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Inert Metal-Modified, Composite Ceramic−Carbon, Amperometric Biosensors: Renewable, Controlled Reactive Layer

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A new type of sol−gel-derived, inert metal-modified, composite, amperometric biosensor is developed. The electrodes are comprised of a dispersion of biochemically and chemically modified graphite powder in a porous, organically modified silicate (Ormosil) network. The percolating carbon dispersion provides electrical conductivity, oxidoreductase enzymes (e.g., glucose oxidase, lactate oxidase, or L-amino acid oxidase) are used for biocatalysis, metallic palladium is used for electrocatalysis of the biochemical reaction product, and the porous organically modified silica provides a rigid skeleton. The hydrophobicity of this composite material guarantees that only a limited section of the electrode is wetted by the aqueous analyte, thus providing a controlled-thickness reactive layer. The thickness of the reaction layer can be tuned by the addition of hydrophilic components. The electrode can be reproducibly renewed by removing its upper layer and exposing a new, thin, porous bioreactive section. The same technology is applicable for the production of thick-film, low-leaching, disposable sensors. In this configuration, the analysis is conducted using a single drop of the analyte applied on the hydrophobic film. The sensors are found to be stable over long periods.

Amperometric biosensors utilize immobilized enzymes for the conversion of target analytes into electrochemically detectable products. Various methods of enzyme immobilization on electrodes have been reported, including immobilization of active proteins in gels,[1−3] cross-linked polymers,[4−8] or conductive salts[9] or mixing into carbon paste and carbon−organic polymer hosts.[10−17] Fouling and contamination of the surface during operation pose a significant hurdle in the long-term use of these materials. Two general methods are employed as countermeasures: Polishable biosensors, which can be renewed by mechanical removal of the outer surface, is one answer and disposable biosensors, which can be discarded after one or several measurements, is another widespread alternative. Renewable amperometric biosensors are currently comprised of either carbon paste or carbon−epoxy materials. Both are nonporous, and therefore only their outermost surface is wetted by the electrolyte and thus accessible to dissolved substrates. Additionally, when the enzyme is attached to the external surface of the electrode, inevitable hydrodynamic fluctuations in its vicinity are reflected in an unsteady, noisy amperometric signal. Incorporation of an additional permeable membrane, which is often employed for obtaining controlled mass transport, is inapplicable for renewable biosensors. Mass-produced, low-cost, screen-printed materials are often used as disposable biosensors.[18] However, incomplete encapsulation of the enzyme and high leachability are still unresolved problems.

The recent finding that biomolecules can be entrapped in a sol−gel-derived matrix triggered active research.[19−21] To date, most of the activity in this field is still directed toward the production of photometric sensors,[22] though some progress in sol−gel-derived biosensors has already been reported.[23−28] Tatsu and co-workers[23] doped glucose oxidase enzyme into tetraethylorthosilicate powder and attached it to a Clark oxygen electrode. Oxygen depletion was used for glucose quantitation. Audebert

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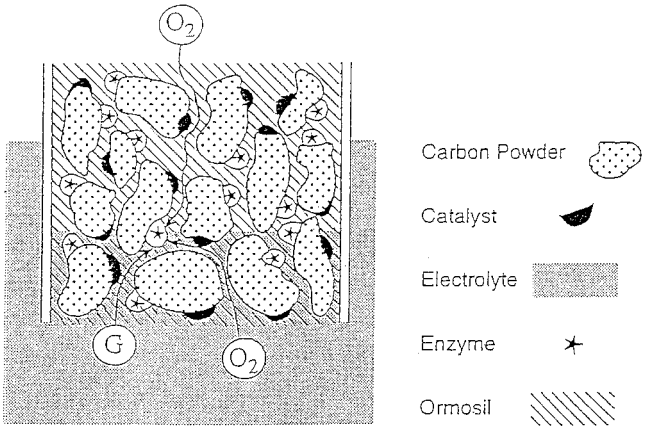

Figure 1. Graphical representation of the palladium-modified enzyme CCE.

and Sanchez[24] doped glucose oxidase along with a mediator, hydroxymethylferrocene, in wet silica gel deposited on a glassy carbon electrode. Prasad and co-workers[3] applied a layer of glucose oxidase between two layers of silica coated on an ITO film, and the resulting sandwich construction was used for biosensing.

Recently, we have introduced composite carbon−ceramic electrodes (CCEs)[29−32] which can be bulk modified by organic, inorganic, or biochemical species. We used these materials for the development of tetrathiafulvalene-mediated glucose biosensors,[33] which, however, had only limited storage and in-use stability. In this article, we introduce the Pd-modified, enzyme-doped carbon−Ormosil (organically modified silica) composite material and demonstrate how the properties of these sensors, including stability and rate-limiting step, can be altered by minor changes in their preparation protocol. The Pd-modified hydrophobic carbon−ceramic biosensors can be schematically visualized as shown in Figure 1. Palladium- and redox enzyme-loaded carbon powder percolates through a porous, hydrophobically modified silcate network. The biochemical reaction occurs in a thin, flooded section, confined to the outermost section of the electrode due to the hydrophobicity of the network. The substrate (e.g., dissolved lactate or glucose or L-amino acid) penetrates from the solution side, and oxygen can be introduced from either the liquid side or the gas side. Generated hydrogen peroxide is electrocatalytically oxidized on the palladium sites.

