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Catalytic Materials, Membranes, and Fabrication Technologies Suitable for the Construction of Amperometric Biosensors

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A selection of recently available catalytic carbon powders were assessed and compared with the more conventionally used platinized material. Their suitability for incorporation in amperometric biosensors is discussed. In conjunction with this study, methods of applying membranes to the surfaces of these devices were investigated. Advanced fabrication technologies, potentially suitable for scale-up of sensor production, such as screen printing and ink-jet printing, were used for manufacture of the complete sensor structure. Hydrogen peroxide-sensing electrodes and glucose biosensors were produced as model systems, demonstrating the advantages of these approaches. The commercially available rhodinized carbon MCA4 produced a high current density at low potentials over a plateau region (300−400 mV vs SCE). In addition, direct oxidation of glucose (seen with platinized carbon) was not observed at the chosen potential of +350 mV. Further interference studies using fermentation media highlighted its suitability as an electrode material for use in complex samples. Ink-jet printing proved to be a successful method for the deposition of Nafion membranes of defined and reproducible geometry.

A number of approaches to the construction of amperometric biosensors have been adopted since the conception of the first device by Clark in 1962.[1] The first biosensors were based on the Clark oxygen electrode, detecting the depletion of oxygen resulting from the action of an oxidase enzyme.[2] Alternatively, hydrogen peroxide, generated as a result of the enzymatic reaction, can be detected and related to the analyte concentration.[3] Further refinements involved the use of mediators to circumvent the reliance of the sensor on the local oxygen concentration, to reduce the operating potential and hence enhance selectivity.[4] In addition, the use of metals such as platinum and rhodium, coated onto carbon electrodes as a fine dispersion, has been widely adopted.[5,6] This results in a highly catalytic surface as long as the particle size of the deposited metal is comparable to that of the electrical double layer.[7] In this instance, the difference in the electronic work functions of platinum and carbon result in an increase in the electron density on the platinum.

Several methods for the incorporation of the metal into the carbon matrix have been reported. These include electrochemical deposition using, for example, cyclic voltammetry,[8] sputtering[9] and straightforward mixing of the metal in a carbon paste.[10] The conventionally preferred working electrode material, platinized carbon, is able to reduce greatly the oxidation potential of hydrogen peroxide. Unfortunately, this material is also highly electrocatalytic for a large number of other substances, such as glucose and other reducing sugars. In complex samples, the presence of such interfering compounds will clearly compromise specificity. To overcome this drawback, selectivity can be enhanced by the use of membranes, typically constructed from polymers such as cellulose acetate, polyurethane, Nafion, and a variety of others.[11] Membranes are also a convenient way of extending the linear range of a biosensor. They form a diffusion barrier, and substrate/product concentration profiles within this layer are, therefore, a function of both diffusion and reaction. One parameter limiting the linear range of such an electrode is the K_m of the enzyme, which exhibits saturation kinetics, and a nonlinear signal is anticipated. If the diffusional effects are dominant, the limitation on the linear range, due to a low K_m value, becomes negligible. In such situations, "apparent" kinetic parameters are often used.[12]

A drawback in the use of membrane-modified electrodes arises from the time-consuming and inconsistent manual fabrication methods typically employed. Automated methods, such as screen printing[13] and ink-jet printing,[14] can be used to accelerate and regulate production. This is an area which is often overlooked when considering the commercialization of such devices.[15]

This paper reports on the suitability of some recently available catalytic carbon powders and examines methods of depositing reproducible membrane structures on screen-printed electrodes constructed of such materials. In addition, this manufacturing process enables biological components such as enzymes to be printed, since it involves a room temperature curing process.

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EXPERIMENTAL SECTION

Electrode Construction and Test Procedure. Screen-printed electrodes were used throughout these investigations and were constructed in planar arrays of eight electrodes. These were fabricated using a DEK 247 screen-printer (DEK Printing Machines, Weymouth, UK) in a multistage printing process whereby a silver conducting track (Electrodag 477SS RFU, Acheson Colloids, Plymouth, UK) was placed on a PVC substrate (Genotherm, Sericol, Surrey, UK). A graphite pad (Electrodag 423 SS, Acheson Colloids) was printed at one end of the silver track, and an insulation shroud (Matt Vinyl White M.V. 27, Apollo Colours, London, UK) was deposited over these layers, such that only a contact pad at one end and a working electrode (8 mm × 2 mm) at the other end were exposed. In each case, the printed layer was cured for 1 h at 40 °C.

