A Spontaneous Growth of a Diaphorase Enzyme Layer over a Gold Electrode for the Catalytic Reduction of NAD^+

Sohyoung Kim, Sei-Eok Yun,† and Chan Kang*

Department of Chemistry, †Department of Food Science and Technology, Institute of Agricultural Science and Technology, Chonbuk National University, Chonju 561-756, Korea

Received June 23, 2001

A diaphorase enzyme electrode for the catalytic reduction of NAD^+, the oxidized form of nicotinamide adenine dinucleotide, has been prepared. The enzyme layer grew spontaneously over an aminoethanethiol self assembled monolayer on a gold plate electrode. The growth was accomplished by simply dipping the electrode covered by the aminoethanethiol monolayer into a solution containing both glutaraldehyde and diaphorase. We suggested that the glutaraldehyde as a cross-linking reagent was attached to the amino groups of the aminoethanethiol monolayer and the diaphorase enzyme molecules were bound to free aldehyde groups of the glutaraldehyde. Further attachments of the enzyme molecules over the bound enzyme molecules continued with the bridging of the glutaraldehyde. In frequency measurements with a quartz crystal microbalance, the frequency decrease was much more than it was for that of the enzyme monolayer formation, and an enzyme layer thicker than a monolayer was formed. The modified electrode was employed to reduce NAD^+, using diffusional methyl viologen as an electron transfer mediator. The NAD^+ was electrocatalytically reduced, and the catalytic current was almost equivalent to that with the multilayered electrode of ten enzyme layers.

Keywords : Diaphorase, NAD^+, Bioelectrocatalysis, Enzyme immobilization, Methyl viologen.

Introduction

Enzyme-catalyzed reduction of NAD^+ (the oxidized form of nicotinamide adenine dinucleotide) has been studied in relation to the production of 1,4-NADH (the reduced form of nicotinamide adenine dinucleotide), which is a coenzyme for organic syntheses using dehydrogenases.[1-4] A system with methyl viologen (MV^{2+}) and diaphorase as a mediator and an enzyme, respectively, has been widely employed in this process,[1,5-9] and other systems with different mediators and enzymes have also been used.[10-12] The mediator transfers electrons between the electrode surface and the enzyme redox sites, and the enzyme converts the substrate into a product.[13,14] In most previous reports, the reduction rate of NAD^+ appeared to be relatively slow based on low catalytic currents in voltammetric measurements.[6,10] The heights of the steady-state catalytic currents were observed to depend on the enzyme concentration under the high concentration of NAD^+.[15]

In mediated enzyme catalytic reactions, the catalytic reactivity is limited either by the concentration of the enzyme or by the concentration of the mediator when the reaction of the enzyme with the mediator is a rate-determining step under the condition of the high concentration of a substrate.[16,17] Increasing the enzyme concentration can increase the overall reaction rate and the immobilization of the enzyme on the electrode is one way to enhance its local concentration on the surface. Enhanced catalytic currents were obtained by coating a film containing enzymes with a fixing agent, such as a polymer,[18-21] or a cross-linking reagent.[6,7] However, difficulty is encountered in controlling the enzyme film thickness on a molecular scale with the film coating method, and electron transfer may be curtailed by the too thick coatings. To avoid this problem, a layer-by-layer construction of an enzyme multilayer by alternately attaching each enzyme layer over the electrode is suggested to control the coating thickness on the molecular-level, and stepwise current enhancements were observed as the enzyme layers accumulated.[22,23] The enzyme multilayers of glucose oxidase,[24-27] horseradish peroxidase,[28] and diaphorase[29] were prepared by this method.

In the present report, we suggest another immobilization method for the diaphorase enzyme layer construction: simply dipping a gold electrode with an aminoethanethiol self assembled monolayer on its surface into a solution containing both a cross-linking reagent and the enzyme. First, the cross-linking reagent was bound to the aminoethanethiol monolayer and followed by the enzyme attachment onto the cross-linking agent. The enzyme layer grew continuously as the enzyme molecules assembled. Glutaraldehyde was used a cross-linking reagent and aldehyde groups on both its ends were expected to react with the amino groups of the aminoethanethiol or the diaphorase enzyme.[30,31] An enzyme layer thicker than a monolayer was formed and the modified electrode showed catalytic activity almost equivalent to the multilayer.

