Biosensors and Bioelectronics 42 (2013) 668–677

Contents lists available at SciVerse ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Microfabricated glucose biosensor for culture well operation ☆

R.M. Pemberton [a], T. Cox [b,1], R. Tuffin [b], I. Sage [b], G.A. Drago [c], N. Biddle [c], J. Griffiths [d], R. Pittson [e], G. Johnson [d], J. Xu [a], S.K. Jackson [a,2], G. Kenna [f], R. Luxton [g], J.P. Hart [a,*]

[a] Centre for Research in Biosciences, Faculty of Health and Life Sciences, University of the West of England, Bristol, UK
[b] QinetiQ Ltd., Malvern Technology Centre, Malvern, UK
[c] Applied Enzyme Technology Ltd., Monmouth House, Mamhilad Park, Pontypool, NP4 OHZ, UK
[d] Uniscan Instruments Ltd., Sigma House, Burlow Rd., Buxton, Derbyshire, SK17 9JB, UK
[e] Gwent Electronic Materials Ltd., Monmouth House, Mamhilad Park, Pontypool, NP4 OHZ, UK
[f] AstraZeneca R&D, Alderley Park, Macclesfield, UK
[g] Institute of Biosensing Technology, University of the West of England, Bristol, UK

ARTICLE INFO

Article history:
Received 12 April 2012
Received in revised form
26 June 2012
Accepted 26 November 2012
Available online 6 December 2012

Keywords:
Glucose microbiosensor
Water-based screen-printing ink
96-Well format
Cell culture

ABSTRACT

A water-based carbon screen-printing ink formulation, containing the redox mediator cobalt phthalocyanine (CoPC) and the enzyme glucose oxidase (GOx), was investigated for its suitability to fabricate glucose microbiosensors in a 96-well microplate format: (1) the biosensor ink was dip-coated onto a platinum (Pt) wire electrode, leading to satisfactory amperometric performance; (2) the ink was deposited onto the surface of a series of Pt microelectrodes (10–500 μm diameter) fabricated on a silicon substrate using MEMS (microelectromechanical systems) microfabrication techniques: capillary deposition proved to be successful; a Pt microdisc electrode of ≥ 100 μm was required for optimum biosensor performance; (3) MEMS processing was used to fabricate suitably sized metal (Pt) tracks and pads onto a silicon 96 well format base chip, and the glucose biosensor ink was screen-printed onto these pads to create glucose microbiosensors. When formed into microwells, using a 340 μl volume of buffer, the microbiosensors produced steady-state amperometric responses which showed linearity up to 5 mM glucose (CV=6% for $n=5$ biosensors). When coated, using an optimised protocol, with collagen in order to aid cell adhesion, the biosensors continued to show satisfactory performance in culture medium (linear range to 2 mM, dynamic range to 7 mM, CV=5.7% for $n=4$ biosensors). Finally, the operation of these collagen-coated microbiosensors, in 5-well 96-well format microwells, was tested using a 5-channel multipotentiostat. A relationship between amperometric response due to glucose, and cell number in the microwells, was observed. These results indicate that microphotolithography and screen-printing techniques can be combined successfully to produce microbiosensors capable of monitoring glucose metabolism in 96 well format cell cultures. The potential application areas for these microbiosensors are discussed.

1. Introduction

The development of miniaturised enzyme biosensors is desirable for the analysis of small sample volumes, for example in multiple array form, and for lab-on-chip applications in various formats. Several technological approaches have been used in attempts to integrate electrochemical glucose biosensors into small easy-to-use assay formats: Wang et al. (2007) have describeda miniaturised capillary electrophoresis microchip device for blood analysis which includes a 300 μm-wide screen-printed gold-coated carbon glucose biosensor suitable for use in the microfluidic system. Two recent publications from groups working on paper-based microfluidic electrochemical systems have described screen-printable biosensor devices capable of quantifying glucose in serum (Dungchai et al., 2009) and in aqueous solution (Nie et al., 2010) for low-cost batch-mode analysis at low cost. More recently, Li et al. (2010) described a lab-on-a-tube approach which used electrochemically-deposited Pt nanoparticles modified with Prussian Blue to form small electrochemical glucose biosensors which operated in chronoamperometric mode for serum samples.

Besides the advantage of small sample volume requirement seen with these flowing-stream, or single time-point devices,

Abbreviations: SPCE, Screen-printed carbon electrode; CoPC, Cobalt phthalocyanine; GOx, Glucose oxidase; Pt, Platinum; MEMS, Microelectromechanical systems

☆This paper was presented at the World Congress on Biosensors 2012.

* Corresponding author. Tel.: +44 117 3282469; fax: +44 1173282904.
E-mail address: John.Hart@uwe.ac.uk (J.P. Hart).
[1] Current address: Institute of Biosensing Technology, University of the West of England, Bristol, UK.
[2] Current address: Centre for Research in Translational Biomedicine, School of Biomedical and Biological Sciences, University of Plymouth, PL4 8AA, UK.