EXPERIMENTAL DETAILS

Reagents. Methyltrimethoxysilane (MTMOS) was purchased from ABCR Inc. (Karlsruhe, Germany). High-purity graphite powder (50 μM, MP-300) was obtained from Bay Carbon Inc. (Bay City, MI). High-purity palladium chloride was the product of Riedel-de Haen, Germany. Poly(ethylene glycol) (PEG) with a molecular weight of 800 was obtained from Sigma. Glucose oxidase (GOx, EC 1.1.3.4 Type VII-S; 250 000 units, from *Aspergillus niger*), lactate oxidase (LOx, EC 1.1.3.2; 39 units/mg, from *Pediococcus* sp.), and L-amino acid oxidase (L-AAOD, EC 1.4.3.2 from *Crotalus adamanteus* venom) were from Sigma. Analytical grade reagents and triply distilled water (resistivity higher than 20 MΩ) were used.

Apparatus. An EG&G PARC Model 273 potentiostat in conjunction with a Watanabe WX 4421 $x-y$ recorder was used for voltammetric measurements. A three-electrode cell with a platinum foil counter and a saturated calomel electrode (SCE) was used. Unless otherwise specified, all experiments were conducted at room temperature, ~20 °C. BET surface area measurements were carried out using Micromeritics Gemini II 2370 surface area analyzer. Measurements were carried out using nitrogen as the adsorbent, and the desorption isotherm was used for quantitation. Water contact angle measurements were made using a telescope and goniometer (Rame-Hart Inc., NRL C.A. Model 100-00230).

Preparation of Pd-Modified, Enzyme-Loaded Electrodes. *Preparation of Modified Carbon Powder*. The required amount of $PdCl_2$ was dissolved in water, and the resulting solution was mixed with graphite powder. Water was then evaporated by stirring the solution overnight at room temperature. This procedure yielded graphite powder impregnated with $PdCl_2$. Subsequently, Pd^{2+} was reduced under hydrogen at 75 atm for about 20 h. The powder was then thoroughly washed to remove the HCl produced in the reduction reaction; presence of acid is detrimental to enzymic activity. Palladium loading was 1 wt % with respect to graphite. Higher Pd loadings did not improve the electrocatalytic activity considerably. Next, 100 mg of Pd-modified graphite powder was mixed with 0.5 mL of distilled water containing 25 mg of GOx (or 3.5 mg of LOx or 5 mg of L-AAOD) at 4 °C. The mixture was allowed to dry in a desiccator at 4 °C.

Preparation of the Ormosil Sol. Two different protocols were employed to prepare the sol−gel precursors. In the first preparation (these electrodes are denoted as type A), 0.8 mL of MTMOS was mixed with 0.5 mL of methanol, 0.5 mL of water, and 0.1 mL of 0.5 M HCl. This mixture was sonicated for 10 min to ensure uniform mixing and left at 4 °C for a day. The second procedure (for preparation of type B electrodes) involved mixing 0.8 mL of MTMOS with 0.5 mL of water and 0.1 mL of 0.1 M HCl and subsequent sonication for 10 min. Methanol was not added in this protocol, though some methanol was released by the condensation reaction. In some electrodes, hydrophilic modifiers (PEG) were introduced to the colloidal solution in order to control the thickness of the wetted section of the electrode (these electrodes are denoted by the prefix PEG).

Electrode Molding. Approximately 0.1 g of the modified graphite powder was mixed with 0.6−0.7 mL of Ormosil sol and subsequently molded in 2−4 mm i.d. glass tubes. The electrodes were dried either at 4 °C or at room temperature (only when specified). Gelation and drying took about 1 day at room temperature and 3 days at 4 °C. The resulting bulk-modified enzyme electrodes (denoted as Pd-GOx/CCE, Pd-LOx/CCE, Pd-L-AAOD/CCE, or PEG/Pd-GOx/CCE) were polished with 400 and 600 grit polishing paper and thoroughly rinsed with triply distilled water. Electrical contact was achieved by placing a copper wire through the back of the electrode during the initial stages of drying. Some electrodes were stored in a refrigerator at 4 °C, and others were stored at room temperature (when specified).

A similar preparation procedure was used for the production of enzyme-encapsulated CCE without palladium catalyst (type C). Unless otherwise specified, the results shown here are for the

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Table 1. Physical Characteristics of CCEs

CCE type[a]	surface area (m^2/g)[b]	water contact angle, (deg)	double layer capacitance (mF/cm^2)	bulk density (g/cm^3)	skeletal density (g/cm^3)
blank	nd	80	0.7	0.94	1.70
A, GOx/CCE without Pd	nd	70	6.8	0.96	1.73
A, Pd-GOx/CCE	nd	60	12	0.95	1.68
B, GOx/CCE without Pd	9.2	72		0.96	1.68
B, Pd-GOx/CCE	10	66	4.75	0.96	1.70
B, PEG/Pd-GOx/CCE	42	50	10.6	0.99	1.65

[a] All CCEs except "blank" contain 250 mg of glucose oxidase/g of graphite. Blank contains 1 wt % Pd and no enzyme. A and B represent different preparation protocols as given in the text. Pd electrodes contain 1 wt % Pd. PEG/Pd-GOx/CCE electrode contains 125 mg of PEG/g of graphite. [b] nd, not amenable for nitrogen adsorption analysis.

enzyme electrodes prepared using a sol without methanol (type B electrodes), with or without PEG modifier.

