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Electrochemically Mediated Electrodeposition/ Electropolymerization To Yield a Glucose Microbiosensor with Improved Characteristics

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A procedure is described that provides for electrochemically mediated deposition of enzyme and a polymer layer permselective for endogenous electroactive species. Electrodeposition was first employed for the direct immobilization of glucose oxidase to produce a uniform, thin, and compact film on a Pt electrode. Electropolymerization of phenol was then employed to form an anti-interference and protective polyphenol film *within* the enzyme layer. In addition, a stability-reinforcing membrane derived from (3-aminopropyl)trimethoxysilane was constructed by electrochemically assisted cross-linking. This hybrid film outside the enzyme layer contributed to the improved stability and permselectivity. The resulting glucose sensor was characterized by a short response time (<4 s), high sensitivity (1200 nA/mM·cm^2), low interference from endogenous electroactive species, and working lifetime of more than 50 days.

The enzyme electrode, or more particularly the glucose electroenzymatic biosensor, has served for more than 25 years as a valuable clinical tool for detecting and monitoring diabetes. A majority of glucose sensors, especially those used in in vivo applications, are based on the rate of glucose oxidase-catalyzed oxidation of glucose by dioxygen, where the rate of the reaction is measured by monitoring the formation of hydrogen peroxide or the consumption of oxygen. The fabrication of such a sensor involves the controlled deposition of a permselective polymer layer used to eliminate interferences such as ascorbate, urate, and acetaminophen, an enzyme layer, and an outer layer that renders the sensor response mass transfer rather than kinetically controlled and that also provides a biocompatible interface with the surrounding environment.[1] The use of thick-film techniques, including screen printing, has been demonstrated successful in the preparation of sensors with reasonably reproducible characteristics, and this approach has been applied, for example, in the electrochemically based sensors used for self-monitoring of blood glucose as marketed by Abbott (Medisense)[2] and others. If, however, it is desired to employ a cylindrical geometry or to prepare a sensor array,[3] then the reproducible deposition of the various functional layers becomes significantly more complicated. Thus, it would be of considerable advantage to control the preparation of the sensor electrochemically, especially when the sensing elements in an array are themselves electrochemically addressable. This would also allow for the deposition of different enzymes in various parts of the array.

Electropolymerization makes it possible to generate a coating on small electrodes of complex geometry and to do so precisely in one or two rapid and simple steps.[4] In general, electrochemically mediated fabrication of biosensors is accomplished in two ways: First, a polymer layer is formed directly on the electrode, and polymers formed from such monomers as pyrrole,[5] aniline,[6] tyramine,[7] *o*-aminophenol,[8] and *o*-phenylenediamine[9] have been used to create a permselective layer before or after the application of enzyme solution and cross-linking with glutaraldehyde.[10] A second approach involves the entrapment of enzyme in a growing polymer network by copolymerization of enzyme and monomer.[11] In some cases, a monomer unit is attached to the enzyme to facilitate this process.[12] Yacynych employed a copolymer of 1,3-diaminobenzene and resorcinol as the preferred film for blocking interferences from the surface of carbon or partially platinized

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carbon electrodes.[10a] Vadgama and co-workers found that electropolymerized 4-aminophenol and then phenol constituted an exceptionally selective film against acetaminophen and ascorbate in glucose biosensors.[13] Curulli et al.[14] reported that poly(1,3-diaminobenzene/catechol) was the most efficient polymer to prevent the interference of acetaminophen. It has been pointed out[15] that electropolymerized films have significantly different characteristics when formed on different electrode materials and under different electropolymerization conditions. Our experience has shown that these approaches lead typically to sensors of moderate activity but often high selectivity, but that both of these essential characteristics deteriorate quickly with time (over a period of several days). The fact that the diffusion of enzyme and monomer cannot proceed at the same rate makes it difficult to enrich the composite layer with enzyme without at the same time degrading the permselective properties of the polymer.