A range of commercially available metalized carbon powders (Table 1) were obtained from ETEC and MCA Services (Royston, UK) and investigated. The MCA powders are proprietary materials consisting of highly dispersed precious metals and containing promoters. For studies of the response of the catalytic materials to hydrogen peroxide, the powders were mixed with a 2.5% (w/v) hydroxyethyl cellulose (HEC; Fluka, Gillingham, UK) solution dissolved in 0.1 M, pH 7.0 phosphate buffer. The metalized carbon powder was incorporated into the HEC solution in the ratio 1:2 (w/w). The ink was mixed using a rotary stirrer until a uniform consistency was achieved (~1 h at 25 °C). Finally, the resultant ink was screen-printed onto the graphite pad. Despite this layer being slowly soluble in the test solution, it remained bound to the working electrode surface for sufficient time to enable the analysis to be carried out.

Experiments were carried out in a batch mode using a three-electrode amperometric system consisting of a saturated calomel reference electrode (SCE; Russell, Auchtermuchty, Fife, UK), a 2 mm diameter platinum wire counter electrode, and the working electrode as described above. All experiments were performed under stirred conditions at 25 °C using 0.1 M sodium phosphate buffer containing 0.1 M potassium chloride at pH 7.0. Aliquots of hydrogen peroxide were added to the buffer solution, and the dynamic current response was monitored and recorded using an Autolab electrochemical analyzer (EcoChemie, Utrecht, Netherlands), which was controlled by the software package GPES3.

Enzyme Electrode Construction. Having determined the preferred composition of the electrode base material, the next stage was to incorporate the enzyme into the structure. Glucose oxidase (Glucox PS, ABM, Stockport, UK) was chosen as a model system. Two sensor configurations were investigated. First, the enzyme was mixed with the catalytic powder to form a printable ink (electrode types A). Second, the enzyme was printed over the catalytic powder in a separate layer (electrode types B).

The following ink formulations were used: type A, 1 g of 2.5% (w/v) HEC in phosphate buffer (pH 7.0) solution, 100 mg of glucose oxidase, and 400 mg of catalytic graphite powder; type B, the catalytic graphite layer deposited as described earlier and containing no glucose oxidase. The enzyme was subsequently incorporated into an ink consisting of 1 g of 3% HEC, 100 mg of glucose oxidase, and 400 mg of T10 graphite (Acheson Colloids).

After printing, all electrodes were left to dry for 2 h at room temperature. Because the organic binder is soluble in aqueous solution, an outer protective membrane was required. We investigated two such materials, Nafion (Aldrich, Gillingham, UK), a persulfonic charge exclusion material, and cellulose acetate (Aldrich), a size exclusion barrier. Three techniques were used to apply these membranes: ink-jet printing, dip-coating, and spin-coating. Ink-jet printing requires a charged solution for deposition and is therefore suitable for applying the Nafion layer. Cellulose acetate solutions, which are nonconducting, require the presence of a charged species, which can be provided by the inclusion of an organic salt such as tetrabutylammonium toluenesulfonate (TBATS). Dip-coating and spin-coating are not subject to this constraint and were suitable for the application of both membrane materials.

Nafion was supplied as a 5.0% (w/v) solution, dissolved in a mixture of lower aliphatic alcohols and water. It was necessary to dilute this to reduce the viscosity for printing and coating. Water was added to make a final concentration of 1% (w/v) for ink-jet printing and 0.5% (w/v) for spin-coating and dip-coating. Cellulose acetate was prepared as a 2.0% solution in acetone.