Thick-Film Sensors. The CCEs can also be used in thick-film form amenable for mass production.[18] Thick films (~0.5−1 mm thick) of type B GOx-modified Pd-CCE were spread-coated on microscope glass slides and allowed to dry in ambient conditions for 3 days. Sensing was carried out using a single drop of the analyte applied by a Gilson pipet or an accurate syringe. The CCE film served as the working electrode and was connected to the potentiostat via a copper lead (the silver paste contact was a few millimeters away from the wetted section). Miniature reference (SCE) and counter electrodes (Pt) were carefully inserted into the drop with the aid of a microscope. The drop was stabilized by the hydrophobicity of the film, and measurements could be undertaken for ~20−30 min (depending on the rate of evaporation rather than on the instability of the drop itself).

RESULTS AND DISCUSSION

Physical Characteristics. The Pd metal-modified carbon−ceramic enzyme electrodes were black, rigid, and porous. They did not shrink upon drying, and the adhesion to the glass capillary was found to be good. The resulting electrodes contained ~25% (wt) graphite, which is well above the percolation threshold.[32] Table 1 shows some physical characteristics of the carbon−ceramic electrodes used in the present study. The BET surface area, as measured by the nitrogen adsorption, was 9−10 m^2/g for the type B electrode. The poly(ethylene glycol)-containing xerogel yielded a still higher surface area (42 m^2/g), which is in agreement with previous studies showing that PEG additives increase the surface area of silica xerogels. The material prepared by the sol using methanol and under relatively higher acidic conditions (type A) did not yield any measurable surface area. This is in accordance with previous findings that a low-pH process yields dense xerogels which cannot be adequately quantitated by nitrogen adsorption experiments.[34] Indeed, the nitrogen adsorption isotherms of type A electrodes exhibited open hysteresis loops, indicating nonequilibrium conditions and microporous structure.

Despite the marked differences in the specific surface area of the various preparation procedures, the bulk and skeletal densities of the four electrodes were identical (within experimental error). The reason for this obvious discrepancy is the bimodal size distribution of the CCEs, comprised of <~1 nm micropores and submicrometer-dimension (>0.1 μm) macropores. The macropores contribute most of the void fraction of the CCEs but have little effect on their specific surface area. The macropores were probably formed by the collapse of micropores during the evaporation of the solvent. Unlike homogeneous silicates, which would shrink considerably during drying, the graphite particles in the CCE contribute localized rigid structures that prevent homogeneous shrinkage, yielding bimodal distribution of micro- and macropores.

Wettability. Neither highly hydrophobic nor totally hydrophilic matrices are desirable for sensing applications. In the former, the analyte cannot approach the embedded enzyme, and the latter exhibits a large background current. The xerogels prepared using MTMOS precursor were highly hydrophobic, and water was rejected from their outermost surface. When chemical modifiers such as metal dispersion, water-soluble polymers, and proteins (including the enzyme itself) were added to the material, the resulting electrodes became more hydrophilic. The change in water contact angle manifests the wettability and, in turn, the hydrophobicity of the material. Blank electrodes exhibited the highest water contact angle (Table 1). The contact angle and the corresponding hydrophobicity decreased with the increase in enzyme and Pd loading. The lowest contact angle was obtained for the PEG-modified electrodes (PEG/Pd-GOx/CCE).

The changes in hydrophilicity can also be followed by electrochemical measurements. An increase in wetted area increases the wetted conductive surface accessible to the electrolyte and also the corresponding electrochemically active area and capacitive currents. The unwetted surface area does not contribute to the capacitive or Faradaic currents. The observed capacitance (C) calculated from the background current (i) in cyclic voltammetry ($C = i/v$, where v is the scan rate) can be used as a semiquantitative measure of the wetted section. Figure 2 depicts the dependence of observed capacitance on enzyme loading for the Pd-containing CCEs (type A). A near-logarithmic dependence was observed up to 250 mg of enzyme/g of Pd-modified carbon, where a tendency for saturation was observed. A similar logarithmic dependence of the capacitance on Pd loading was observed for Pd/CCE without proteins.[31] The exposed surface area of the type B electrodes, used as a model electrode here, was roughly 25 times larger than that of the blank CCEs containing neither Pd nor enzyme and ~6 times larger than those of the electrodes containing palladium but no enzyme.

