

Electropolymerized Thin Bilayers of Poly-5-amino-1-naphthol and Poly-1,3-phenylenediamine for Continuous Monitoring Glucose Sensors

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Received December 24, 2002

A highly interferent-resistive membrane, poly-5-amino-1-naphthol (poly-5A1N), underlaid beneath enzyme-embedded poly-1,3-phenylenediamine (poly-*m*-PD) network for miniaturized continuous monitoring glucose sensors. The enzyme layer was prepared from a mixed solution of glucose oxidase (GOx) and *m*-PD monomer by simple electrolysis. The mass change of poly-5A1N was monitored by electrochemical quartz crystal microbalance (EQCM) *in situ* and the corresponding thickness was measured. Successive electropolymerization of poly-5A1N and poly-*m*-PD create a several tens nm-thick bilayer showing excellent selectivity for H₂O₂ and low activity loss of immobilized enzymes.

Key Words : Glucose sensor, Electropolymerization, Continuous monitoring

Introduction

A representative example of the achievements in practical biosensors is the commercialization of glucose sensors. Despite steady studies on *in vivo* monitoring with implanted probes for clinical use, many problems remain unsolved because such sensors must fulfill additional strict requirements, e.g. excellent biocompatibility, minimal size and long term stability.^{1,2} In particular, high reliability in terms of the sensitivity to glucose concentration and the interference protection are essential for the clinical applications.¹ Recently, characterization of a few successful sensors has been reported,³ and one of them is on the way to commercialization for clinical use.^{4,5} Now, the progress of continuous monitoring biosensors seems to have reached the phase that the problems of mass production need to be addressed.⁶

Over the decades electropolymerization techniques for biosensor fabrication have attracted much attention as a new way to easily prepare an H₂O₂- or O₂-selective layer in or on which enzymes are immobilized.⁷⁻⁹ This methodology has many major advantages over the conventional solvent casting method.⁹ First of all, it is possible to fabricate a film regardless of the geometry and area of the electrode so that uniform and thin membranes are formed on microelectrodes and silicon-machined electrode array.¹⁰ When water-soluble monomers are used, enzymes in neutral aqueous solution can be easily immobilized by simple electrolysis even without any covalent linkage. The other is that the electronically controlled procedure offers high reproducibility. Because the thickness of the membranes is finely controlled and reproduced by electrochemical techniques, the final products offer a more quantitative response. As a result, many electropolymerized membranes have been reported.⁷⁻⁹ Non-conducting membranes such as poly-phenylenediamines have been of particular interest because their thin, dense films

lead to both fast response and high H₂O₂- or O₂-selectivity.¹¹ Moreover, these polymers are inert against reactive chemical species (H₂O₂).^{12,13} A recent report shows that substituted naphthalenes are promising candidates as a monomer of highly selective non-conducting polymer.¹⁴ Especially, 5-amino-1-naphthalene (5A1N) exhibits excellent properties for an inner membrane such as nearly perfect elimination of interference and prolonged durability.¹⁴

In this study, we have suggested the bilayer composed of poly-5A1N and poly-*m*-PD as a thin membrane composite for miniaturized glucose sensors. Both of those polymers are amenable to electropolymerization in aqueous phases and form ultra-thin inner membranes. The serial electrochemical deposition was confirmed by electrogravimetric *in-situ* monitoring using electrogravimetry and ellipsometry. This pair of ultrathin films allows high permeability of H₂O₂ while effectively rejecting possible interfering species such as ascorbic acid and acetaminophenol.

Experimental Section

Reagents. Glucose oxidase (E.C. 1.1.3.4, high purity, 250,000 unit/g, without O₂ type II-S, *Argellius niger*) L-ascorbic acid, L-cysteine, D-fructose, acetaminophen, uric acid and bovine serum were used as received from Sigma. D-glucose, 1,3-phenylene diamine (*m*-PD, 99+%), and 5-amino-1-naphthol (5A1N, 97%) purchased from Aldrich were used without further purification. Deionized water produced by a Nano Pure System (Barnsted) with high resistance (~18 MΩ) was used.