Ink-Jet Printing. A Biodot printer (Biodot, Diddington, UK) was used for all ink-jet experiments. Such a printer operates by forcing fluid under pressure through a small nozzle (75 μm). As the fluid passes through the print head, a drive rod actuated by an oscillating piezoelectric crystal produces a shock wave which breaks the jet into a series of regular droplets. This occurs at a frequency of ~64 000 Hz, dependent on the modulation voltage. Printing is achieved by charging individual droplets via a charge electrode which is controlled by a microprocessor. The printed pattern is constructed in a dot matrix format.

An array of 16 × 21 droplets of membrane solution (total printed volume, 0.47 μL) was printed onto the electrode surface such that it completely covered the active area and overlapped the insulation shroud by ~2 mm. The amount of solution was varied by repeatedly passing the electrode array under the print head.

Spin-Coating. The electrodes (individually) were mounted on a purpose-built spigot and rotated at 2000 rpm. An aliquot (10 μL) was pipetted onto the surface of the working electrode, which was left to rotate for 15 min, allowing the solvent to evaporate, leaving a thin membrane deposit. All spin-coated electrodes were prepared using this approach.

Dip-Coating. The enzyme electrode was immersed in the membrane solution and immediately removed. Following immersion, the electrodes were suspended vertically and left to dry for 30 min (allowing the solvent to evaporate).

Glucose Biosensor Test Procedure. Glucose stock solutions (0.1 M) were all prepared in 0.1 M, pH 7.0 phosphate buffer containing 0.1 M KCl. These solutions were allowed to mutarotate overnight prior to use. All glucose experiments were carried out under the conditions described earlier for hydrogen peroxide determination. pH optimization was carried out using five electrodes which were tested at 25 °C in the appropriate buffer under stirred conditions, with the working electrode potential poised at +350 mV (vs SCE). The following buffers were used:

pH 5–5.5	0.1 M sodium acetate
pH 6–7.5	0.1 M sodium phosphate
pH 8–8.9	0.1 M Tris-HCl

When the electrode had reached a steady state, a 5 mM concentration of glucose (prepared in the appropriate buffer) was added to the buffer, and the electrode response was monitored. Three measurements were made at each pH.

Table 1. Catalytic Graphite Analysis: Electrochemical Response to Hydrogen Peroxide in the Range 0–2.5 mM at 25 °C and pH 7.0

material	hydrogen peroxide response (μA/mM) and correlation coefficient (r, in parentheses) at the indicated applied potentials				
	100 mV	200 mV	300 mV	400 mV	500 mV
ETEC (10% Pt)	−23.4 (−0.9975)	13.9 (0.9995)	29.1 (0.9998)	29.3 (0.9997)	
MCA1 (5% Ni, 5% Cr)	−0.1 (−0.9165)	0.1 (0.9165)	0.8 (0.9820)	2.7 (0.9750)	5.1 (0.9979)
MCA2 (5% Pt)	−22.3 (−0.9992)	8.2 (0.9997)	33.1 (0.9937)	41.7 (0.9934)	
MCA3 (0.1% Pt)	−2.0 (−0.9943)	4.6 (0.9991)	14.8 (0.9987)	22.9 (0.9989)	
MCA4 (5% Rh)	−10.9 (−0.9961)	15.0 (0.9973)	27.3 (0.9925)	26.8 (0.9982)	
MCA5 (0.1% Rh)	no response	no response	no response	no response	0.8 (0.9586)
MCA6 (5% Ru)	−13.1 (−0.9971)	−11.2 (−0.9985)	−9.1 (−0.9991)	−0.3 (−0.9846)	52.8 (0.9976)
MCA7 (0.1% Ru)	no response	no response	no response	no response	7.6 (0.9952)
MCA8 (10% Pd)	−14.3 (−0.9994)	7.9 (0.9984)	23.5 (0.9998)	31.0 (0.9998)	
MCA9 (5% Pt, 5% Pd)	−30.4 (−0.9993)	4.4 (0.9898)	28.9 (0.9982)	44.5 (0.9985)	
MCA10 (5% Pd, 0.5% Ru)	−22.8 (−0.9990)	2.0 (0.9999)	27.4 (0.9998)	36.6 (0.9995)	
MCA11 (5% Ni, 5% Cr, 5% Pd)	−1.8 (−0.9948)	0.9 (0.9853)	3.7 (0.9935)	7.4 (0.9988)	
MCA12 (10% Ag)	no response	no response	no response	no response	no response