Another way of increasing the exposed surface area is by incorporating readily leachable, water-soluble components in the matrix and dissolving them out by immersing the electrode in an electrolyte. This leaves wide open channels for the penetrating

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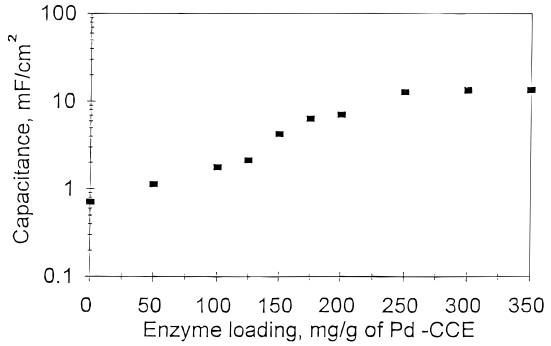

Figure 2. Dependence of the observed capacitance of Pd-modified CCE (type A) on glucose oxidase loading. Cyclic voltammetric studies were carried out at 10 mV/s scan rate in pH 5.7 phosphate buffer.

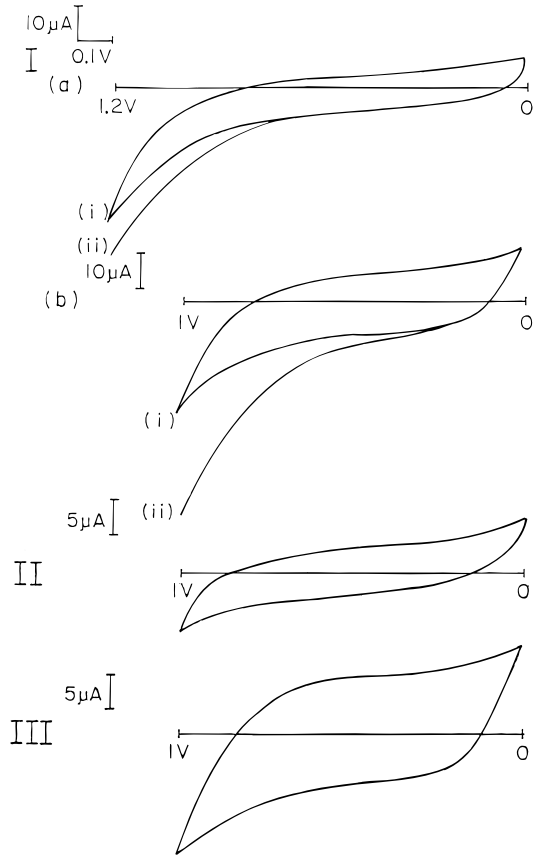

Figure 3. Cyclic voltammograms of CCEs in phosphate buffer, pH 5.7; scan rate, 10 mV/s; geometric area, 0.07 cm^2. (I) Type A electrodes, (a) GOx/CCE and (b) Pd-GOx/CCE, (i) in the absence of glucose and (ii) with 6 mM glucose. (II) Type B electrode, Pd-GOx/CCE, in the absence of glucose. (III) Type B electrode, PEG/Pd-GOx/CCE, in the absence of glucose.

electrolyte, thereby increasing the wetted section inside the matrix. A typical example is the use of poly(ethylene glycol). The poly(ethylene glycol)-containing CCE matrix (type B, PEG/Pd-GOx/CCE) showed an observed capacitance of 10.6 mF/cm^2, which is approximately twice as large as those of electrodes not containing PEG (type B, Pd-GOx/CCE) (Figure 3). The impact of increased wetting on the sensor response is demonstrated and discussed later.

Response of Pd Metal-Modified GOx/CCE. Figure 3 shows the cyclic voltammograms of the bulk-modified GOx/CCE and Pd-GOx/CCE in the presence and in the absence of glucose in the solution. A delay time of 5 min was used between successive

