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Diamond-based glucose sensors

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Abstract

A series of diamond-based glucose sensors, based on the interaction of glucose with the enzyme glucose oxidase (GOD), has been produced. For each sensor, the sensitivity to glucose was assessed and, with some devices, the range of glucose concentrations over which the sensor showed a linear response was determined. The sensing electrode formed one electrode of an electrochemical cell, and a tungsten counter electrode formed the other. All the sensors were diamond-based, with the nature of the working electrode being the distinguishing feature. The first device was a diamond–platinum–GOD sensor. However, this device was prone to interference from other electroactive chemicals in the blood such as vitamin C and acetaminophen.

To minimise the metal content of the sensors, two further sensors were produced using heavily boron doped diamond as the conducting electrode in place of the platinum. In the first case, the GOD was immobilised on to the surface of the diamond by electrochemical deposition, and in the second, the GOD was "wired" directly to the electrode by covalent bonding to the electrode surface. © 1998 Elsevier Science S.A.

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1. Introduction

According to recent statistics, there are between 100 and 120 million sufferers of diabetes (diabetes mellitus) world-wide [1], of which approximately 10% [2] are dependent on insulin therapy. Treatment of this disease involves daily blood tests to measure the blood sugar level and hence determine the appropriate dose of insulin. Currently, the blood sugar tests involve the extraction of a drop of blood and subsequent analysis by an external glucose sensor. These techniques, as well as being uncomfortable for the patient, do not provide a continuous measure of the blood sugar levels.

There is a large volume of research in the area of implantable glucose sensors [3–8]. These devices are intended to operate inside the body for either temporary or long-term placement. As such, the materials of construction in contact with the body are required to be chemically stable and biocompatible [9]. Diamond fulfils these criteria. The most promising sensor designs predominately operate by measuring a current produced by the interaction of glucose with the enzyme glucose oxidase [10].

$$\beta\text{-d-glucose} + \text{GOD (FAD)} \rightarrow \text{gluconic acid} + \text{GOD (FADH}_2\text{)} \quad (1)$$

$$\text{GOD (FADH}_2\text{)} + \text{X (oxid)} \rightarrow \text{GOD (FAD)} + \text{X (red)}. \quad (2)$$

In order for the enzyme to return to its active state, its redox centre, flavine adenine dinucleotide (FAD), must be reoxidised. The oxidant (X) is most commonly oxygen, which is reduced to hydrogen peroxide. This forms the basis of a first generation glucose sensor [10]. Alternatively, second-generation sensors involve a sequence of charge-transfer pathways [3] to the electrode surface.

Platinum, glassy carbon and carbon fibres [11] are electrode materials most commonly used in the construction of both first- and second-generation devices [4]. In first-generation sensors, the enzyme is physisorbed on

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to the electrode surface, along with a cross-linking agent to stabilise the enzyme layer [10]. Hydrogen peroxide diffuses through the sensing layer to the electrode where it is oxidised, thus generating the output current. In the second-generation sensors, the enzyme is chemically modified to contain charge transfer agents, for example ferrocene derivatives and, in some cases, is actually covalently bonded to the electrode [12]. Whilst the construction of first generation devices is simple, second-generation devices exhibit distinct advantages. In blood, there are species, for example urea and vitamin C, that are oxidised at an electrode at a lower over-potential than hydrogen peroxide, thus resulting in interference in the analysis. However, second-generation devices do not rely on the oxidation of hydrogen peroxide. Therefore, they operate at a lower potential and, as a result, the sensor is more selective. Various polymer membranes have been developed, e.g. Nafion [13], to act as a glucose or hydrogen peroxide selective barrier between the in-vivo environment and the electrode. Whilst this approach has proven successful, concern has been raised over the use of these polymer membranes, due to possible rejection reactions of the materials in the body [14].

The use of metal and amorphous carbons *in-vivo* has the potential to produce toxic particulates in the body, due to degradation reactions. Diamond is particularly well suited to this application due to its inherent biocompatibility, conductivity when doped and electrochemical stability in extreme environments [15].

In this work, we have designed and constructed four glucose sensors and investigated the feasibility of incorporating diamond-like carbon membranes. The first design (sensor A) was based on a traditional first generation sensor, with a platinum-based working electrode on a diamond substrate, with the enzyme immobilised on to an evaporated platinum electrode. In the second set of devices, the immobilised enzyme was deposited on to a boron-doped electrically conducting diamond layer (sensors B and C). The final sensor (D) was based on a second-generation glucose sensor. The diamond was chemically modified via oxidation of the surface, to provide a more chemically reactive platform for the attachment of the enzyme.

