Oxygen-Rich Oxidase Enzyme Electrodes for Operation in Oxygen-Free Solutions

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Abstract: The oxygen dependence of oxidase enzyme electrodes is successfully addressed through the use of carbon paste biosensors with fluorocarbon pasting liquids. Due to the high oxygen solubility in perfluorocarbons, such pasting liquids provide an internal supply of oxygen, and efficient operation of first-generation oxidase electrodes under oxygen-deficit conditions. In particular, the use of poly(chlorotrifluorethylene) (Kel-F) oil results in an identical response up to 4×10^{-2} M glucose, in the presence and absence of oxygen. Such oxygen independence compares favorably with that reported for mediator or wired enzyme electrodes. The internal supply of oxygen of a Kel-F/carbon paste enzyme microelectrode is not depleted over a prolonged operation under oxygen-deficient conditions. The oxygen effect upon the kinetic parameters of the immobilized enzyme is examined for a variety of pasting liquids. Such attention to the oxygen demand addresses numerous practical biosensor applications, involving low or fluctuated levels of oxygen, including in-vivo monitoring of glucose.

Oxidase-catalyzed enzymatic reactions play a major role in the development of enzyme-based biosensors.[1,2] The large number of commercially available oxidases opens up the prospects for the detection of important substrates (such as glucose, lactate, or cholesterol) in relevant clinical or food matrices. As the immobilized enzyme relies on the use of oxygen as the cosubstrate, the operation of these enzyme electrodes suffers from problems due to restricted solubility of oxygen (that limits the enzymatic reaction) and variations in the oxygen level.[3] For example, implantable glucose sensors often suffer from the low oxygen availability in the subcutaneous tissue. Common routes for minimizing the strong oxygen dependence include the replacement of oxygen with a non-physiological electron acceptor,[4] or the use of a proper membrane coverage that improves the surface availability of oxygen (relative to the substrate).[3,5,6] A less common route involves a cumbersome geometry with a backside external oxygen supply.[7,8]

We report here a novel strategy, based on oxygen-rich carbon-paste enzyme electrodes, for minimizing oxygen effects in oxidase-based biosensors. We demonstrate that using fluorochemical pasting liquids, possessing very high oxygen solubility, provides an internal supply of oxygen and allows efficient operation of first-generation enzyme electrodes under *severe* depletion of oxygen and a wide range of substrate concentrations.

Carbon paste electrodes (CPEs), consisting of a mixture of graphite powder and an organic pasting liquid (commonly mineral oil), represent an attractive approach for the preparation of reagentless biosensors.[9,10] The pasting liquid not only serves for filling the crevices between the graphite particles but also results in an electrode that is fundamentally different from those (e.g., Pt, Au) commonly used for amperometric transduction.

It is well-known that the solubility of oxygen is many times greater in some organic solvents than in water.[11,12] In particular, due to the very high oxygen solubility in fluorochemicals (resembling that of hemoglobin), such solvents have been used as oxygen transporters and blood substitutes in humans and animals.[13−16] Taking advantage of this remarkable oxygen solubility we constructed novel oxygen-insensitive first-generation oxidase electrodes based on fluorochemical carbon pastes. The new electrodes satisfy the oxygen demand internally, and eliminate the signal dependence on the oxygen level.

Experimental Section

Apparatus. All experiments were carried out with the BAS CV-27 voltammetric analyzer (BAS, W. Lafayette), in connection with a BAS X−Y-t recorder. The enzyme electrode, reference electrode (Ag/AgCl, Model RE-1, BAS), and platinum wire auxiliary electrode joined the 10 mL cell (Model VC-2, BAS) through holes in its Teflon cover. A magnetic stirrer provided the convective transport during the amperometric measurement.

Electrode Preparation. Enzyme electrodes were prepared by mixing 10 mg of GOx with 100 mg of carbon paste containing 75%