Electrochemical Quartz Crystal Microbalance and Electrochemistry. Electrochemical experiments were performed with a Windows-driven electrochemical analyzer (Model CH660, CH Instruments Inc., Austin, TX 78733). Ag/AgCl (3 M KCl) and platinum wire were reference and counter electrode, respectively. A home-made electrochemical quartz crystal microbalance (EQCM) was used to monitor the growth of thin polymeric films. The quartz crystal was

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mounted perpendicular to the solution surface in the teflon cell. A quartz plate (1 inch diameter, 1 × 1 mm thickness) was placed in the window (16 mm dia.) opposite the quartz crystal (gold electrode on the quartz plate, area: 0.22 cm²). The quartz crystals having a fundamental frequency of 10.0 MHz were purchased from International Crystal Manufacturing Co., Inc. (Oklahoma City, catalog no. 03210). The circuit for quartz crystal was slightly modified that developed by Buttry and coworkers.¹⁵ A model 568 digital function generator (AMEL Co.) combined with a home-made Wenking-type potentiostat converted the current to voltage, which was read by a data acquisition card (AT-MIO-16XE-50, National Instrument) installed in the computer. The data acquisition program was written in LabVIEW software (National Instrument, version 5.0). Mass to frequency ratio was calibrated by monitoring frequency change during copper deposition from a 0.05 M CuSO₄ and 0.1 M H₂SO₄ solution and the result was 1.06 ng/Hz.

Thickness Measurements. The thickness of the thin polymeric films was measured using an ellipsometer (AutoEL-II, Rudolph Research Co., Flanders, NJ 07836), which employs 632.8 nm (He-Ne laser) as an incident beam. The resolution of polarizer or analyzer was 0.05 deg. The incident angle was 70 (±0.02) deg and the time for measurement was 10 s. The refractive indices of the poly-5A1N (*n*₂) and gold substrate (*n*₃) were determined using a fitting program written in LabVIEW software which processes pairs of Δ and Ψ measured by the ellipsometer. Pictures of polymer surfaces in micrometer scale were obtained with a scanning electron microscope (FEG XL30, Phillips Inc., 5600 MD Eindhoven, The Netherlands).

Fabrication of Sensors. The method described in the literature was adopted to prepare the electrode body and perform electrochemical surface pretreatments.⁵ A teflon-coated platinum-iridium alloy wires (full diameter, 0.24 mm, length: 4 cm) was employed as the electrode body of the needle-type sensor. The teflon skin at 5 mm from the end of the wire was peeled off so that the area of the exposed bare Pt-Ir surface was 2.8 mm² (5 mm in length). Epoxy resin (araldite), which was dried for 30 min, sealed off the end of the wire. The bare electrode was anodized in 0.5 M H₂SO₄ at +1.6 V for 30 s, followed by 25 cycles of potential sweeps between 0 and +1.2 V at 100 mVs⁻¹. The electrode body was immersed into 5 mM 5A1N solution, which was prepared by dissolving 5A1N in 0.1 M NaCl balanced to pH 1.0 with concentrated HCl solution.¹⁴ Applying a constant potential of +0.65 V vs. Ag/AgCl (3 M KCl) for 30 min resulted in a very thin and nearly invisible inner membrane of poly-5A1N. The coated electrode was rinsed with nanopure deionized water and then immersed into an enzyme solution containing 0.1% GOx and 5 mM *m*-PD in phosphate buffered saline (PBS) containing 0.1 M Na₂HPO₄ and 0.15 M NaCl balanced to pH 7.4. The GOx-containing poly-*m*-PD membrane was electropolymerized by six consecutive potential cycles between +0.5 and +1.2 V vs. Ag/AgCl at 5 mVs⁻¹. The concentrations, scan rate and the number of cycles in electropolymerization with 5A1N and *m*-PD were

optimized to maximize sensitivity to D-glucose and to minimize interference in PBS.