2. Chemicals

Glucose oxidase (EC 1.1.3.4) from *Aspergillus niger* (with an activity of 128 U mg^{-1}), bovine serum albumin, glutaraldehyde, and Sephadex G-15, were all purchased from Sigma Chemical Co. and used without further purification. The DEC [1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride], Na-HEPES [sodium 4-(-2-hydroxyethyl)-1-piperazineethanesulfonate] and the APTES [3-aminopropyl-triethoxysilane] were purchased from Avocado Research Chemicals Ltd. Ferrocenedicarboxylic acid and the ferrocenemonocarboxylic acid were from Lancaster Synthesis.

3. Experimental

3.1. Diamond film growth

The polycrystalline diamond films were deposited by microwave plasma [16] chemical vapour deposition. Sections of (100) p-type silicon (2 cm^2) were abraded using synthetic diamond dust (0.75–1.50 μm) for 10 min and then cleaned by a sequence of ultrasonic washes using acetone, methanol, Decon solution and then finally de-ionised water. The films were grown using a carbon monoxide (70 sccm), methane (2.5 sccm) and hydrogen (85 sccm) gas mixture at a total pressure of 45 Torr, a substrate temperature of 650 °C and at a microwave power of 800 W.

3.2. Doping of the diamond films

The as-grown diamond was selectivity implanted at 137 °C with ^{11}B from a boron trifluoride source gas at an energy of 50 keV (EMF, University of Edinburgh). The implantation energy was low to ensure a shallow implantation, with an estimated penetration depth of 125 nm. To achieve a high electrical conductivity, the sample was exposed to a dose of 5.1×10^{15} atoms cm^{-2}. From the estimated range and distribution, the concentration of boron atoms in the diamond lattice was calculated to be of the order of 1×10^{20} atoms cm^{-3}. The implanted diamond sample was annealed at 850 °C under argon (74.4 Torr) for 1 h. The surface conductivity of the diamond was measured using a standard four-point probe method. A value of 5.27×10^{-3} S was determined at 25 °C compared with the value of $< 3.6 \times 10^{-8}$ S for the undoped sample.

The stability of the implanted boron in the diamond lattice was assessed by immersing samples of doped diamond into deionised water at 37 °C for 4 weeks. At the end of this period, the concentration of B was determined to be 4 ppb, which is well below the concentration allowable, 100 ppb, in drinking water [17].

3.3. Electrochemical measurements

All the test solutions for the sensors were prepared using 0.1 M phosphate-buffered saline (PBS) solution. Current measurements were performed at a constant voltage using a Hewlett-Packard 4041B picoammeter. In each measurement, the counter electrode consisted of a tungsten probe without a reference electrode.

3.4. Construction of sensor A—first-generation sensor on a diamond substrate

The glucose sensor was fabricated using previously published enzyme immobilisation techniques [6] on a diamond substrate [18]. Firstly, a platinum working electrode was thermally evaporated through a mask on to the substrate (2×1 cm^2). The platinum was then electrochemically oxidised with 2.5% potassium dichromate in 10% nitric acid solution, at a potential of 2.5 V for 10 min. The oxidised electrodes was then treated with APTES in a 20% solution in toluene at 80 °C and then with a 2% solution of tetrachloro-*p*-quinone in toluene at 40 °C. The glucose oxidase was immobilised on to the modified electrode, by placing a few drops of GOD in phosphate buffer solution on to the electrode for 2 h at room temperature. The working electrode was rinsed in sodium chloride solutions to remove any unimmobilised GOD. The sensor performance was assessed using buffered glucose solutions from 10 to 20 mM at a potential of 0.7 V.

Five of the platinum electrodes were prepared by evaporation on to sections of undoped diamond. The platinum was then electrochemically oxidised under the conditions previously described. Four of the electrodes were coated with DLC of various thicknesses in the range 10–250 Å. The DLC was deposited in an r.f. plasma reactor (30 W) using a methane (45 sccm) and argon (5 sccm) gas mixture at a total pressure of 100 mTorr, a temperature of 20 °C and at a bias voltage of −195 V. The average growth rate was 94 Å min^{-1}. The effectiveness of the membrane action of the DLC was assessed by measuring the current produced with the interferent chemicals listed earlier and hydrogen peroxide solution (15 mM) at a potential of 0.8 V.