The mass changes on the surface and the catalytic current responses were compared for the modified electrode prepared by the present immobilization method and the multilayered enzyme electrode.

*To whom correspondence should be addressed. Tel: +82-63-270-3420, Fax: +82-63-270-3408; e-mail: chankang@moak.chonbuk.ac.kr

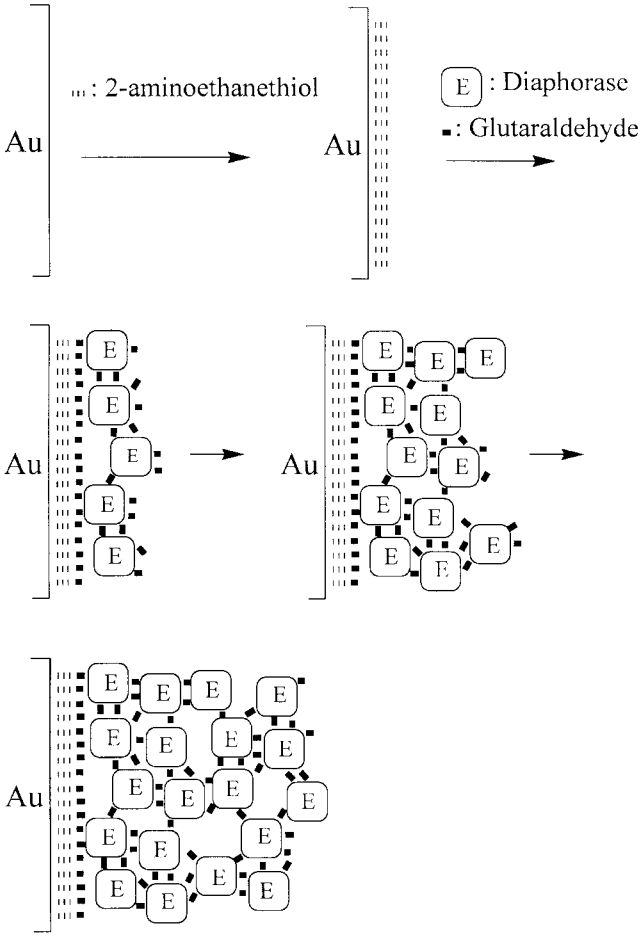
Experimental Section

Materials. Diaphorase (from porcine heart, E.C. 1.8.1.4), β-nicotinamide adenine dinucleotide (NAD^+), 8.0% glutaraldehyde, methyl viologen (MV^{2+}), and 2-aminoethanethiol were purchased from Sigma. A 0.1 M phosphate buffer solution (pH 7.0) was prepared for enzyme immobilization and electrochemical measurements. Other commercial reagent grade chemicals were used as received. Deionized water was further purified by a purification train (Milli-Q, Millipore Co.) and used to prepare solutions.

Apparatus and procedures. Electrochemical experiments were performed in a two-compartment cell closed with a Teflon cap. A gold plate working electrode (area, 0.32 cm^2), platinum auxiliary electrode, and gas bubbling tube were fitted through the cap, and a reference electrode was immersed in another compartment separated by glass frit. A BAS 50 electrochemical analyzer connected to a personal computer was used to perform electrochemical measurements. Potentials were measured and quoted with respect to a Ag/AgCl (3 M NaCl) reference electrode with a potential of 0.22 V *vs* NHE. All electrochemical experiments were conducted at the temperature of 40 °C of the cell solution dipped in a thermostat. A quartz crystal microbalance (QCM) EQCN 1000 system (SHIn Co., Korea) was employed to measure frequency changes according to minute mass changes on the gold surface during the enzyme immobilization. A 9 MHz quartz crystal resonator with a gold surface (0.2 cm^2) was used. During the QCM measurements, the temperature was stabilized at 23 °C (± 0.5 °C).