Results and Discussion

Electropolymerization of 5A1N. 5A1N electropolymerized in the presence of perchlorate, ClO₄⁻, in acetonitrile exhibits a semiconducting conductivity, 4.9 × 10⁻⁷ S/cm, in the dry state.¹⁶ This material can be also electrochemically polymerized in aqueous perchloric acid solution, and the film shows highly reversible redox behavior around +0.23 V vs. saturated calomel electrode (SCE).¹⁷ However, the conducting property almost disappears in aqueous hydrochloric acid. Figure 1 shows that 5A1N in acidic aqueous media is oxidized and polymerized on platinum-iridium alloy electrode surface. The first scan exhibits an oxidation peak at +0.65 V, and oxidation currents upon subsequent scans become much smaller than that upon the first scan in the same potential region. This implies that a polymer film with appreciable resistance is covering the electrode surface. The film growth on the gold-deposited electrode was monitored by EQCM, as shown in Figure 2(A).¹⁸ The mass increases very rapidly at the beginning of electropolymerization, after which the rate of film growth gradually slows. The amount of poly-5A1N on the electrode reaches 100 ng for less than 2 s. Figure 2(B) indicates the thickness change, which was independently measured by ellipsometry, for different electropolymerization times.¹⁵ The thickness does not initially grow as fast as the mass does, suggesting that extremely thin poly-5A1N film is unusually dense. The density of poly-5A1N film at 500 s is 2.3 g/cm³. As the film grows, the change of density slows (to 1.4 g/cm³ at 1000 s and 1.3 g/cm³ at 3000 s) with a negligible change after that. This result suggests that the density of electropolymerized poly-5A1N is far from uniform and that the polymer network closer to electrode surface is denser. Presumably, the dense layer

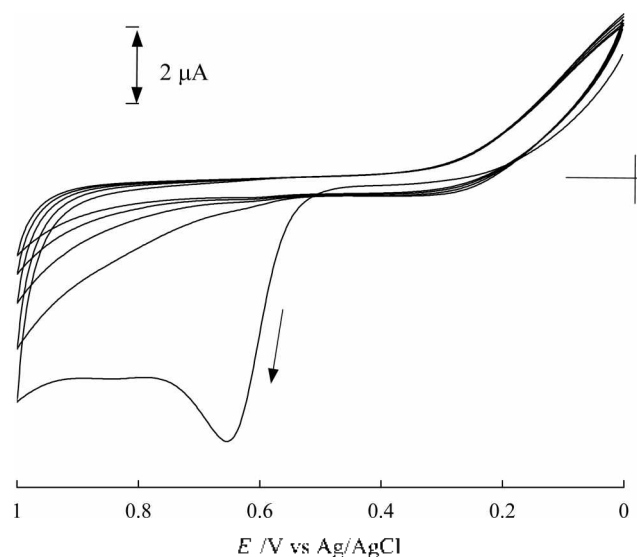


Figure 1. Cyclic voltammograms of 5 mM 5A1N, 0.1 M NaCl solution at pH 1.0. Scan rate is 50 mVs⁻¹.

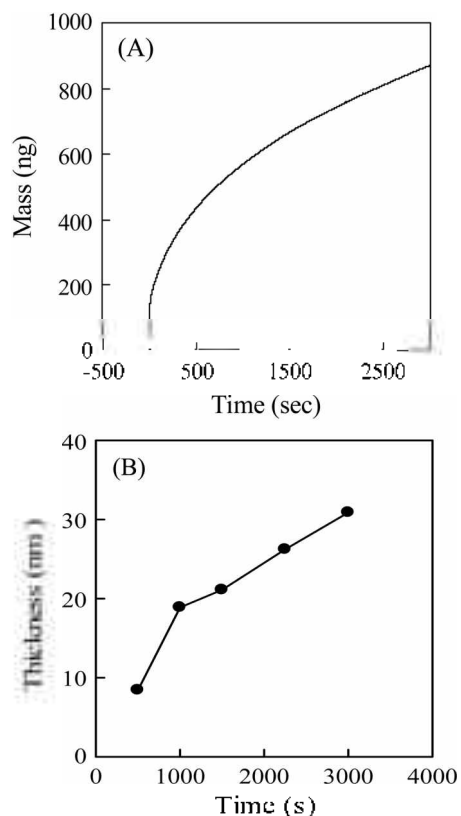


Figure 2. Mass (A) and thickness (B) change measured by EQCM and ellipsometry, respectively, during the electropolymerization of 5A1N. A constant potential of +0.65 V vs. Ag/AgCl is applied in 5 mM 5A1N, 0.1 M NaCl solution at pH 1.0.

close to electrode surface likely plays a crucial role in determining H_2O_2 -sensitivity. Murphy assumed the density of the film is $1\text{--}1.5\text{ g/cm}^3$ at 900 s and estimated the thickness of poly-5A1N, 66–98 nm on the basis of charge.¹⁴ Figure 2(A) and (B) shows that the thickness of poly-5A1N is only *ca.* 18 nm and the density is 1.4 g/cm^3 upon the electropolymerization at 900 s. The mass calculated from frequency change is $0.55\text{ }\mu\text{g}$ whereas simultaneous measurement of charge due to oxidation of 5A1N monomers is $1.3\text{ }\mu\text{g}$, assuming the number of electrons is 2.¹⁴ This reveals that more than a half of the oxidized monomers on the electrode surface do not participate in the formation of the film and diffuse into the bulk solution. Therefore, it is believed that the assumption on density in the Murphy report is reasonable but the thickness of the poly-5A1N film was overestimated.