3.5. Sensors B and C—minimum metal content

Two designs of sensor were compared. In the first case, the working electrode consisted of boron-doped diamond. The second sensor comprised doped diamond patterned with an array of platinum dots 1 mm in diameter. In both sensors, the enzyme was immobilised on to the electrode surface as follows. Firstly, a solution of 5% by weight solution of GOD and bovine serum albumin (BSA) was prepared in phosphate buffer saline (pH 7.0). The electrodes were immersed into the solution and biased at 3.5 V for 2 h. This voltage gave the maximum current density at the electrode before the solution started to foam and denature the enzyme. The electrodes were removed from the solution and rinsed quickly in DI water, before being immersed, secondly, into a 25% solution of glutaraldehyde (GA) in phosphate-buffered saline for 45 min. The electrodes were then rinsed once again in DI water and left to dry in air. In the first step of the immobilisation process, the GOD and BSA were dissolved in a buffer with a pH greater than their isoelectric points. Therefore, the negatively charged proteins (GOD and BSA) were drawn to the positively charged electrode [19]. In the second step, the GA cross-links the protein layer forming a water insoluble barrier.

Testing of the sensors was carried out at a potential of 0.7 V using buffered glucose solutions of varying concentration (1–13 mM). The normal blood sugar concentration lies between 3 and 8 mM. The current was measured with time for each concentration, as well as for the control buffer solution.

3.6. Preparation of sensor D—second-generation sensor based on diamond

The diamond surface was chemically modified in the following manner. A section of annealed boron doped diamond was heated in air at 600 °C for 10 min to oxidise the surface of the film and subsequently examined using Fourier Transform infra-red spectroscopy (FTIR). The diamond was then heated to 111 °C in a 20% v/v solution of APTES in toluene for 8 h. Following the treatment with APTES, the film was rinsed with hot (60 °C) toluene, and then immersed in toluene in an ultrasonic bath for 1 h. Following the latter treatment, the silanised film was examined by FTIR to monitor the resulting surface modification.

Ferrocenedicarboxylic acid (137 mg) and DEC (96 mg) was dissolved in 6 ml of 0.1 M Na–HEPES. The silanised diamond film was immersed in this solution for 20 h at room temperature. The film was then soaked in deionised water for 5 days and then placed in fresh deionised water in an ultrasonic bath for 1 h.

The glucose oxidase was modified using a procedure adapted from Heller et al. [20]. Ferrocenemonocarboxylic acid (120 mg) was dissolved in 4 ml of 0.15 M Na–HEPES solution. The solution was cooled to 0 °C, and its pH adjusted to 7.2 using 2 M HCl. To this turbid solution, DEC (149 mg) was added, followed by urea (1.21 g), and the pH was maintained at 7.1 ± 0.1 by the addition of either HCl or Na–HEPES. Glucose oxidase (88 mg) was added, and the solution was stirred at 0 °C for 2 h and then left in a sealed container for 20 h at 2 °C. The suspension was centrifuged, and the supernatant liquid was passed through a 0.45-μm filter. The orange liquid was then further purified through a column of Sephadex G-15, which had been soaked in 0.085 M sodium hydrogen phosphate solution (pH adjusted to 7 by conc. HCl). The modified enzyme was eluted in the first orange fraction.

The modified enzyme was attached to the diamond surface as follows. Modified glucose oxidase in 1 ml of phosphate buffer was mixed with DEC (22 mg) and urea (180 mg) and cooled to 0 °C. The modified dia-

mond film was immersed in the latter solution at 3 °C for 21 h and subsequently rinsed in deionised water.

The sensor was tested using 6.1 mM buffered glucose solution and PBS solution. The current was measured at a variety of voltages between 0.4 and 0.6 V. All the measurements were made in a nitrogen atmosphere.

4. Results

4.1. Sensor A—first-generation sensor on diamond film support

It can be seen in Fig. 1 that the sensor current was linearly related to the glucose concentration in the range 10–20 mM. The sensitivity of this device was low (0.8 nA mM^{-1}), compared to other published data [8,21]. The low output and sensitivity could be due to the shape of the electrode and poor enzyme loading.

