, *18*, 2239.
- Smalley, J. F.; Chalfant, K.; Feldberg, S. W.; Nahir, T. M.; Bowden, E. F. *J. Phys. Chem. B* **1999**, *103*, 1676.

- (8) Xu, J. S.; Bowden, E. F. *J. Am. Chem. Soc.* **2006**, *128*, 6813.
- (9) Lei, C.; Shin, Y.; Liu, J.; Ackerman, E. J. *J. Am. Chem. Soc.* **2002**, *124*, 11242.
- (10) Kumar, C. V.; Chaudhari, A. *J. Am. Chem. Soc.* **2000**, *122*, 830.
- (11) Zhou, H.-X.; Dill, K. A. *Biochemistry* **2001**, *40*, 11289.
- (12) Zhou, H.-X. *Acc. Chem. Res.* **2004**, *37*, 123.
- (13) Yau, S.-T.; Thai, I.; Strauss, E.; Rana, N.; Wang, G. *J. Nanosci. Nanotechnol.* **2006**, *6*, 796.
- (14) Hecht, H. J.; Kalisz, H. M.; Hendle, J.; Schmid, R. D.; Schomburg, D. *J. Mol. Biol.* **1993**, *229*, 153.
- (15) Wang, G.; Yau, S.-T. *Appl. Phys. Lett.* **2005**, *87*, 253901.
- (16) Zhong, D.; Zewail, A. H. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 11867.
- (17) Akhtar, M. S.; Ahmad, A.; Bhakuni, V. *Biochemistry* **2002**, *41*, 3819.
- (18) Zolda, G.; Zubrik, A.; Musatov, A.; Stupa, M.; Sedla, E. *J. Biol. Chem.* **2004**, *279*, 47601.
- (19) Bard, A. J.; Faulkner, L. R. *Electrochemical Methods*, 2nd ed.; John Wiley & Sons, Inc.: Hoboken, NJ, 2001.
- (20) Chi, Q.; Zhang, J.; Dong, S.; Wang, E. *J. Chem. Soc., Faraday Trans.* **1994**, *90*, 2057.
- (21) Willit, J. L.; Bowden, E. F. *J. Electroanal. Chem.* **1987**, *221*, 265.
- (22) Brett, C. M. A.; Brett, A. M. O. *Electrochemistry: Principles, Methods, and Applications*; Oxford University Press: Oxford, U.K., 1993.
- (23) Chi, Q. J.; Zhang, J. D.; Dong, S. J.; Wang, E. K. *Electrochim. Acta* **1994**, *39*, 2431.
- (24) Laviron, E. *J. Electroanal. Chem.* **1979**, *101*, 19.
- (25) Zayats, M.; Katz, E.; Baron, R.; Willner, I. *J. Am. Chem. Soc.* **2005**, *127*, 12400.
- (26) Xiao, Y.; Patolsky, F.; Katz, E.; Hainfeld, J. F.; Willner, I. *Science* **2003**, *299*, 1877.
- (27) Zayats, M.; Katz, E.; Willner, I. *J. Am. Chem. Soc.* **2002**, *124*, 2120.
- (28) Bourdillon, C.; Demaille, C.; Guerin, J.; Moiroux, J.; Saveant, J. M. *J. Am. Chem. Soc.* **1993**, *115*, 12264.
- (29) Bourdillon, C.; Demaille, C.; Moiroux, J.; Saveant, J. M. *J. Am. Chem. Soc.* **1993**, *115*, 2.
- (30) Tominaga, M.; Kusano, S.; Nakashima, N. *Bioelectrochem. Bioenerg.* **1997**, *42*, 59.
- (31) Jiang, L.; McNeil, C. J.; Cooper, J. M. *Chem. Commun.* **1995**, 1293.
- (32) Kamin, R. A.; Wilson, G. S. *Anal. Chem.* **1980**, *52*, 1198.
- (33) Swoboda, B. E. P.; Massey, V. *J. Biol. Chem.* **1965**, *240*, 2209.
- (34) Godet, C.; Boujtita, M.; Murr, N. E. *New J. Chem.* **1995**, *23*, 795.