Effect of Electropolymerization Time on Sensitivity and Selectivity. No noticeable difference between the potentiostatic and cycling methods for electropolymerization was found in terms of both sensitivity and selectivity. The inner membranes of poly-5A1N were made by the potentiostatic method, which is more convenient. To assess the effect of the electropolymerization time, sensitivity to H_2O_2 and selectivity over representative electro-active species, ascorbic acid (AA) and acetaminophen (AP), were investigated with Pt-Ir/poly-5A1N. Figure 3(A) illustrates the height of current

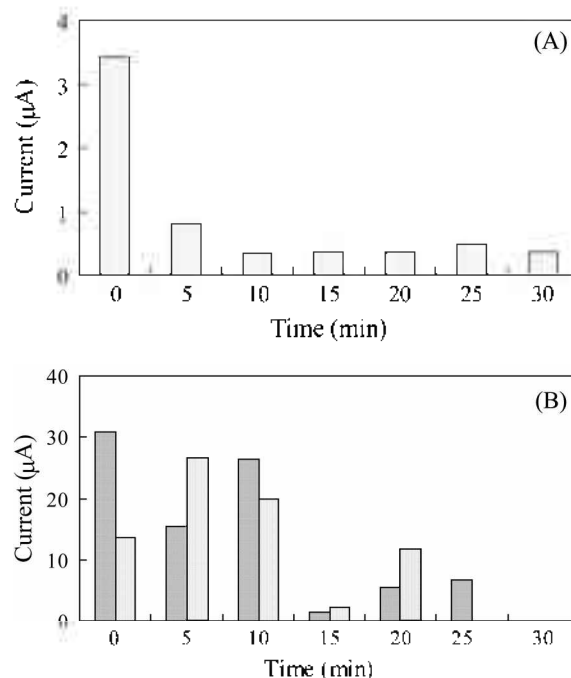


Figure 3. The effect of electropolymerization time on the responses of Pt-Ir/poly-5A1N to 6 mM H_2O_2 (A), 0.11 mM AA and 0.17 mM AP (B). Dotted and slashed columns in (B) stand for AA and AP, respectively.

responses to H_2O_2 in the solution as the electropolymerization time increases. The sensitivity steeply drops in the case of Pt-Ir/poly-5A1N for 5 min and no further appreciable decrease is detected for electropolymerization times longer than 10 min. On the other hand, Figure 3(B) shows that the interference by AA and AP, decreases as the electropolymerization time is prolonged. Dependence of selectivity on electropolymerization time is markedly reduced for longer than 15 min. Both results imply that an error of a few seconds at an electropolymerization time longer than 15 min causes no significant deviation in the film properties. When the electropolymerization in 5A1N solution continues for 30 min, interference due to both AA and AP is completely suppressed. The response to H_2O_2 is still comparable to that for 10 min. Therefore, the optimal electropolymerization time was determined as 30 min and the resulting poly-5A1N has 24 nm of thickness and 1.4 g/cm^3 of density.

Consecutive Electropolymerization of Bilayer. Figure 4 shows that *m*-PD is oxidized on uncoated Pt-Ir electrode (A) and on a poly-5A1N coated electrode (Pt-Ir/poly-5A1N) (B).²⁰ The oxidation peak of *m*-PD at a poly-5A1N coated electrode appears at more positive potential than that at a uncoated Pt-Ir electrode by about +0.4 V.²¹ The current at a poly-5A1N coated electrode is much smaller. The voltammetric behavior in Figure 4(B) is reminiscent of oxidation and polymerization of 1,2-phenylenediamine on Pt/poly-pyrrole electrode.¹³ Despite substantially lowered current and shift of oxidation potential, 6 potential cycles between 0 and +1.2 V at scan rate of 5 mVs^{-1} yield a very reproducible poly-*m*-PD membrane on the poly-5A1N membrane. Potential cycling in this potential range is superior to the potentiostatic