Sensors incorporating the DLC membranes reduced the measured current in the presence of the listed interferent chemicals. However, since the relative response as a function of DLC film thickness varied with the particular molecule, it was concluded that the DLC was not acting as a inert membrane. Further work is in progress to establish the electrochemical stability of the material.

4.2. Sensors B and C

Sensor B, which is a metal free device, initially showed a high sensitivity to glucose (for 8 mM glucose, I = 1.6 μA) compared to buffer solution (I = 0.1 μA). However, after about an hour's usage, the current rapidly dropped and became unstable. This failure was accompanied by the visual formation of a surface film on the diamond. However, there was no obvious difference between the surface structure of the surface film and the doped diamond when viewed by scanning electron microscopy (SEM). The surface conductivity of the film was measured using the four-point probe technique; the conductivity had decreased from 5.27×10^{-3} S to 7.86×10^{-5} S within this area. Experiments were performed in an attempt to explain the phenomenon. Firstly, the sensor was biased at 0.7 V in the presence of the each component (in deionised water), i.e. buffer solution, hydrogen peroxide, gluconic acid, and glucose. The concentrations of these solutions were at a level comparable to what they would be at during the normal operation of the sensor in 10 mM glucose solution. However, none of these chemicals alone caused the formation of the insulating film, but when the sensor was operated under normal conditions, the surface film was formed.

The surface composition of the insulating layer was examined by laser ionisation mass analysis (LIMA). The negative-ion LIMA spectra, from both the insulating and the conductive regions, showed the characteristic peak pattern [22] resulting from carbon atom clusters and phosphate groups from the enzyme of on the surface. There were no discernible differences between the spectra. The positive ion spectra for the same regions contained peaks due to sodium and boron and, as with the negative-ion spectra, there were no significant differences between the spectra.

Sensor C, the minimum metal content device, showed a strong and repeatable response to glucose compared to sensor B. A typical current versus time plot can be seen in the inset of Fig. 2. There is sharp drop off in current initially, which is a characteristic of the enzyme immobilisation technique [23]. Subsequently, there is a gradual increase in current with time. It was observed that the transition point, where the gradient changes from negative to positive, occurs at a time that is characteristic of the glucose concentration. A linear correlation between transition time and concentration can be seen in Fig. 2. The sensitivity of the device was calculated to be 10.5 s mM^{-1}.

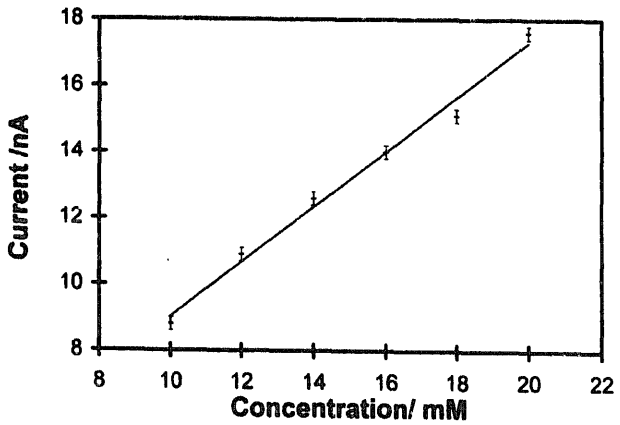

Fig. 1. Dependence of the current from sensor A on the concentration of glucose in buffered aqueous solution.

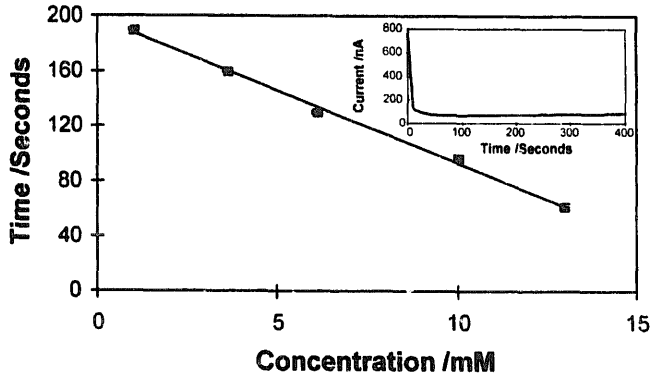

Fig. 2. Graph of transition time versus concentration of the glucose solution for sensor C. In the absence of glucose the current continuously decreased (up to 900 s). Inset: a typical current versus time plot for a 10 mM glucose solution.