A diaphorase enzyme multilayer over the gold electrode, for comparison with the present system, was layer-by-layer constructed, according to the literature.[29] A gold plate (0.32 cm^2) with an aminoethanethiol self assembled monolayer was immersed into a 0.5% glutaraldehyde solution and then transferred to another 9.6 u/mL diaphorase solution. After one turn of this process, one enzyme layer was coated and more layers were accumulated as the same procedures were repeated. The other construction method that the diaphorase enzymes assembled over the surface was illustrated in Scheme 1. First, a self assembled monolayer was formed by immersing a clean gold plate (0.32 cm^2) into a 100 mM aminoethanethiol solution (1 h).[32] The electrode was then transferred to a pH 7.0 phosphate buffer solution containing both 0.2% glutaraldehyde and 0.1 u/mL diaphorase. The electrode was allowed to remain for several hours in the solution for the layer to grow to an appropriate level. The surface of the gold electrode was cleaned by dipping it in a 6 M HNO_3 solution, polished with diamond slurry and 0.3 micron alumina powder, and finally sonicated.

The QCM measurement during the immobilization of one enzyme layer[29] was made for comparison with the present method. The quartz resonator with the gold surface on which an aminoethanethiol self assembled monolayer was previously formed was immersed for 2 hours in a solution containing only glutaraldehyde (0.5%). The solution of the cell was exchanged with a new phosphate buffer (pH 7.0), and the



Scheme 1. The spontaneous growth of a diaphorase enyzme layer.

resonance frequency was allowed to stabilize in the range of ± 2 Hz. An aliquot of concentrated diaphorase solution was added to make the final enzyme concentration 4.8 u/mL, and the change of the frequency was further monitored. For the enzyme layer formation by Scheme 1, initially the gold surface of the resonator with an aminoethanethiol monolayer was exposed to air without any solution in the cell, and a small aliquot of the diaphorase enzyme was added into the bottom. At this stage, the enzyme solution was not in contact with the gold surface of the crystal and no adsorption occurred. The cell was then filled with another phosphate buffer solution containing glutaraldehyde to make the concentration of the enzyme and glutaraldehyde, 0.1 u/mL and 0.2%, respectively. Just after the addition of the glutaraldehyde solution, the measurement for the frequency change started.

Results and Discussion

Confirmation of the enzyme layer formation over the gold electrode surface. The attachment of the enzymes over the electrode was confirmed by measuring frequency changes with a quartz crystal microbalance (QCM). Figure 1 illustrates a comparison of the frequency changes between the two different enzyme immobilization methods as described in

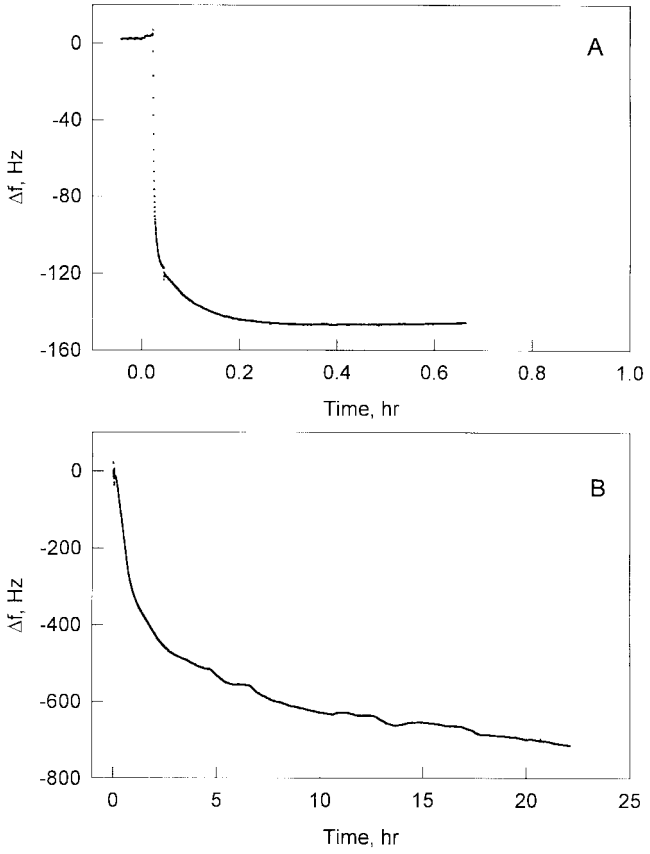


Figure 1. (A) QCM frequency changes for the immobilization of diaphorase enzyme. The measurement for the immobilization started in a 0.1 M pH 7.0 phosphate buffer solution containing 4.8 u/mL diaphorase. A gold surface previously covered with glutaraldehyde over an aminoethanethiol monolayer was employed. (B) The measurement for the immobilization started in a solution containing both 0.1 u/mL diaphorase and 0.2% glutaraldehyde. The gold surface with an aminoethanethiol monolayer was used. The zero point in the x-axis indicates the time when the enzyme immobilization started.