Interference Studies. To validate the potential use of the selected catalytic material as a suitable base for practical biosensors, interference studies were carried out by studying the direct oxidation of glucose and the electrode response in fermentation media. For these experiments, the non-enzyme electrodes were used. All experiments were carried out under the conditions described above, with the potential interferent solutions being added in place of hydrogen peroxide. The first six media investigated were sterile samples in which no cells had been grown. Following this, tests were carried out in cell-free extracts obtained during two bacterial fermentations and one yeast fermentation. The media were prepared according to the following (amounts given in grams per liter):

medium 1 (medium for yeast)	
KH_2PO_4	12
$MgSO_4 \cdot 7H_2O$	5
$FeCl_3 \cdot 6H_2O$	0.2
yeast extract	20
hycase	20

medium 2 (medium for bacteria)	
$CaCl_2 \cdot 4H_2O$	0.1
$MgCl_2 \cdot 6H_2O$	2
K_2SO_4	5
NaCl	2
KCl	10
yeast extract	20
hycase	20
K_2HPO_4	2

medium 3 (LB medium)	
bactotryptone	10
yeast extract	5
NaCl	10

medium 4 (brain–heart infusion medium)	
calf brain	1.25
beef heart infusion solids	5
proteose peptone	10
NaCl	5
dextrose	2
Na_2HPO_4	2.5

medium 5 (reinforced clostridial medium)	
yeast extract	3
lablemco powder	10
peptone	10
soluble starch	1
dextrose	5
cysteine hydrochloride	0.5
NaCl	5
sodium acetate	3
agar	0.5

medium 6 (MRS broth)	
bacteriological peptone	10
beef extract	8
yeast extract	4
Na_2HPO_4	2
sodium acetate	5
triammonium citrate	2
$MgSO_4 \cdot 4H_2O$	0.2
$MnSO_4 \cdot 4H_2O$	0.05
Tween 80	1

Fermentation Broths. Fermentations were carried out in 250 mL shake flasks, containing 50 mL of inoculated media. The organisms grown were *Escherichia coli*, *Lactobacillus plantarum* LPCO-10, and *Saccharomyces cerevisiae*.

E. coli was grown with shaking at 37 °C in nutrient broth consisting of 10 g/L beef extract, 10 g/L peptone no. 1, and 5 g/L NaCl. Samples were taken at regular intervals over a period of 7.5 h.

L. plantarum was grown in a stationary mode at 30 °C in medium 6 (outlined above). Once more, samples were taken over a 7.5 h period.

S. cerevisiae was grown in a stationary mode at 30 °C in a medium consisting of 7 g/L yeast extract, 10 g/L mycological peptone, and 20 g/L glucose. Aliquots were removed at intervals over a 24 h period.

RESULTS AND DISCUSSION

Selection of Electrocatalytic Material. The criteria used to select the catalytic material were based on (i) high current density, thus maximizing the signal; (ii) low operating potential, with a response plateau, such that small fluctuations in the applied potential have a negligible effect on precision; (iii) low interference from other electroactive compounds, a problem highlighted earlier for metal/carbon electrodes; and (iv) linear response in the working range.

Electrochemical Response to H_2O_2. The response to hydrogen peroxide over a range of concentrations between 0 and 2.5 mM was determined amperometrically for each of the electrode types over a series of applied potentials between 100 and 500 mV. Results of these measurements are summarized in Table 1. From this, it can be seen that several catalytic carbons show significant electrocatalytic activity. This is characterized by a high positive slope value at low potentials. From these data, it is apparent that the following materials exhibited the highest current densities in this range: ETEC Pt–C, MCA2, MCA3, MCA4, MCA8, MCA9 and MCA10.

The electrodes which exhibited the best plateau responses were ETEC Pt–C and MCA4, which both displayed this feature between 300 and 400 mV. Electrodes which produced a significant signal were all linear over the tested hydrogen peroxide concentration range.