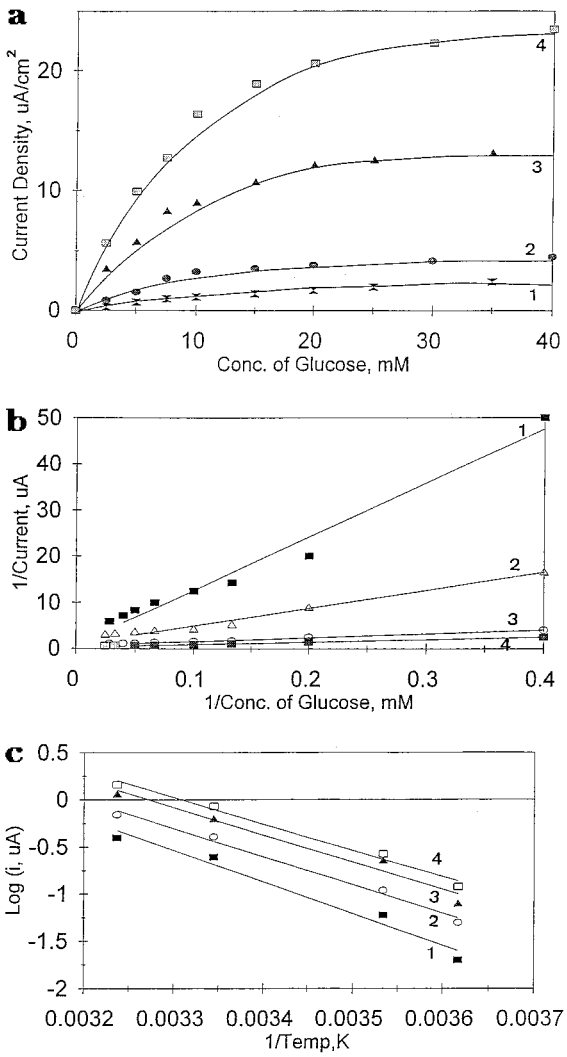

Figure 4. (a) Steady-state calibration curves of type B Pd-GOx/CCEs at 0.5 V vs SCE, corresponding to (1) 3.5, (2) 10, (3) 26, and (4) 36 °C. (b) Lineweaver–Burk plots for type B Pd-GOx/CCEs, corresponding to (1) 3.5, (2) 10, (3) 26, and (4) 36 °C. (c) Arrhenius plots for type B Pd-GOx/CCEs, corresponding to (1) 2.5, (2) 5, (3) 10, and (4) 20 mM glucose concentrations.

cycles to accumulate a substantial quantity of hydrogen peroxide in the porous network. The anodic wave, corresponding to the oxidation of hydrogen peroxide, was shifted by ~0.4 V by the Pd electrocatalysis. In all cases, the response dropped down to the base value when the electrolyte was changed to phosphate buffer without glucose. Also, the base response was restored when the electrolyte was purged with nitrogen. Steady-state glucose calibration curves at 0.5 V vs SCE at various temperatures are given in Figure 4a (similar calibration plots were obtained at a bias of 0.3 V as well). The cyclic voltammograms and the steady-state responses were found to be independent of solution agitation. Hence, the external mass transport limitation to the overall rate can be ruled out. The electrode response can therefore be controlled by either kinetics of the biochemical reaction, mass transport of the substrate inside the porous matrix, or a combination thereof. The limiting step can be resolved by evaluating the governing rate law.

Type B electrodes exhibited typical Michaelis–Menten kinetics at all temperatures, with the linearity range restricted to a low range of glucose concentrations. The increase in temperature

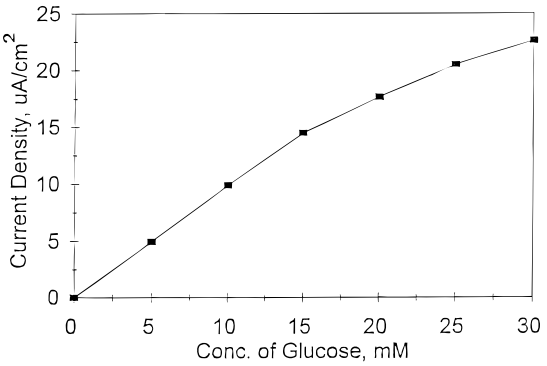

Figure 5. Steady-state calibration curve of type A Pd-GOx/CCE at 0.5 V vs SCE.

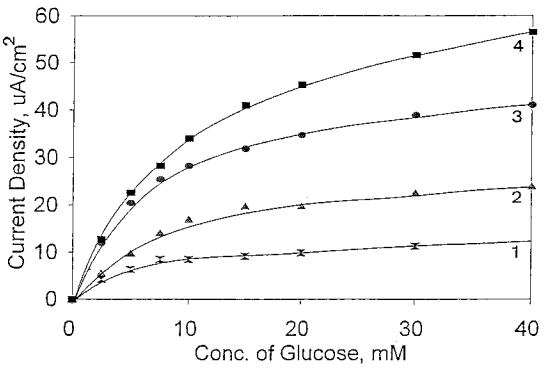

Figure 6. Steady-state calibration curves of type B PEG/Pd-GOx/CCEs at 0.5 V vs SCE, corresponding to (1) 3.5, (2) 10, (3) 26, and (4) 36 °C.

increased the response of the sensor, as expected. The maximum response was obtained at 36 °C, after which the enzyme lost its activity during prolonged operation. The apparent Michaelis−Menten constants can be determined following the Lineweaver−Burk type equation,[35]

$$1/i_{ss} = (K_M'/i_{max})(1/C) + (1/i_{max}) \quad (1)$$

where i_{max} and i_{ss} are the current measured under substrate saturation and the steady-state current for a given substrate concentration (C), respectively. The linear fit is depicted in Figure 4b. The apparent K_M' determined in the present studies works out to be 20 ± 2 mM for the entire 0−36 °C temperature range (degrees of freedom, $n - 2 = 5$; $R^2 = 0.97$ at 3.5 °C, 0.98 at 10 °C, and >0.99 at 26 °C and at 36 °C). The effective activation energy (E_a) can be evaluated using the log(i) vs $1/T$ plot (Figure 4c), and it works out to be 12 ± 1 kcal/mol K (for the range 3.5−36 °C). The enzyme immobilization process usually alters the microenvironment of the enzyme active site. This might affect the intrinsic characteristics of the enzyme. However, the activity coefficients determined for the CCEs prepared in the present study agree well with the reported values for the GOx in the solution phase (E_a = 9 kcal/mol K and K_M' = 33 mM[36,37]).