In this paper, we describe the formation of an electrochemically generated polyphenol film that has been formed within a previously electrodeposited enzyme layer. This procedure provides both the functions of enzyme entrapment and interference rejection. Polyphenol, as a typical nonconducting polymer, is generally continuous, insulating, and self-limiting. Because it can be made free of defects such as pinholes,[16] it has been used for corrosion protection,[17] for permselective films,[18] and as pH sensors.[19] Films can be grown under electrochemical control from aqueous buffered solutions at physiological pH. Vadgama and co-workers[20] showed that polyphenol films are permselective for peroxide over ascorbate, uric acid, and acetaminophen, thus making it useful for assays of clinical significance. This selectivity appears to be based primarily on size exclusion.[21] Once the composite film is formed, a silane film, (3-aminopropyl)trimethoxysilane, is deposited under applied potential to create lateral stabilization.[22] The final step in sensor construction involves the application of a polyurethane outer layer, chosen to control glucose and oxygen fluxes in order to optimize linearity of sensor response and minimize dependence on oxygen tension.

EXPERIMENTAL SECTION

Reagents. Glucose oxidase (GOx, EC 1.1.3.4.) was obtained from Biozyme Laboratories International Ltd. Phenol and (3-aminopropyl)trimethoxysilane (3-ATS) were purchased from Fluka. N-propyltrimethoxysilane was obtained from United Chemical Technologies (Bristol, PA). D-Glucose (Glu) (from Sigma) solutions were allowed to mutarotate for 24 h before use. Acetaminophen (AP), L-ascorbic acid (AA), and uric acid (UA) were obtained from Aldrich and prepared immediately before testing, as they are subject to oxidative decomposition in solution. Teflon-coated platinum (Pt(90%)/Ir(10%)) wire (0.17-mm diameter) was purchased from Medwire Corp. (Mount Vernon, NY). Polyurethane was obtained from Thermedics Inc. A 0.05 M phosphate buffer was prepared from the corresponding phosphate salts. Phosphate-buffered saline (PBS; pH 7.4) was prepared from phosphate salts (0.1 M) and sodium chloride (0.15 M).

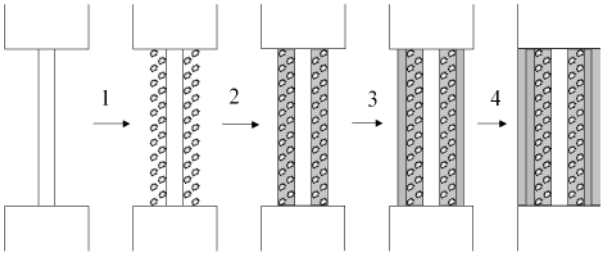

Figure 1. Scheme for the preparation of the GOx biosensor. Step 1: electrodeposition of GOx. Step 2: electropolymerization of phenol. Step 3: electrochemical cross-linking of (3-aminopropyl)trimethoxysilane. Step 4: coating of polyurethane outer membrane.

Apparatus. Amperometry was performed using a model 814 electrochemical detector (CH Instruments) connected to a Dell (L500r) computer. Electrochemical quartz crystal microbalance (EQCM) measurements were made on a model 422 Electrochemical Analyzer (CH Instruments). For EQCM experiments, a 14-mm-diameter platinum-coated AT-cut crystal (frequency 7.995 Hz) was used (International Crystal Manufacturing Co. Inc., Oklahoma City, OK). Each crystal had an etched (3−5 μm roughness) surface, and Pt was directly deposited on it (thickness 100 nm; electrode area 0.196 cm^2). Pt wire and Ag/AgCl were used as counter and reference electrodes, respectively.

Sensor Preparation. One end of a 5-cm-long Teflon-coated Pt wire (0.17-mm diameter) was stripped 1 mm to expose the metal surface as sensing cavity. This was achieved by first putting a circular cut in the Teflon coating (35 μm thick) 5 mm from the tip and then sliding the Teflon out to create a cavity of 1-mm length. The excess Teflon at the tip was trimmed off and the tip was sealed off with epoxy glue (Super Glue Corp.). The area of the sensing cavity of 1 mm was 0.534 mm^2. The other end was stripped by 1 cm to provide a connection to the potentiostat. The sensing cavity of the Pt−Ir wire was cleaned by dipping into 1 M HNO_3/1 M HCl and 1 M NaOH solution for 20 min, respectively. Copious rinsing with deionized water followed. A three-electrode system was employed with the Pt wire working electrode, Ag/AgCl (3 M NaCl) reference electrode, and a large Pt wire as counter electrode.

The procedures for preparing a GOx electrode are shown in Figure 1.