Direct electrooxidation of glucose has been previously described.[16] Generally, the anodic oxidation was carried out using

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Table 2. Electrode Material Response to Hydrogen Peroxide and Glucose

material	hydrogen peroxide	glucose
ETEC	+++	++
MCA1	+	+++
MCA2	++	−
MCA3	+	+
MCA4	+++	−
MCA5	+	+
MCA6	+++	++
MCA7	++	+
MCA8	++	+
MCA9	++	−
MCA10	++	+
MCA11	++	−
MCA12	+	−

[a] −, no noticeable response; +, slight response; ++, significant response; +++, strong response.

either pure (Au, Pt) or adatom (Pb, Tl, Bi)-modified noble metal electrodes. Similar behavior has been reported at platinized carbon electrodes. The effects of direct glucose oxidation may cause problems due to electrode fouling and may complicate the biosensor kinetics, leading to a poorly defined relationship between the glucose concentration and the recorded signal. It is also likely that if glucose is electrochemically active at these electrode surfaces, then other organic compounds, such as alcohols and carbohydrates, will also exhibit this characteristic.[6] Hence, the electrodes were tested for their sensitivity to direct glucose oxidation using cyclic voltammetry. The potential was cycled between 0 and 700 mV at a scan rate of 50 mV/s in the buffer solution described above, in buffer containing 1.0 mM hydrogen peroxide, and finally in buffer containing 25.0 mM glucose. The magnitude of the response in each case, which represents a qualitative interpretation of the cyclic voltammograms, is shown in Table 2.

It is apparent from these results that MCA4 offers the best response to hydrogen peroxide with no noticeable interference from the direct oxidation of glucose. The cyclic voltammograms of the MCA4 material in buffer, buffer + hydrogen peroxide, and buffer + glucose are shown in Figure 1. As can be seen, the scans for the buffer and the buffer + glucose are virtually identical. The scan when hydrogen peroxide is present, however, clearly shows a large electrocatalytic oxidation of hydrogen peroxide. As a result of these experiments, it was concluded that MCA4 is the best of the materials tested. It offers a significant advantage, when compared with the commonly used platinized carbon material, in that it does not suffer interference from direct oxidation of reducing sugars, and hence it was used for all subsequent experiments.

Results of Interference Studies. A three-electrode amperometric cell, as described earlier, was poised with an applied potential of +350 mV. Initially, the electrodes were immersed in 20 mL of phosphate buffer (pH 7.0) under stirred conditions at 25 °C. When the electrode response had reached a steady current (~60 s), buffer was removed and replaced with the same volume of the appropriate media or fermentation sample over a dilution range between 1:20 and 1:1. Following the addition of the media sample, hydrogen peroxide was added to ensure that the operational characteristics of the electrode were unimpaired. For all of the samples tested, no significant interference was observed.