The linear range of an enzymatic reaction that follows Michaelis−Menten kinetics corresponds to $0.1K_M'$,[38] which leaves a very narrow linear region (as, indeed, is the case for the type B electrodes). The linear range of the electrode can be increased by incorporating an additional resistance for diffusion of the analyte, so the overall kinetics would then be controlled by a diffusion process or a combination of reaction and diffusion.

The porous silica skeleton of type A electrodes (produced using methanol under more acidic preparation conditions) is microporous, with very minute mesopores (N_2 adsorption BET measurements did not yield a measurable surface area (Table 1) and is expected to provide a barrier for diffusion). Indeed, it was found that the linearity range of the glucose calibration curve was about 15 mM in type A electrodes (Figure 5). The response time described below also supports the same conclusion. The response of the PEG-containing CCE, which has a larger active section as compared to electrodes without modifier (see CV curves in Figure 2), was governed by an intraelectrode diffusion process, which is

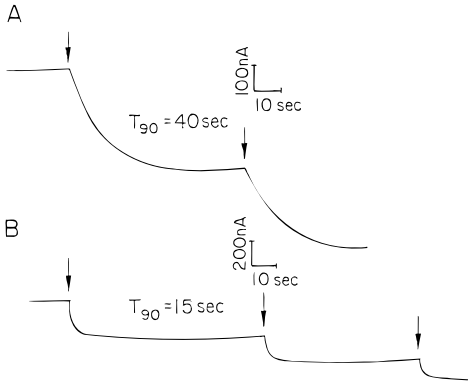

Figure 7. Typical response times of type B electrodes (at 0.5 V): (A) PEG/Pd-GOx/CCE and (B) Pd-GOx/CCE. Arrows indicate the additions of 5 mM glucose. The response was independent of solution agitation.

affected by both the enzymatic kinetics and the diffusion of the substrate.[39,40] This is indicated by the fact that saturation of the response was not reached even at 40 mM glucose and also by a wider distribution of K_M' at different temperatures (Figures 4b and 6).

Response Time. The response times of the various CCEs are largely dependent on the structure and size of their active sections. Kinetically controlled type B electrode is expected to respond much more quickly than the diffusion-controlled electrodes. Figure 7 shows a typical dynamic response to a step change in the glucose concentration for PEG-modified and unmodified type B Pd-GOx/CCEs. The CCE without PEG responded quickly to additions of glucose; the T_{90} was 15 s. The CCE containing PEG had a response time of about 40 s, reflecting the thicker inner reaction layer and the longer path length of the analyte. The response time of the type A CCE sensor was an order of magnitude larger (~180 s). Figure 7 also shows that the increased response time of the PEG-modified electrodes is accompanied by an enhancement of the signal due to the larger wetted section, which also implies a larger amount of reactive enzyme.

External mass transport did not play any role, as verified by the indifference of the response to stirring of the solution. However, when operating at very low overpotentials (0.2−0.3 V

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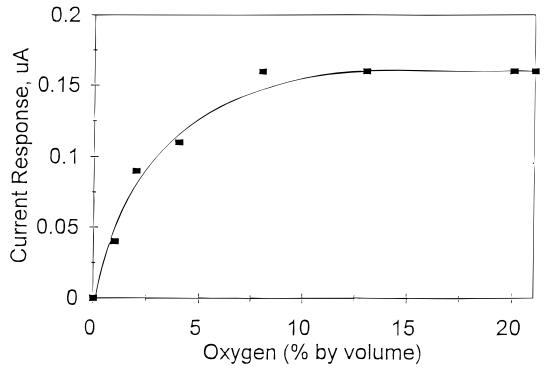

Figure 8. Effect of oxygen on the response of type B Pd-GOx/CCE. The back side of the electrode was exposed to different partial pressures of oxygen. Glucose concentration was 5 mM. The electrolyte was nitrogen saturated.

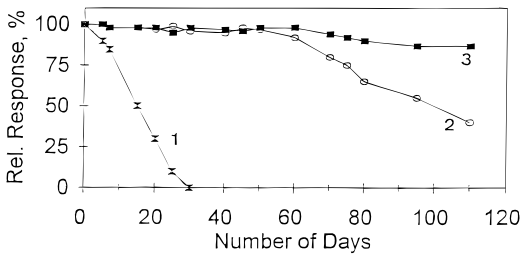

Figure 9. Stability of the CCE biosensors. Effect of preparation and storage conditions. (1) Type A Pd-GOx/CCEs, stored at 4 °C. (2) Type B Pd-GOx/CCEs, stored at 20 °C. (3) Type B Pd-GOx/CCEs, stored at 4 °C.

vs SCE), we frequently encountered situations where switching on the stirrer decreased the observed signal. This is explained by slow hydrogen peroxide conversion, leading to the formation of a hydrogen peroxide external diffusion layer. The concentration gradient in this layer becomes steeper with the external stirring, thus increasing the escape of H_2O_2 to the solution and thereby decreasing the signal.