Step 1. A 10 mg/mL GOx solution in pH 7.0 phosphate buffer (0.05 M, containing 0.02% (v/v) Triton X-100) was used for the electrodeposition of GOx on the Pt−Ir electrode. A potential of 1.3 V (vs Ag/AgCl) was applied to the Pt electrode for 1 h.

Step 2. The Pt electrode deposited with GOx was put into 40 mM phenol solution in pH 7.0 buffer (this solution was degassed with argon for at least 20 min before the experiment and the argon atmosphere was maintained during the experiment) and 0.9 V (vs

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Ag/AgCl reference) was applied to the electrode for 15 min to induce the electropolymerization reaction.

Step 3. The Pt electrode was dipped into 10 mM 3-ATS solution and 0.6 V (vs Ag/AgCl reference) was applied for 15 min to enhance the cross-linking of ATS onto the polyphenol film. By adjusting the variables involved in the preparation of the enzyme electrodes, one can control their analytical performance with regard to both glucose measurement and interference removal.

Step 4. Finally, polyurethane film was loaded onto the electrode by dip-coating the electrodes with 3% (w/w) polyurethane (PU) solution in 98% tetrahydrofuran (THF)/2% dimethylformamide (DMF) (w/w). Most of the organic solvent was allowed to evaporate on the loop before the film was coated onto the electrodes. For the electrochemical measurements, the sensor was dipped into a cell consisting of 5 mL of pH 7.4 PBS buffer at room temperature and a potential of 0.65 V (vs Ag/AgCl) was applied for the amperometric glucose detection with stirring. The background current was allowed to stabilize before measurement.

RESULTS AND DISCUSSION

Electrodeposition of GOx on the Pt Electrode. The most obvious advantages of the electrodeposition of enzyme are the ease of control and the possibility of performance under mild conditions, making this procedure very suitable for a range of biomolecules. Electrodeposition of GOx (together with BSA) on electrodes has been accomplished by constant current[23] or fixed potential.[24] The latter approach was found more effective in our work. Variation of the applied potential has been shown to influence the characteristics of enzyme electrodes. It was reported that a large amount of GOx could be electrodeposited in the oxygen evolution region (above 1.0 V) (vs Ag/AgCl)).[23b] We found a potential of 1.3 V to be optimal for the electrodeposition. The reasons for this conclusion have been described elsewhere.[25]

Acetaminophen is often used as a performance standard because it is a difficult electrochemical interference to eliminate. It is therefore chosen as a primary test of electrochemical specificity. The choice of 1.3 V for the electrodeposition potential proved to give the highest ratio of enzyme activity (measured by the current due to peroxide oxidation) and also high selectivity against acetaminophen (Glu/AP) as shown in Figure 2. EQCM experiments showed that the extrapolated mass increase on the Pt-coated quartz was 280.36 ng/mm^2. Considering the electrode area of 0.534 mm^2 and a GOx molecular diameter of 8∼9 nm,[26] the thickness of the GOx layer was ∼0.5 μm under the electrodeposition conditions of 1.3 V for 1 h, the conditions used in the present study. It was found that a biosensor prepared by GOx adsorption showed only very small response to glucose (Figure 2), which meant the physisorption of GOx on the electrode could be neglected compared with electrodeposition at 1.3 V.

Selectivity of Pt Electrodes Modified with Polyphenol and 3-ATS Films. It was the goal of this work to find more stable

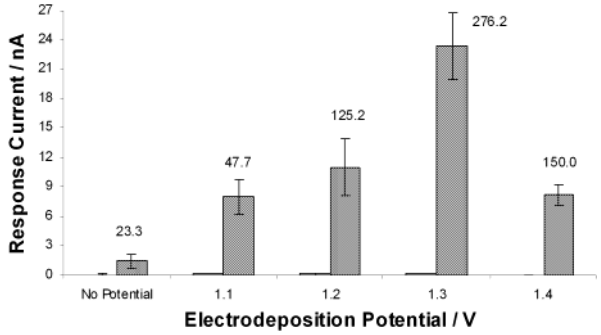

Figure 2. Effect of electrodeposition potential for GOx on peroxide response of the electrode ($n = 3$). The numbers given define the ratio of average I_{Glu}/I_{AP}. Peroxide response measured at 0.65 V vs AgCl/Ag reference. Electrodes polyphenol and silane treated. Right histogram, response to 5 mM glucose; left histogram, response to 0.1 mM AP.