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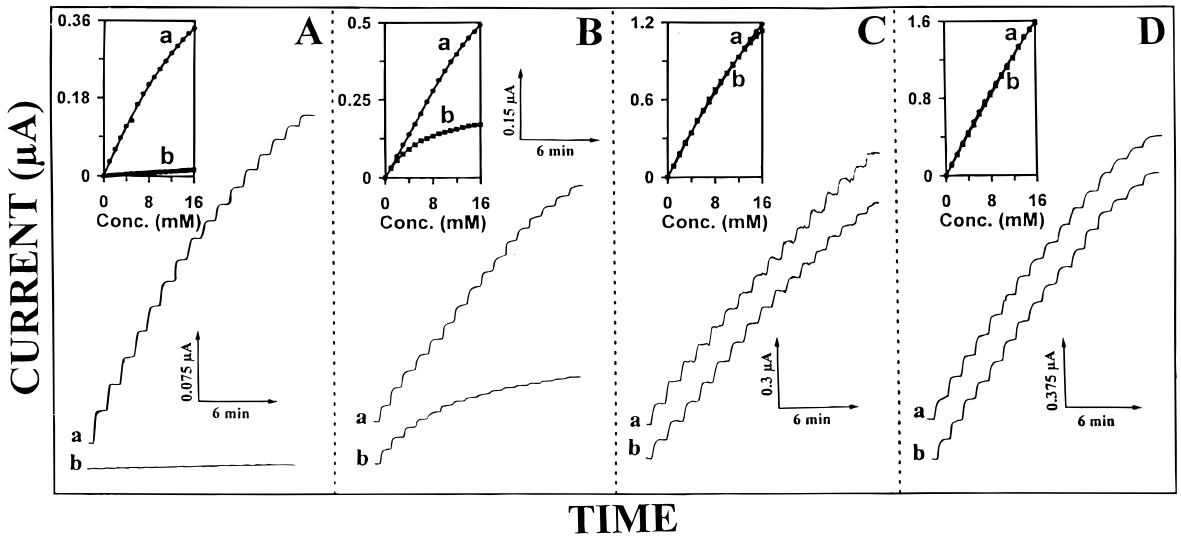

Figure 1. Current−time recordings for successive 1×10^{-3} M increments of glucose, obtained in the presence (a) and absence (b) of oxygen, at poly(phenylenediamine) (PPD)/glucose-oxidase(GOx)-coated platinum electrode (A), and GOx-modified carbon paste electrdoes (B−D). Pasting liquid: mineral oil (B), mineral oil/Nafion (1:2 ratio) (C), and Kel-F oil (D). The pastes were prepared by mixing 10 mg of GOx with 100 mg of carbon paste containing 40% graphite powder (Rh-on-C) and 60% pasting liquid; a 75:25 oil/graphite ratio was employed in D. Nafion (5% in ethanol) mixed with mineral oil for C. The polymer-based biosensor was prepared by growing the PPD/GOx layer electrochemically for 15 min at +0.65 V using a quiescent solution containing 5mM *o*-phenylenediamine and 1000 U/mL GOx. Disk electrode diameter, 3 mm; operating potential, +0.6 V (vs Ag/AgCl); a 10 mL phosphate buffer (0.05M, pH 7.4) solution, stirred at 300 rpm. Insets display the resulting calibration plots.

Kel-F oil and 25% graphite powder (Rh-on-C, Aldrich). Mixing proceeded for an additional 30 min. A portion of the resulting paste was packed tightly into the cavity (3 mm diameter, 2 mm depth) of a BAS voltammetric electrode (Model MF-2010). The electrode surface was smoothed on a weighing paper. Carbon paste biosensors based on other pasting liquids or formulations were prepared in a similar fashion. The Kel-F/GOx-carbon paste microelectrode was prepared by packing the paste into the end of a 6-cm long Teflon tube (0.2 mm i.d, 0.6-mm o.d). The paste filled the tip to a height of 8 mm, with electrical contact to its inner end made with a 0.1-mm diameter copper wire.

Chemicals. All solutions were prepared from double-distilled water. Glucose, glucose oxidase (EC 1.1.3.4, Type X−S, Aspergillus Niger, 135 000 U/g), *o*-phenylenediamine, sodium acetate, and glucose (all reagent grade from Sigma) were used without further purification. Kel-F oil (No. 10) was purchased from Ohio Valley Specialty Chemical (Marieta, OH). The rhodium-on-carbon (5% Rh), mineral oil, heptane, and Nafion solution (5% w in a mixture of lower aliphatic alcohols and water, ER 1,100) were obtained from Aldrich. The paraffin oil (PX0047-1) and dodecane (DX2415-1) were purchased from EM Science. All measurements were performed in a 0.05 M phosphate buffer solution (pH 7.4).

Procedure. Oxygen removal was accomplished by purging the solution with helium for 40 min; a helium atmosphere was subsequently maintained over the solution. Experiments were performed by applying the desired potential (usually +0.60 V), stirring the solution at 300 rpm, allowing the transient background current to decay to a steady-state value (in the presence of the blank solution), and spiking the glucose substrate. All experiments were conducted at room temperature.