In the minimal metal sensor, electrochemical oxidation of the hydrogen peroxide occurs at the platinum electrode. In the metal-free design, there is no platinum to aid the oxidation of the peroxide, so the formation of the insulating film might be due to the build-up of hydrogen peroxide at the diamond surface. The change in the appearance of the surface within the insulating area could be a result of corrosion of the diamond as a scale that could not be observed by SEM. It should be noted that there was no formation of an insulating surface film on the doped diamond between the platinum dots. As yet, the nature of the surface film in sensor B is not understood, and it has not been proven possible to replace the platinum electrode entirely with boron doped diamond. However, the amount of platinum necessary for the operation of the sensor can be minimised by depositing a matrix of platinum dots on to boron-doped diamond substrate.

4.3. Results from sensor D—the second-generation device

The diamond surface was thermally oxidised so that it was terminated by an oxide layer, which is more chemically active than the hydrogen terminated surface. This termination was confirmed by reflectance FTIR, a sharp peak at 1070 cm^{-1}, that can be assigned to a C–O stretch, was observed in the spectrum of the oxidised film. The oxide surface was reacted with APTES at 111 °C. The presence of amine terminal groups was confirmed by reflectance FTIR, peaks in the spectrum corresponding to -NH_2 (3400–3200 cm^{-1} N–H stretch, 1300 cm^{-1} C-N stretch) were observed in the treated films. This amine terminated surface was then reacted with ferrocenedicarboxylic acid using a coupling agent, DEC, to form the amide linkage and *O*-acylisourea.

Glucose oxidase is a large biomolecule, nearly 86 Å in diameter [24]. The redox centres—the coenzyme FAD units—are buried deep in the centre. As a result, direct electrical communication between the FAD centres and the working electrode is impossible. The insertion into the enzyme structure of electron relay sites, ferrocenemonocarboxylic acid, enables the charge to reach the electrode. The redox couple of ferrocenecarboxylate/ferrociniumcarboxylate occurs at a standard potential of 0.51 V (versus SHE) [25]. This altered diamond surface was then reacted with the modified enzyme in a similar manner to form another amide linkage between the free carboxylic acid group attached via ferrocenedicarboxylic acid to the diamond surface and an NH_2 residue in the GOD.

The sensor's output current was measured over the region 0.4–0.6 V, which encompasses the redox couple of the ferrocene complex. All the measurements were performed in a nitrogen environment. The results (Fig. 3) show that the sensor's optimum operating voltage in the presence of glucose lies above 0.5 V. This confirms that the electronic pathway taken by the electrons is via the ferrocene charge transfer centres and not via the oxidation of hydrogen peroxide.

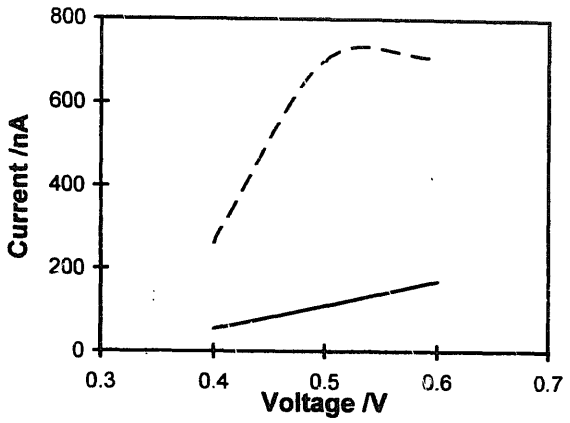

Fig. 3. Output of sensor D for phosphate-buffered saline (PBS) (——) and 6.1 mM glucose (- - -) in PBS.

5. Conclusions

Various designs of glucose sensors have been investigated. Whilst sensors A, B and C are easily fabricated, they will be susceptible to adverse reactions with interferents in the blood stream due to their reliance on the oxidation of hydrogen peroxide. In sensor D, we have managed to modify the diamond surface chemically to allow the attachment of a large biomolecule, and produce a glucose sensor based on mediated electron transfer from the enzyme to the electrode. The possible use of DLC as an interferent barrier has been examined, and the results have proved to be inconclusive. Finally, we have shown that diamond can be used in glucose sensors both as a substrate for the working metallised electrodes and, when doped, as a working electrode.

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