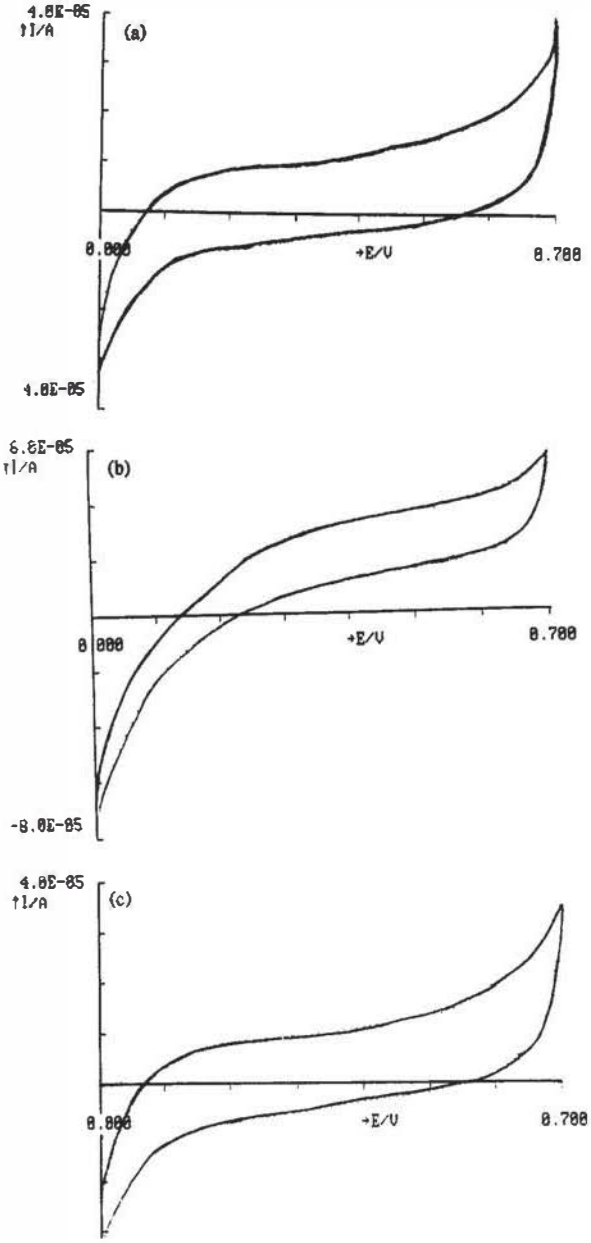

Figure 1. Cyclic voltammograms of MCA4-based electrodes. (A) Response to 0.1 M, pH 7.0 phosphate buffer. (B) Response to 1.0 M hydrogen peroxide in phosphate buffer (as in A). (C) Response to 25.0 mM glucose in phosphate buffer (as in A).

This suggests that this electrode material may be well suited to operation in complex samples, where potential electrochemical interferences may be present, such as in fermenters.

Enzyme Electrodes: Initial Membrane Test Results. Table 3 illustrates the mean responses from five of each type of electrode to a 5 mM addition of glucose. Electrode types A and B are as defined earlier. All of the electrodes fabricated using dip-coating as the method of membrane application produced very small currents and exhibited poor reproducibility.

It can be seen that type A electrodes (enzyme and catalytic powder in the same layer) produced significantly higher currents and were found to be more stable than those of type B. Furthermore, producing electrodes via this approach eliminates the need for a separate step during manufacture. Nafion-coated

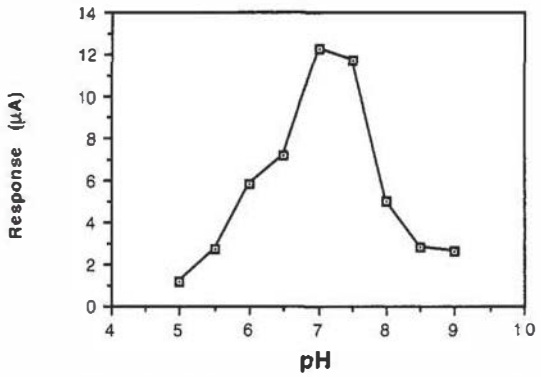

Figure 2. pH profile of glucose biosensor response at a glucose concentration of 5 mM at 25 °C under stirred conditions.

Table 3. Results of Membrane Fabrication Studies

electrode and membrane type	response to 5 mM glucose (μA) mean	SD	comments
type A/ink-jet Nafion (8 passes)	15.8	1.2	low noise
type B/ink-jet Nafion (8 passes)	4.8	0.4	noisy
type A/spin-coated cellulose acetate	10.7	1.0	noisy
type B/spin-coated cellulose acetate	1.8	0.6	very noisy
type A/spin-coated Nafion	17.7	1.2	noisy
type B/spin-coated Nafion	3.5	1.0	very noisy

electrodes produced higher currents with less noise than those coated with cellulose acetate. Ink-jet printing proved to be the most controllable method of applying the membranes. Spin-coated, Nafion-covered electrodes produced marginally higher currents, but the response was significantly noisier. From these results, it was decided that all future electrodes would be constructed in the type A format, with initial work being carried out using an ink-jet-printed Nafion membrane.