Results and Discussion

Figure 1 compares the amperometric response to successive additions of 1×10^{-3} M glucose as obtained in the presence (a) and absence (b) of oxygen, using conventional polymer-based (A) and carbon-paste (B) enzyme electrodes, and utilizing fluorochemical-containing carbon paste bioelectrodes (C, D). As expected for oxygen-deficit conditions, the polymer-entrapped enzyme electrode does not respond to the substrate additions (A,b), while the mineral-oil carbon paste biosensor displays greatly reduced signals (B,b). In contrast, the inclusion of the perfluoropolymer Nafion in the mineral oil (C) or use of the Kel-F (poly(chlorotrifluoroethyene)) oil binder (D) dramatically minimizes this oxygen dependence, and results in a similar response for nondeaerated and helium-saturated solutions. Despite the absence of oxygen, no reduction in the upper limit of linearity of the fluorocarbon-based electrodes is observed, with linearity prevailing up to about 1×10^{-2} M, and only a slight curvature thereafter. Apparently, these oxygen-rich fluorocarbon environments supply sufficient oxygen to satisfy the enzymatic reaction even under severe oxygen deprivation. Indeed, the Kel-F/GOx/CPE displayed a total oxygen independence up to a glucose concentration of 4×10^{-2} M (not shown). Such oxygen independence is superior to that of most effective mediators (ferrocene)[4] or wired (osmium)[17] bioelectrodes, which display some oxygen competition for the enzyme. A nearly oxygen independence was obtained also using perfluorononane ($CF_3(CF_2)_7CF_3$) as the pasting liquid, but the resulting paste was too dry and required a large liquid/graphite ratio (10:1).

While the solubility of oxygen within fluorocarbons is approximately 25-fold higher than that in water, oxygen solubilities in short-chain hydrocarbons are about 10 times higher than in water, and 2-fold larger than in mineral oil.[12,16,18] Hence, carbon-paste enzyme electrodes based on heptane or dodecane pasting liquids displayed a greatly reduced oxygen dependence in comparison to their mineral oil counterpart (not shown). However, their oxygen insensitivity was inferior to that of fluorocarbon-based biosensors. Overall, the Kel-F CPE displayed the most favorable oxygen independence, sensing performance and surface consistency.

Lineweaver−Burk plots for the different fluorocarbon and hydrocarbon carbon paste biosensors were essentially linear (for glucose concentration in the range 5 to 60 mM, with and without oxygen; not shown). The resulting values of maximum current

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Table 1. Kinetic Parameters of Carbon Paste Glucose Biosensors Based on Different Pasting Liquids[a]

	$K_{m,app}$ (mM)		I_{max} (mA)	
pasting liquid	nondeaerated sample	deaerated sample	nondeaerated sample	deaerated sample
mineral oil	27.4	24.5	1.1	0.3
silicone grease	35.9	25.1	1.1	0.2
dodecane	41.5	40.2	2.4	1.8
heptane	42.8	43.1	5.2	4.5
Kel-F oil	49.3	52.5	6.9	6.5
perfluorononane	49.4	49.2	12.1	12.0
mineral oil/ Nafion (1:2)	38.6	35.5	1.6	1.3

[a] Conditions as in Figure 1, except of a 5−60 mM glucose concentration range.

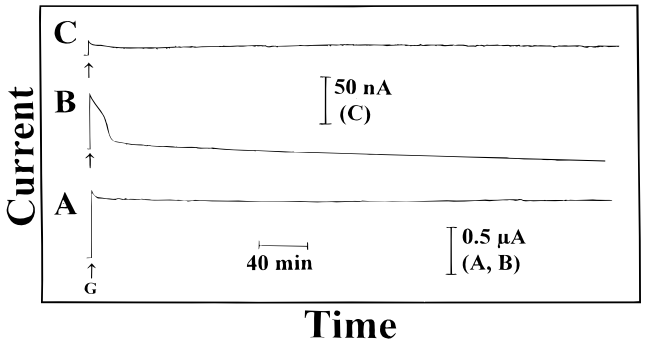

Figure 2. Amperometric response to a 5×10^{-3} M glucose addition to an deoxygenated buffer solution at Kel-F/GOx/CPEs (A,C) and mineral-oil/GOx/CPE (B). Electrode diameter: 3 mm (A,B); 0.25 mm (C). Other conditions, are as in Figure 1D(b).