Oxygen Supply. Biosensors are often used in biological applications such as glucose estimation in brain or subcutaneous tissue, where the oxygen availability is low, or for environmental monitoring, where oxygen tension is subject to unpredictable changes. In these cases, external oxygen supply is desirable to facilitate the enzymatic reaction. The porous structure of CCEs is particularly suitable for such applications. The top side of the electrode can be left open, and oxygen from the atmosphere can permeate through the bulk of the electrode to the reactive layer. Such a configuration is often used for gas electrodes in fuel cell applications, and therefore the terminology "fuel cell type enzyme electrodes" may be suitable here. The effect of oxygen partial pressure at the back of a glucose electrode operated in a fuel cell type mode is shown in Figure 8. When operated under oxygen-deficient conditions (i.e., the electrolyte is bubbled with nitrogen), the sensor response was saturated at an oxygen level of 0.08 atm (at the back of the electrode). A similar dependence was observed for air-bubbled solution. The glucose sensors operated under fuel cell mode of operation were still slightly dependent on the level of dissolved oxygen and lost ~25% of the response upon deaeration of the solution with nitrogen. We anticipate that optimized electrode configuration (e.g., thicker reactive layer) will totally eliminate the signal dependence on the level of dissolved oxygen.

Stability of the Sensors. The ceramic−carbon material encapsulates the enzyme effectively, and no leaching of the enzyme from the CCE was observable, even after prolonged immersion (>24 h) in aqueous solutions. The stability of the glucose sensor in a continuous operation mode (E = 0.5 V vs SCE) revealed that the signal (for 5 mM glucose) was constant, in ambient temperature conditions, at least for 6 h without any decrease. The test was terminated after this time period. The long-term stability of the Pd-GOx/CCE biosensors was followed by storing different electrodes at different temperatures and observing the dynamic response for glucose at regular intervals. The electrodes were polished before each test, and the signal was observed in 5 mM glucose solution (which is well within the dynamic range) without recalibration. The stability of the electrodes tested in the present study is given in Figure 9. Type A electrodes (prepared using methanol and more acidic conditions) retained some response for about a month, with steady deterioration. The reasons for the low stability were found to be (1) high acidity of the sol during the preparation stages and (2) use of methanol as a solvent for the sol−gel process. Indeed, we were quite successful in preparing very stable sensors. The electrodes prepared using the sol without methanol were stored at two different conditions, one batch at 4 °C and another at room temperature, 20 °C. Both of these batches were stored in undesiccated, open beakers. The CCEs stored at room temperature were found to retain full response for 2 months and only then started deteriorating slowly, probably due to the beginning of hot summer in Israel. CCEs stored at 4 °C were found to retain complete response for about 70 days, and subsequently the response was stabilized with a small decrease and continued to be stable for 120 days. We suspect that the small deterioration was due to exposure of the electrode to higher temperatures during the period of experimentation. The stability studies are still in progress.

Repeatability. The fact that the electrodes are bulk modified and only a thin section is wetted by the electrolyte leaves them amenable for surface renewal whenever the sensor surface is contaminated. A mere mechanical polishing was sufficient to get a fresh bioactive section. Both the background current during cyclic voltammetry and the absolute response when glucose was added remained constant after polishing. Renewal repeatability tests reveal <3% relative standard deviation for repeated surface polishing (16 times) of the same electrode. The remarkable renewal repeatability is obvious from Figure 9 as well, since each data point represents operation with a renewed surface (with no recalibration). This also indicates the uniformity in the distribution of the enzyme as well as the void fraction throughout the CCE matrix. The electrochemical response was also indifferent to the depth of immersion of the electrode in the electrolyte. Both the cyclic voltammetric background current and the steady-state signal remained constant when the depth of immersion was varied between 2 and 35 cm. Higher heights were not checked, as they are impractical. Several bulk-modified, surface-renewed, amperometric biosensors were reported in the literature, but carbon−ceramic biosensors are, to the best of our knowledge, the first renewed reactive layer electrodes. This is attributed to the brittleness of the inorganic matrix, which is highly resistive to plastic deformations, and thus the porous structure is not clogged by the mechanical polishing step. The interelectrode reproducibility of the glucose sensor was determined by following the steady-state signal for five different electrodes from the same batch. The standard deviation worked out to be <10%.

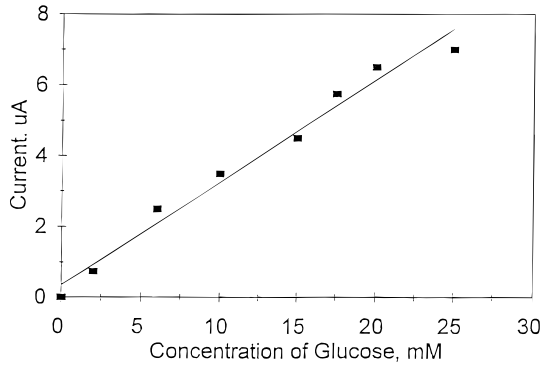

Figure 10. Calibration curve for glucose on a thick-film Pd-GOx/CCE glucose sensor. Sample volume, 15 μL.