the experimental section. Figure 1A is a measurement with the electrode surface covered by an enzyme monolayer, and Figure 1B was obtained with the electrode surface covered by an enzyme layer according to Scheme 1. In Figure 1A, the zero time in the x-axis is the point of the injection of the diaphorase enzyme. Before the injection, the frequency change was negligible, but the frequency decreased abruptly just after the injection, reaching a steady state in about 30 minutes. The decreasing pattern depended on the concentration of the enzyme and how it was injected, but the frequency changes through the completion of enzyme immobilization were almost constant in repeated experiments. The average was estimated as 150 Hz, which is equivalent to the mass change of 8.2×10^2 ng/cm^2 at the surface.[33,34] For the measurements of monolayer immobilization of other enzymes, 500-1000 ng/cm^2 was usually obtained and the mass change in the present measurement was also considered to be a monolayer immobilization.[26,35] For Figure 1B, the frequency change was monitored during the immobilization in the solution of glutaraldehyde and diaphorase mixture. The zero time in the x-axis of Figure 1B is the point at which the glutaraldehyde solution was added to the aliquot of the diaphorase. The decrease of the frequency indicates the adsorption of both the glutaraldehyde and the enzyme. The rate of frequency decrease was much lower than in Figure 1A because of the lower concentration of the enzyme (notice the different time scales between Figure 1A and Figure 1B). The frequency in Figure 1B decreased continuously over 20 hours, and the frequency change is several times larger than that in Figure 1A. This result indicates that the enzymes assembled to form a layer according to Scheme 1, and more enzymes were immobilized than in the monolayer. The continuously decreasing frequency, even after 20 hours, implies that the enzyme layer was still growing at the moment when the measurement was stopped. However, the frequency decreased rapidly initially but the growing rate of the layer gradually decreased with time. This means the immobilization was inhibited as the layer grew. This decreasing immobilization rate may be explained by the electrostatic repulsion from the bound enzyme molecules. In the pH 7.0 phosphate buffer, the charge of the protein surface of the enzyme is expected not to be neutral. As the enzyme layer grows, the electrostatic repulsive force becomes stronger and further attachment of the enzymes is more difficult.

In this enzyme layer growing method, if the concentrations of the glutaraldehyde and the enzyme are high, the enzyme molecules can be polymerized in the solution. Hence, those concentrations need to be lowered to avoid the cross-linking reactions in the solution. At the electrode surface, however, the density of the amino groups of the aminoethanethiol monolayer is high and the reaction rate with the glutaraldehyde is expected to be higher. The enzyme molecules then react with free aldehyde groups of glutaraldehyde attached on the surface since their concentration is also higher than in the solution phase. The glutaraldehyde and the enzymes from the solution bind consequently and the enzyme layer over the surface continuously grows. The enzyme layer becomes thick, and more enzymes than in the monolayer are confined on the surface. For the voltammetric measurement, the enzyme layer was allowed to grow 12 hours, which gave roughly 4-5 times more enzymes of the monolayer on the electrode, as indicated in Figure 1B. In fact, the amount of the enzyme at this moment should be less than the estimated amount, considering the glutaraldehyde molecules connecting the enzymes.

Catalytic reduction of NAD^+ using the enzyme immobilized electrode. Figure 2 is a comparison between voltammograms for the catalytic reduction of NAD^+ with Au electrodes on which an enzyme multilayer (Figure 2A)[29] and the enzyme layer, according to Scheme 1 (Figure 2B), were formed. For Figure 2A, the diaphorase enzyme multilayer was constructed as described in the experimental section. The voltammogram of Figure 2A-a was measured with a bare gold electrode in a solution containing 1.0 mM MV^{2+} and 12.0 mM NAD^+, and it is the same as that obtained from the solution of 1.0 mM MV^{2+} only. NAD^+ itself was not reduced at all at about -0.7 V, and a more negative potential of -1.0 V was required to reduce it.[15] Figure 2A-b is a voltammogram after one diaphorase