Investigation of the pH Profile. The graph shown in Figure 2 depicts the electrode response over a pH range between 5.0 and 9.0. Obviously, the optimum applied potential for hydrogen peroxide detection and the activity of the enzyme will be influenced by the pH. However, for simplicity of associated instrumentation of an analytical device based on this technology, it is useful to operate at a fixed potential. Glucose oxidase operates over a wide pH range (2.7–8.5) and the optimum is usually reported to lie between 4.8 and 6.0 for the free enzyme utilizing dioxygen as the electron acceptor.[17] Numerous publications describing glucose biosensors have described a shift in the pH optimum exhibited due to the use of artificial electron acceptors.[18] Immobilization is another factor which may alter the operating characteristics of the enzyme.[19] Hence, the observation that the optimum value is 7.0 is a function of the electrode and its mode of operation at a constant voltage and is not necessarily an indication that the behavior of the enzyme has been altered.

Calibration Curves. A series of enzyme electrodes (five of each), prepared using different amounts of Nafion (between 2.35 and 22.09 μL, calculated by multiplying the mean droplet volume

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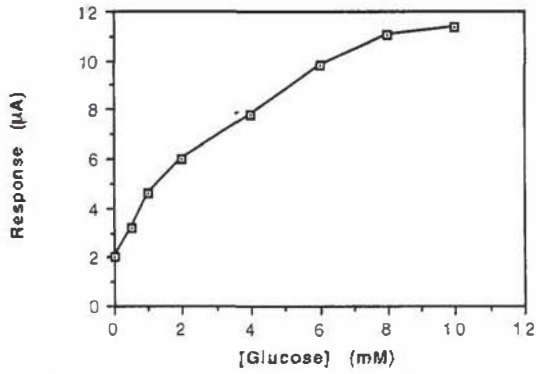

Figure 3. Typical calibration plot for a screen-printed glucose biosensor with an ink-jet-printed Nafion membrane.

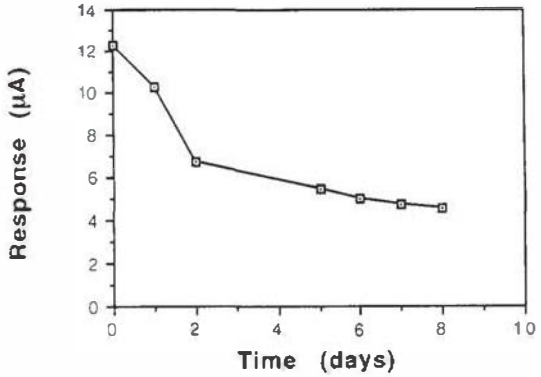

Figure 4. Biosensor stability over an 8 day period. Sensor stored dry at 4 °C between measurements. Plot depicts biosensor response to a glucose concentration of 5 mM at 25 °C under stirred conditions.

by the number of printed droplets) which was ink-jet-printed onto the sensor surface, were tested for their response to a range of glucose concentrations from 0 to 15 mM. Generally, linear responses were obtained at the lower glucose concentrations, with the linear range extending as the amount of Nafion deposited was increased. Above 22.09 μL, the current response declined significantly, indicating that the membrane thickness restricted the flux of glucose to the immobilized enzyme. A representative calibration graph is shown in Figure 3.

Stability. Using the same operating conditions as those described above (phosphate buffer, pH 7), an electrode was tested for stability over 8 days. Three electrodes were tested against a 5 mM glucose concentration daily over this time. When not in use, the electrodes were stored in buffer at 4 °C. Figure 4, which depicts the response of a typical enzyme electrode, indicates that an initial sharp decline in activity is followed by a more gradual decrease and that after 8 days, the sensor was still producing a significant response.

Nafion is a perfluorosulfonic ion-exchange membrane material which differs from conventional ion-exchange materials in that it is not a cross-linked polyelectrolyte, but a thermoplastic polymer with pendant sulfonic acid groups partially or completely neutralized to form salts. This also provides a negatively charged surface. It is believed that the membrane, by virtue of the electrostatic interaction between enzyme and membrane, is helping to retain the enzyme electrostatically as well as physically. Furthermore, this immobilization procedure may lead to a greater rigidity, enhancing physical resilience of the enzyme. Another effect may

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