electropolymerized films, so that the biosensor performance was limited by the lifetime of the enzyme, not by the stability of the polymer film. Our results showed the permselective behavior of the films was greatly influenced by the monomers employed and electropolymerization conditions. Thicker films will exclude the interferences, but they will also lower sensitivity to the analyte and increase response time. We found polyphenol film very selective against acetaminophen and also stable long term when considering the balance of selectivity and sensitivity, as noted previously.[27] Figure 2 shows the sensor response to 5 mM glucose as a function of the electrodeposition potential of the enzyme. It will be noted that, at 1.3 V, the ratio of the response I_{Glu}/I_{AP} is 276, largely due to the high enzyme activity resulting from the electrodeposition. The exclusion of AP, therefore, does not depend strongly on the deposition conditions for the enzyme. These results were obtained on sensors without the PU membrane, which will reduce the response to glucose by a factor of less than 10 without significantly affecting the AP response. So the selectivity for the complete sensor should be at least 28:1.

It was observed that modification of a Pt electrode by polyphenol or polyphenol and then 3-ATS films resulted in a loss of sensitivity for hydrogen peroxide by factors of 6.7 and 8.2, respectively (Table 1). However, a much greater decrease was observed for interferences such as acetaminophen, ascorbic acid, and uric acid. Furthermore, the electrode modified with polyphenol/3-ATS was more selective than electrodes modified with only polyphenol, especially against acetaminophen. It is assumed that the small faradaic current from interferences occurs either by long-range slow electron transfer across the membrane or by electron transfer at a few microscopic defect sites in the membrane.[28]

Stability of GOx Electrodes. Figure 3 shows the response of sensors to glucose and acetaminophen for a period of over 50 days. These sensors have no mass transfer-limiting PU membrane and therefore are a somewhat more accurate reflection of how the enzyme activity varies with time. These sensors do possess the 3-ATS film. It will be noted that the response increases initially and then reaches a somewhat lower and relatively constant value. The response to acetaminophen increases slightly over this period of time. The electropolymerization mechanism of phenol is based

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Table 1. Selectivities of Pt Electrodes[a] Modified with Polyphenol (PPH) and (3-Aminopropyl)trimethoxysilane (3-ATS)

analyte (0.1 mM)	response (nA)			ratio		selectivity	
	at bare Pt (A)	at Pt/PPH (B)	at Pt/PPH/ATS (C)	A/B	A/C	B[b]	C[b]
H_2O_2	475 ± 32	71 ± 4	58 ± 4	6.7	8.2	1	1
acetaminophen	185 ± 4	0.16 ± 0.03	0.05 ± 0.002	1154	3692	172	449
ascorbic acid	154 ± 14	0.17 ± 0.02	0.09 ± 0.01	906	1711	135	208
uric acid	128 ± 11	0.18 ± 0.02	0.10 ± 0.01	710	1278	106	155

[a] A poential of 0.65V vs AgCl/Ag was applied to the Pt electrodes ($n = 5$) in pH 7.4 PBS solution at room temperature. [b] Selectiviy of hydrogen peroxide over interferences. There is no enzyme applied to the electrodes.

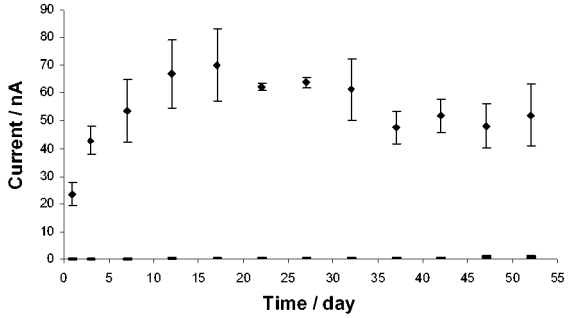

Figure 3. Stability of GOx electrodes ($n = 5$) without the outer membrane. Lower response curve due to AP. (◆) Response to 5 mM glucose; (—) response to 0.1 mM AP.