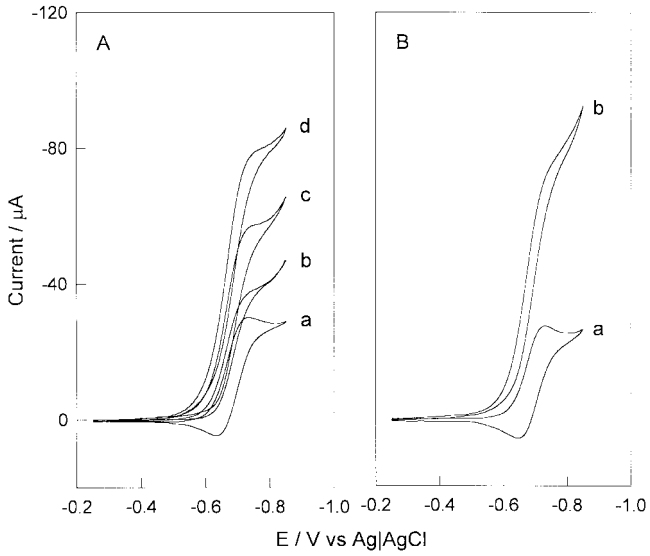


Figure 2. Cyclic voltammograms in a solution of 1.0 mM MV^{2+} and 12.0 mM NAD^+. (A) A gold plate electrode was employed as the layer-by-layer enzyme immobilizations proceeded: (a) a bare gold electrode, (b) one enzyme layer coated electrode, (c) five layers coated electrode, and (d) ten layers coated electrode. (B) (a) A bare gold electrode and (b) the modified electrode prepared by the method as described in Scheme 1 were used as working electrodes. Supporting electrolyte: 0.1 M pH 7.0 phosphate buffer (40 °C, under Ar). Scan rate: 5 mV/s.

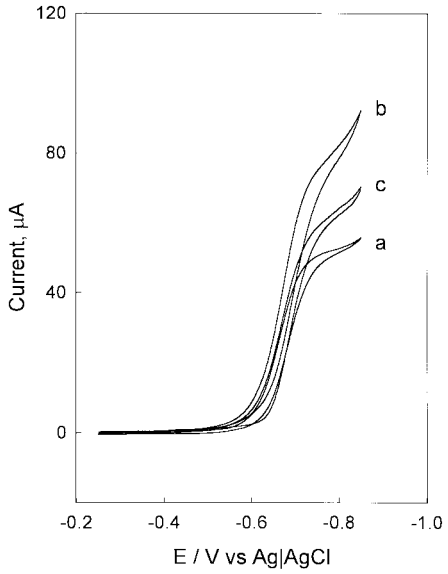


Figure 3. Cyclic voltammograms in a solution of 1.0 mM MV^{2+} and 12.0 mM NAD^+ with a gold plate electrode after the enzyme was formed according to Scheme 1. The immersing times were 6, 12, and 24 hours for a, b, and c, respectively. Other conditions were the same as Figure 2.

enzyme layer was coated, and the enhancement of a current was observed. The increase of the current was due to the catalytic reduction of NAD^+ by the attached diaphorase enzymes, and the dissolved methyl viologen mediated electrons into the enzyme from the electrode. Stepwise current enhancements were obtained as more layers were accumulated. The voltammograms of Figure 2A-c and 2A-d are the results with the coatings of five and ten layers, respectively. For Figure 2B, an Au electrode, which was previously immersed for 12 hours in the solution of 0.2% glutaraldehyde and 0.1 u/mL diaphorase, was employed to observe the catalytic effect for the NAD^+ reduction. Figure 2B-a is a voltammogram with a bare gold electrode in the same solution as in Figure 2A, and the same result as in Figure 2A-a was obtained. However, when the electrode where the diaphorase enzyme layer was formed as described in Scheme 1 was used, a large enhancement of the current was measured as in Figure 2B-b. The current height of Figure 2B-b is almost close to that obtained with the electrode of the ten layer coatings of Figure 2A-d. In the QCM measurement, the amount of immobilized enzyme at 12 hours in Figure 1B was approximately four times that in the monolayer. The higher than expected activity in Figure 2B-b may be explained by the different activities in the different structures of the enzyme layers formed by the two different methods. Other controlled experiments were carried out and it was confirmed that the enhancements of the currents were due to the catalytic reduction of NAD^+ since the current with the modified electrode in the solution containing only methyl viologen was the same as that with the bare gold electrode. The results in Figure 2 were reproducibly obtained with the electrodes prepared under rigorously controlled conditions.