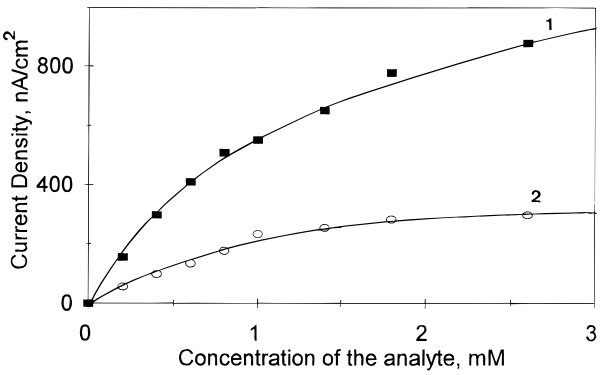

Figure 11. Calibration curves for lactate (1) and L-phenylalanine (2) on a type B Pd-LOx/CCE (1) or Pd-L-AAOD/CCE (2) operated at 0.5 V vs SCE; phosphate buffer electrolyte, pH 7.4 for lactate and pH 7.0 for L-phenylalanine.

Thick-Film Sensors. Thick-film disposable sensors are another, well-accepted way to overcome the problem of contamination of the surfaces of modified electrodes. Other well-documented benefits include sample minimization, no need for a sterilization step, and the possibility of mass production.[18] Type B Pd-GOX/CCE films spread-coated on glass slides were used exclusively in these experiments. The enzyme was found to be uniformly distributed over the entire area of the glass slide, and the electrochemical activity at different locations of the film was the same. Again, similar to the rod electrodes, no leaching of the enzyme was observed after prolonged (24 h) immersion in the solution.

The thick-film sensors cannot operate in a steady-state mode since the substrate is gradually depleted from small drops during measurement. Periodic cyclic voltammetric studies were conducted in order to find the dependence of the cyclic voltammetric response on the waiting time between application of the drop and measurement. It was found that the response is saturated after ~10−15 min. Figure 10 demonstrates feasibility of the single-drop mode of analysis. The figure shows a typical calibration curve based on the observed current at 0.8 V vs SCE taken during 10 mV/s cyclic voltammetric measurements (potential range was 0−1 V vs SCE) in a single drop of the analyte. Each data point was taken after thoroughly rinsing the electrode with distilled water, drying the surface at ambient conditions, and then applying a fresh 15 μL drop containing glucose using a syringe and waiting for 10 min before application of the potential sweep. A linear calibration plot was observed in the range 0−20 mM glucose ($n - 2 = 6$; $R^2 = 0.97$).

Lactate and L-Amino Acid Sensors. To show that the CCE concept is applicable to other redox enzymes, we used lactate oxidase and L-amino acid oxidase as demonstrating cases. Lactate level in blood is specific to lymphomas, muscle diseases, sacromas, etc. The fermentation of wine, beer, fruit juice, etc. is followed by monitoring the lactate level. The sensing of amino acids is of importance for various applications. Diseases such as diabetes are associated with defects in amino acid metabolism.[41] Amino acids are also found as constituents of fermented food. Derivatization is usually necessary for the estimation of amino acids by common detection methods, like spectroscopy and electrochemistry, but this step can be avoided using the specificity of enzymes in biocatalysis.

The mechanism of the lactate sensor is similar to that of glucose sensing, i.e., oxidation of lactate to pyruvate, reduction of oxygen, formation of hydrogen peroxide, and subsequent electrocatalyzed oxidation of the hydrogen peroxide on the electrode. A steady-state calibration curve for lactate on a B type, Pd-modified CCE sensor is shown in Figure 11. The calibration plot reveals that Michaelis−Menten kinetics was obeyed in this case as well. The apparent K_M' value determined using the Lineweaver−Burk type equation was ~1.7 mM ($n - 2 = 6$; $R^2 = 0.99$). The lactate sensor was found to be stable over a period of 3 weeks, and the stability tests are still in progress.

Figure 11 also shows the calibration curve for L-phenylalanine at 0.5 V vs SCE, using Pd-CCE modified with L-amino acid oxidase. The enzymatic reaction is

$$\text{amino acid} + H_2O + O_2 \rightarrow \text{2-oxyacid} + NH_3 + H_2O_2 \quad (2)$$

and the hydrogen peroxide is detected at the CCE. The sensor was found to be stable for over a week. Again, Michaelis−Menten kinetics was observed. Lineweaver−Burk analysis yielded a K_M' value of ~1.43 mM ($R^2 = 0.99$; $n - 2 = 6$).

CONCLUSIONS

The Pd metal-modified, carbon−ceramic biosensors constitute a new type of renewable surface biosensors with a controllable size reaction layer. The same type of material is also useful for the production of thick-film disposable sensors, wherein a sample drop applied on the hydrophobic surface of the electrode is used. The size of the reactive layer can be modified using hydrophilic chemical additives such as PEG, and thus the rate-limiting step, the absolute level of the signal, and the response time can be altered. Adequately prepared electrodes, using a preparation protocol with no methanol and minimal concentration of hydrochloric acid, yield excellent stability and repeatability.

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