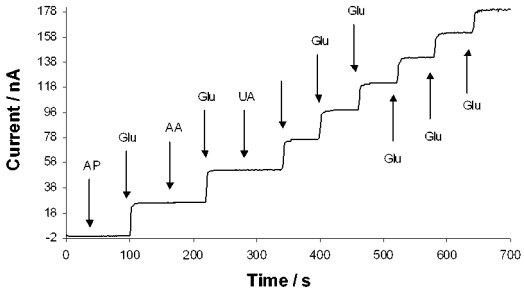

Figure 4. Current−time curve for a GOx electrode with outer membrane upon sequential addition of 0.1 mM AP, 5 mM glucose, 0.1 mM AA, and 0.1 mM UA.

on simple radical initiation.[29] It was previously reported that polyhydroxyl compounds tended to stabilize the activity of the enzyme,[30] a possible contributing factor to improved performance.

The presence of the 3-ATS film resulted in a significant improvement in the stability of the polyphenol film. The effectiveness of the 3-ATS deposit was significantly enhanced if a potential of 0.6 V was applied compared with no potential applied (data not shown). The fact that enhancement is specifically linked to an electrochemical reaction suggests that intermediates produced by phenol oxidation must be playing a key role. At the specified applied potential, the 3-ATS cannot be oxidized. As Table 1 also indicates, the presence of the 3-ATS film significantly improves the permselectivity of the polyphenol. When *N*-propyltrimethoxysilane was substituted for 3-ATS, the enhancement of permselectivity and stability was significantly reduced. Thus, although it is clearly important to form a network by cross-linking of the silane function to form an Si−O−Si network, the key must be the reaction of the silane amine function. Oxidation of phenols can result in the production of quinoid structures, and the reaction of primary amines with them via Michael addition is well known. Indeed, the formation of quinoid structures will result not only in the reaction of the silane with oxidized forms of phenol, but the reaction of protein primary amines is also possible.[31]

AFM measurements[25] of the electrodeposited enzyme layer before and after electropolymerization show no apparent increase in the thickness of the enzyme layer (480 nm). This suggests that the polymer layer is not thicker than the enzyme layer and can form around the enzyme molecules.

Performance of the Glucose Biosensor with a Mass Transfer-Limiting Outer Membrane. Figure 4 shows a typical current−time curve for the biosensor with PU outer membrane upon the injection of glucose and various electroactive interferences.

The responses to 0.1 mM AA, UA, and AP were essentially negligible. The responses of electrodes to AA and UA were lower than AP, which corresponded to the well-known observation that AP was the most serious electrochemical interference so far encountered in the course of sensor development. There is indeed some deterioration of the permselectivity with the complete sensor compared to just the electropolymerized layer. There are two reasons for this: (1) In the complete sensor, the enzyme is buried *in* the permselective layer as opposed to being on top of it. (2) There is a slight disruption of the inner membrane due to the solvent (THF and DMF) used to deposit the external membrane.

The biosensor demonstrated a 90% response to glucose within less than 4 s. This behavior was expected because the growth of the nonconducting polyphenol film was largely self-limiting and the resulting deposit very thin (usually 10−100 nm).[15,29,32] In a recent study of electropolymerized poly(phenol) films, Arrigan and Bartlett[36] showed by scanning force microscopy that such films deposited under conditions similar to our work yielded thicknesses of 3 nm. Electropolymerized films formed in the presence of a monolayer of an enzyme gave a thickness comparable to the enzyme molecular dimensions. Although this work was performed on highly oriented pyrolytic graphite, the conclusions are similar

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Table 2. Comparison of GOx Electrodes Prepared with Different Electropolymerized Films

types of GOx electrode	detection potential (mV)	sensitivity (nA/(mM·cm^2))	T_{95}[b] (s)	linearity range (mM)	ref
Pt/GOx/PPH/PATS/PU[a]	650[c]	1205 ± 217	4	25	*e*
Pt/RH-GOx/PC	400[d]	~800		15	15
Pt/PMPS/GOx(BSA/GA)/PU	600[c]	42	60	15	22
Pt/PPy$_{ox}$/GOx(BSA/GA)	700[c]	1189	1.2	12	33
Pt/PPy-GOx	700[c]	283		6	34
Pt/PPy-GOx/oPPD	700[c]	283		6	35

[a] $n = 8$. PPH, polyphenol; PATS, poly((3-aminopropyl)trimethoxysilane); PU, polyurethane; PMPS, poly((3-mercaptopropyl)trimethoxysilane); BSA, bovine serum albumin; GA, glutaraldehyde; PPY, polypyrrole; PPy$_{ox}$, overoxidized polypyrrole; RH, redox hydrogel; PC, polycarbonate; oPPD, poly(*o*-phenylenediamine). [b] T_{90}, response time which is defined as the time neemed to reach 90% of the maximum response. [c] Versus Ag/AgCl. [d] Versus SCE. [e] This work.