The amount of the enzyme to be confined was controlled by adjusting the immersion time of the electrode in the solution of the mixture in Scheme 1. The thickness of the layer increased with the immersion time and an optimal time for the layer growth was decided by a trial and error. In Figure 3, the catalytic current heights were compared with the electrodes where the enzyme layer thickness was varied by immersion time. From the result of QCM measurement, as in Figure 1B, a thicker enzyme layer was expected with longer immersion time. The three voltammograms of Figure 3a, b, and c were obtained after the electrodes were immersed in the same solution used in Figure 1B for 6, 12, and 24 hours, respectively. The voltammogram of Figure 3b gave the best result and lower catalytic currents were measured at the electrodes for times shorter and longer than 12 hours. The deficiency in the amount of the enzyme might have resulted from the low current with the shorter immersion time, and, with the too thick enzyme layer in the other case, the diffusion of the mediator or the substrate through the layer might be hindered.

Another factor influencing the result was the concentrations of glutaraldehyde and diaphorase in the solution for the formation of the layer. The concentrations finally used to prepare the electrode for Figure 2B-b were employed after several trials with various other combinations. The low concentrations of glutaraldehyde and diaphorase required more time to obtain results similar to that of Figure 2B-b. The stability of the prepared electrode was tested. After the measurement, the electrode was immersed in a phosphate buffer and stored in a refrigerator. The voltammograms were measured during the following days and the catalytic current

gradually decreased. The half-life time of the enzyme electrode was roughly estimated as four to five days.

Conclusions

The present study demonstrates a relatively simple method to prepare an enzyme electrode for bioelectrocatalysis. The enzyme multilayer formation has been known as a useful way to confine more enzymes over the electrode than the monolayer formation. The step-by-step immobilization of each enzyme layer affords the advantage that the enzyme layer thickness can be controlled on a molecular level.[29] However, long repeated trials are required for the multiple enzyme layer coatings. With the present method, however, by simply dipping the electrode into the solution and waiting until the layer grows to appropriate thickness, an almost similar catalytic current for the NAD^+ reduction to the multilayered electrode of ten enzyme layers was obtained. Similarly, a glucose oxidase enzyme layer was formed by dipping a gold electrode in a solution of glucose oxidase, glutaraldehyde, and (2-ammonioethyl) ferrocene iodide.[36] A glucose oxidase enzyme layer was immobilized on the surface and precipitates due to an enzyme polymerization formed in the solution. In the present system, by employing low concentrations of the cross-linking reagent and the enzyme, precipitate formation could be avoided. The kinetics of the enzyme layer formation could be observed and the enzyme layer thickness could be controlled with immersion time. The same principle may be applicable to the construction of other types of enzyme-modified electrodes.

Acknowledgment. This work was supported by grant No. 1999-124-00-001-1 from the Korea Science & Engineering Foundation (KOSEF).