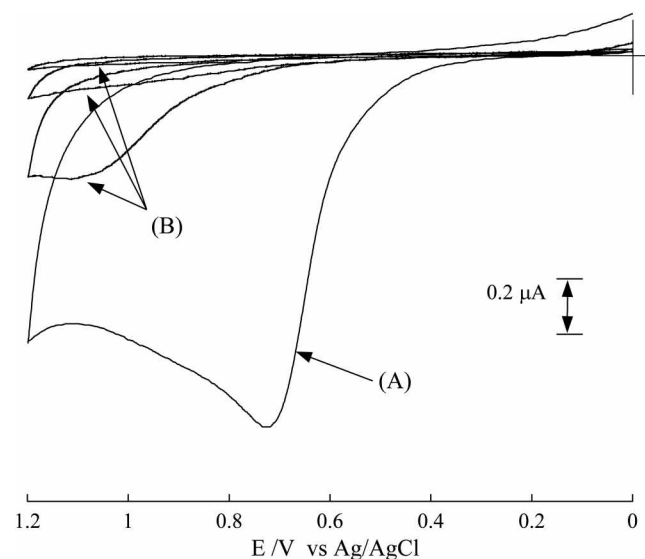


Figure 4. Cyclic voltammograms of 5 mM *m*-PD with bare (A) and Pt-Ir/poly-5A1N (B) in PBS at pH 7.4. Scan rate is 1 mVs⁻¹.

method in terms of selectivity. An extremely small oxidation current is observed upon more than 6 cycles. In the presence of GOx, the behavior is almost same as that in the absence of GOx and the response to glucose does not increase when the number of cycles exceeds 6. Thus, further potential cycles cannot raise the amount of loaded enzymes. The uniform surface of Pt-Ir/poly-5A1N/poly-*m*-PD+GOx can be confirmed by scanning electron microscope (SEM). The electropolymerized bilayer is too thin to examine the cross sectional view by SEM. EQCM measurements reveal that the mass of poly-*m*-PD, from which the thickness can be estimated to be *ca.* 8 nm. This result shows that the poly-*m*-PD film is very thin compared to conventional enzyme layers. Poly-5A1N might be suspected to contribute immobilization of enzymes. However, Pt-Ir/poly-5A1N, which has been immersed in GOx solution in PBS, exhibits no response to glucose. This means that GOx is unlikely to adsorb onto poly-5A1N and poly-*m*-PD film plays an essential role of a network entrapping GOx.

Conclusions

First, successive electropolymerization of several tens nm-thick bilayer allows not only high selectivity of poly-5A1N but also easy and reproducible immobilization of GOx without any new problem. Second, electrogravimetry using EQCM is a useful way to see the process of enzyme capture as well as polymer growth *in situ*. The experimental results revealed how exactly thick the thin layer is, what pattern the membrane grows in, and how much enzyme is immobilized

in electropolymerized poly-*m*-PD on poly-5A1N. These results strongly suggest promising applications of electropolymerized thin bilayers for the mass production of continuous monitoring biosensors.

Acknowledgment. This work is the result of research activities of Advanced Biometric Research Center (ABRC) Supported by KOSEF. Ms. R.-A. Jeong, Dr. S.K. Kang, and Prof. H.C. Kim are greatly acknowledged for their valuable supports.

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- The voltammetric behavior with gold electrode is very similar to that with platinum-iridium one in the potential region in Figure 1.
- Assuming the refractive index in air (n_1) is 1, measurements by ellipsometry and fitting processes gave the refractive indices, n_2 (poly-5A1N film) and n_3 (gold substrate), were 0.902-0.02i and 0.15-2.719i, respectively.
- The cyclic voltammograms in Figure 4 were obtained at 1 mV⁻¹ to show the formation of poly-*m*-PD more clearly.
- When a poly-5A1N-coated electrode is immersed in pure PBS, small oxidation current at around 1 V vs. Ag/AgCl is observed in the absence of *m*-PD. Probably, the oxidation of residual monomers in poly-5A1N or further oxidation of poly-5A1N is responsible for this phenomenon. The peak completely disappears upon a second potential scan.