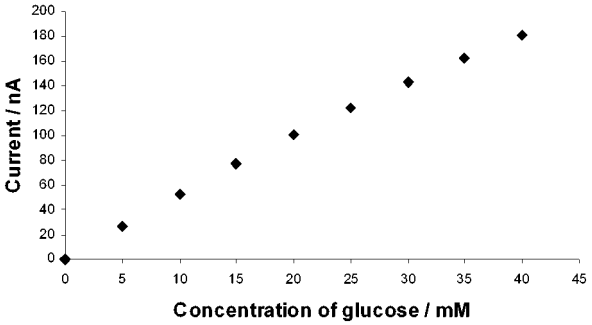

Figure 5. Calibration curve for the GOx electrode response to glucose in pH 7.4 PBS. The applied potential was 650 mV (vs Ag/AgCl (3 mM KCl)).

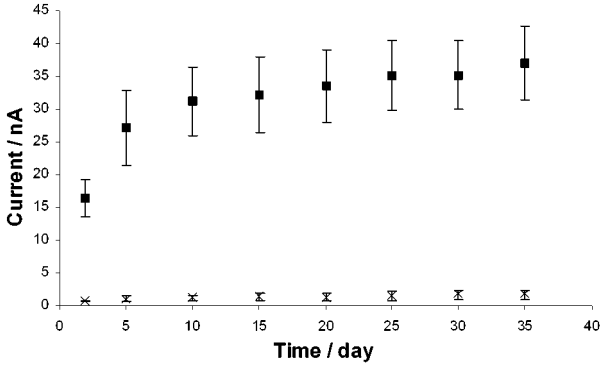

Figure 6. Long-term stability of the GOx electrode ($n = 8$) with PU outer membrane. Conditions same as for Figure 5. (■) Response to 5 mM glucose; (×) response to 0.1 mM AP.

to what we have observed. There is also no evidence of regular surface orientation of enzyme in our case, but electrodeposition does seem to yield somewhat smoother deposits compared to simple adsorption. The thin film also containing the enzyme has another advantage: The closer the enzyme molecules are to the electrode, the more hydrogen peroxide is collected and oxidized to regenerate oxygen. Elevated levels of oxygen in the enzyme layer lead to improved sensor linearity. This is illustrated in Figure 5 where good linearity is observed up to over 20 mM.

The stability of the complete biosensor was explored. Over a period of 35 days, the response of the sensors to glucose (5 mM) and acetaminophen (0.1 mM) was checked periodically and the sensor stored in the interim in pH 7.4 phosphate buffer at 4 °C. The results are shown in Figure 6. Over a period of ~5 days, the sensitivity of the sensors increases, after which a stable value is reached. There is a slight increase in the response to AP, but this is essentially compensated for by the increase in glucose response. The sensitivity and selectivity were stable even after over 6 months. The response data at day 200 showed sensitivities of ~8.5 nA/mM to glucose, and the response to 0.1 mM AP (the values for AA and UA of the same concentration were even smaller) was only 4.2% of that due to 5 mM glucose, the same as the mean value for the first 35 days.

The overall performance of these sensors is compared with previously reported electropolymerization protocols as shown in Table 2. The linearity range and sensitivity are superior to sensors previously reported, and response time is similarly quite short. To our knowledge there is no evidence that the previously described films can maintain their selectivity over extended periods of time, even after conditioning for a few days. By contrast, the sensors in this study showed no change in either sensitivity or selectivity between 35 and 200 days (vide supra).

In summary, we attribute the superior sensor performance characteristics to the ability to prepare a thin membrane serving the dual purpose of stabilizing the immobilized enzyme and providing a stable permselective layer for elimination of electroactive interferences. High sensitivity can be achieved because the enzyme is *deposited first*, thus creating a compact layer. The presence of the enzyme does not interfere significantly with the creation of a polymer layer largely lacking in defects.

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