(I_{max}) and apparent Michaelis−Menten constant ($K_{m,app}$) are shown in Table 1. While similar $K_{m,app}$ and I_{max} values are observed for the fluorocarbon and hydrocarbon CPEs under helium and air saturation, significantly different values are indicated for the ordinary (polymer and mineral-oil) bioelectrodes. The higher values of $K_{m,app}$ (>35 mM) at the fluorocarbon and hydrocarbon bioelectrodes, in comparison with those (26 and 33 mM) reported for the soluble enzyme,[19,20] indicate that their response is controlled partly by mass transport.

A key issue for addressing the oxygen demand of oxidase biosensors is the ability of the internal oxygen reservoir to reliably deliver oxygen over an extended period of time. Figure 2A illustrates such capability in connection with measurements of 5mM glucose in a deoxygenated solution over a prolonged (7 h) continuous operation. The stable response indicates no apparent depletion of oxygen from the internal fluorocarbon reservoir. In contrast, the mineral-oil carbon paste biosensor (with its limited oxygen capacity) displays a rapid, nearly complete loss of the glucose response within the first 20 min of operation in the deoxygenated medium (Figure 2B). Smaller Kel-F/carbon-paste biosensors, relevant to in-vivo glucose monitoring, were also tested (Figure 2C). The 0.2 mm o.d. enzyme microelectrode displays a nearly constant glucose output in the absence of solution oxygen throughout this 7 h operation (with the exception of a 15% initial loss). Apparently, the lower biocatalytic consumption of oxygen—associated with the smaller electrode dimension—"compensates" for its smaller oxygen reservoir. (Note the different current scales.) Hence, a prolonged operation of the microelectrode in deoxygenated medium does not deplete its internal oxygen supply. A further size reduction may be compensated by employing long carbon-paste (i.e., "oxygen") reservoirs. The enzyme microelectrode also displayed a linear, oxygen-independent, response over the 2−25 mM clinically relevant glucose concentration range.

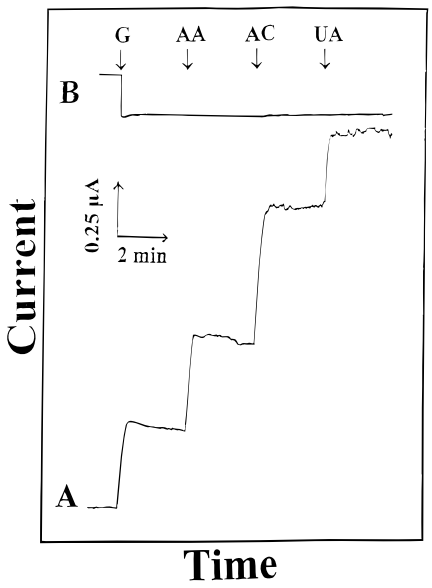

Figure 3. Typical amperograms at +0.6 (A) and −0.05 V (B) for a deoxygenated buffer solution upon addition of 2.5×10^{-3} M glucose (G), followed by 1×10^{-4} M additions of ascorbic acid (AA), acetaminophen (AC), and uric acid (UA). Other conditions, are as in Figure 1D(b).

The confinement of GOx within fluorinated CPEs has no detrimental effect upon the enzyme activity. A nearly identical glucose response was obtained by using the same Kel-F CPE surface (3 mm diameter) over a 120 day period, with intermittent storage at 4 °C. Such Kel-F confinement results also in a remarkable thermal stability, as was indicated from the identical glucose response before and after a 24 h stress at 90 °C. The electrocatalytic action of the metalized (Rh)-graphite transducing component of the Kel-F CPE allows tuning of the operating potential to a region (around 0.0V) where common interferences do not affect the glucose response.[21] Hence, the oxygen independence is coupled to high selectivity (despite the absence of external membrane). As illustrated in Figure 3B, the glucose response (in the absence of oxygen) is not influenced by the presence of ascorbic acid, acetaminophen, or uric acid. At higher potentials, all three oxidizable compounds display a significant response, and the selectivity is compromised (Figure 3A).

Conclusions

We have demonstrated that oxygen-rich carbon-paste bioelectrodes successfully address the oxygen dependence of glucose sensors. The internal flux of oxygen, associated with the fluorocarbon pasting liquid, facilitates the enzymatic reaction under oxygen-deficit conditions. Even though the concept has been presented within the framework of glucose biosensors, it should successfully address the oxygen demand of other oxidase-based devices. Such attention to the oxygen demand fits the requirements of in-vivo monitoring, and addresses numerous practical biosensor applications involving low or fluctuated levels of oxygen. Implantable glucose microsensors, based on the fluorocarbon carbon-paste strategy and different dimensions and geometries, are currently being developed in our laboratory.

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