References

1. DiCosimo, R.; Wong, C.-H.; Daniels, L.; Whitesides, G. M. *J. Org. Chem.* **1981**, *46*, 4622.
2. Gorton, L.; Csoregi, E.; Dominguez, E.; Emneus, J.; Jonsson-Pettersson, G.; Marko-Varga, G.; Persson, B. *Anal. Chim. Acta* **1991**, *250*, 203.
3. Wong, C.-H.; Whitesides, G. M. *J. Am. Chem. Soc.* **1981**, *103*, 4890.
4. Wong, C.-H.; Daniels, L.; Orme-Johnson, W. H.; Whitesides, G. M. *J. Am. Chem. Soc.* **1981**, *103*, 6227.
5. Maeda, H.; Kajiwara, S. *Biotechnol. Bioeng.* **1985**, *27*, 596.
6. Matsue, T.; Chang, H.-C.; Uchida, I.; Osa, T. *Tetrahedron Lett.* **1988**, *29*, 1551.
7. Chang, H.-C.; Matsue, T.; Uchida, I.; Osa, T. *Chem. Lett.* **1989**, 1119.
8. Fry, A. J.; Sobolov, S. B.; Leonida, M. D.; Voivodov, K. I. *Tetrahedron Lett.* **1994**, *35*, 5607.
9. Voivodov, K. I.; Sobolov, S. B.; Leonida, M. D.; Fry, A. J. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 681.
10. Ogino, Y.; Takagi, K.; Kano, K.; Ikeda, T. *J. Electroanal. Chem.* **1995**, *396*, 517.
11. Lacey, A. L.; Bes, M. T.; Gomez-Moreno, C.; Fernandez, V. M. *J. Electroanal. Chem.* **1995**, *390*, 69.
12. Takagi, K.; Kano, K.; Ikeda, T. *J. Electroanal. Chem.* **1998**, *445*, 211.
13. Heller, A. *Acc. Chem. Res.* **1990**, *23*, 128.
14. Degani, Y.; Heller, A. *J. Phys. Chem.* **1987**, *91*, 1285.
15. Kim, S.; Yun, S.; Kang, C. *J. Electroanal. Chem.* **1999**, *465*, 153.
16. Coury, L. A.; Oliver, B. N.; Egekeze, J. O.; Sosnoff, C. S.; Brumfield, J. C.; Buck, R. P.; Murray, R. W. *Anal. Chem.* **1990**, *62*, 452.
17. Coury, L. A.; Murray, R. W.; Johnson, J. L.; Rajagopalan, K. V. *J. Phys. Chem.* **1991**, *95*, 6034.
18. Kashiwagi, Y.; Yanagisawa, Y.; Shibayama, N.; Nakahara, K.; Kurashima, F.; Anzai, J.; Osa, T. *Chem. Lett.* **1996**, 1093.
19. Kashiwagi, Y.; Yanagisawa, Y.; Shibayama, N.; Nakahara, K.; Kurashima, F.; Anzai, J.; Osa, T. *Electrochim. Acta* **1997**, *42*, 2267.
20. Umana, M.; Walier, J. *Anal. Chem.* **1986**, *58*, 2979.
21. Kaku, T.; Karan, H. I.; Okamoto, Y. *Anal. Chem.* **1994**, *66*, 1231.
22. Willner, I.; Katz, E.; Willner, B. *Electroanalysis* **1997**, *9*, 965.
23. Willner, I.; Katz, E. *Angew. Chem. Int. Ed.* **2000**, *39*, 1180.
24. Willner, I.; Riklin, A.; Shoham, B.; Rivenzon, D.; Katz, E. *Adv. Mater.* **1993**, *5*, 912.
25. Riklin, A.; Willner, I. *Anal. Chem.* **1995**, *67*, 4118.
26. Hoshi, T.; Anzai, J.; Osa, T. *Anal. Chem.* **1995**, *67*, 770.
27. Anzai, J.; Takeshita, H.; Kobayashi, Y.; Osa, T.; Hoshi, T. *Anal. Chem.* **1998**, *70*, 811.
28. Ruan, C.; Yang, F.; Lei, C.; Deng, J. *Anal. Chem.* **1998**, *70*, 1721.
29. Kim, S.; Yun, S.; Kang, C. *Electrochem. Commun.* **1999**, *1*, 151.
30. Peters, K.; Richards, F. M. *Ann. Rev. Biochem.* **1977**, *46*, 523.
31. Okuda, K.; Urabe, I.; Yamada, Y.; Okada, H. *J. Ferment. Bioengin.* **1991**, *71*, 100.
32. Willner, I.; Lapidot, N.; Riklin, A.; Kasher, R.; Zahavy, E.; Katz, E. *J. Am. Chem. Soc.* **1994**, *116*, 1428.
33. Buttry, D. A. In *Electroanalytical Chemistry*; Bard, A. J., Ed.; Marcel Dekker: New York, 1991; vol. 17, p 1.
34. Ward, M. D. In *Physical Electrochemistry*; Rubinstein, I., Ed.; Marcel Dekker: New York, 1995; p 293.
35. Willner, I.; Heleg-Shabtai, V.; Blonder, R.; Katz, E.; Tao, G.; Buckmann, A. F.; Heller, A. *J. Am. Chem. Soc.* **1996**, *118*, 10321.
36. Kuwabata, S.; Okamoto, T.; Kajiya, Y.; Yoneyama, H. *Anal. Chem.* **1995**, *67*, 1684.