0956-5663/$ - see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bios.2012.11.032

there is a demand in cell culture assay applications (for instance, drug toxicity testing) for biosensors that can monitor metabolite levels continuously, even in unstirred solutions. These microbiosensors need to be of sufficiently small dimension that they exhibit radial diffusional behaviour and therefore produce a sustained steady-state current responses in the presence of a given analyte concentration; also they do not perturb the system by consuming the glucose in the culture medium. Our previous work on developing screen-printable water-based carbon inks containing both redox mediator (cobalt phthalocyanine; CoPC) and enzyme (glucose oxidase; GOx) showed that glucose biosensors could be screen-printed as thick-film macroelectrodes (Crouch et al., 2005a) and then cut transversely to expose a microband electrode surface (Pemberton et al., 2009a) which exhibited steady-state current behaviour. When suspended in 3 ml culture wells, these microband biosensors could be used to make continuous amperometric measurements in tissue culture medium (Pemberton et al., 2009b) and could monitor changes in the uptake of glucose by mammalian cells in the presence of selected toxins (Pemberton et al., 2011). This system was suitable for small-scale laboratory investigations but impractical for rapid, multiple well analyses. Therefore, with the aim of progressing towards a more integrated biosensor assay system, the work described in this paper addresses the challenge of fabricating glucose microbiosensors that exhibit stir-independent microelectrode behaviour and are compatible with 96-well plate single- or multi-well usage, to monitor metabolic events continuously over 24 h in cell culture.

The approach taken here was to use MEMS technology to create a microfabricated substrate with exposed Pt microelectrode areas and then to functionalise these areas to convert them into working (GOx enzyme+mediator), reference (Ag/AgCl) and counter (bare Pt) electrodes. The working electrode modification was based on screen-printing the previously demonstrated water-based carbon ink containing GOx and CoPC mediator. Since this would be screen-printed onto Pt, some preliminary tests for Pt/ink compatibility were carried out using a dip-coated Pt wire electrode, and Pt microdisks of various diameters, onto which the ink was deposited. The challenge of aligning the screen-printed inks onto microfabricated substrates and their formation into microwells was then addressed. The effect of applying a collagen surface coating to the sensors was investigated by testing the amperometric response of the sensors to glucose. Finally, the operation of these microbiosensors, in 96-well plate microwells, was performed amperometrically in the presence or absence of mammalian cells growing in glucose-containing culture medium.

2. Materials and methods

2.1. Reagents

All reagents were AnalaR Grade. Phosphate buffer supporting electrolyte was prepared in deionised water (Purite R200 system, Purite Ltd., UK) by mixing solutions of NaH_2PO_4 and Na_2HPO_4 to obtain the desired pH then diluting to a concentration of 0.05 M. Type I collagen, from rat tail tendon, was purchased from Sigma-Aldrich (Cat no. C7661). Dulbecco's Modified Eagles Medium (DMEM) containing high (25 mM) glucose (4.5 g L^{-1}) or low (5 mM) glucose (1.0 g L^{-1}), and Hepatocyte medium (glucose-free) were purchased from Sigma-Aldrich, as were foetal bovine serum (FBS), penicillin and streptomycin sulphate solution (pen+strep), and non-essential amino acids. L-glutamine was obtained from GibcoBRL. Standard solutions of β-D-glucose were prepared by mutorotation overnight, and filter sterilised as described previously (Pemberton et al., 2011).

2.2. Biosensor fabrication

The substrate material for preparing microfabricated structures was 0.5 mm-thick silicon, in the form of 11.5×11.5 mm^2 squares (for fabricating microdisks) or 16.5×71.0 mm^2 rectangles (for 5-well microelectrode strips). A process of chemical etching was used to fabricate tracks and pads of Pt onto these chips; a 10 μm-thick dielectric layer of SU8 polymer was then added as an insulating layer, leaving exposed microelectrode regions of Pt pads, suitable for use as counter electrodes, or as base pads for subsequent deposition of active layers to form working or reference electrodes.

Working electrodes were formed from a water-based carbon ink formulation (GEM product code C2041124D3) (Crouch et al., 2005b) which contained cobalt phthalocyanine as redox mediator, plus the enzyme GOx. This ink was dip-coated, drop-coated or screen-printed, as described below. Ag/AgCl reference electrode strips (macroelectrodes) were formed by screen-printing onto PVC substrate (see below). Ag/AgCl microelectrodes were formed by electroplating silver directly onto the Pt pads. The Ag surface was then chloridised by anodisation in constant current mode (20 mA/cm^2) for 30 s in 1 M HCl. Finally the electrodes were rinsed in deionised water.

2.3. Instrumentation

Cyclic voltammetry and amperometry at single working (Pt- or biosensor-) electrodes were conducted using a μAutolab Type II Potentiostat (Windsor Scientific UK) in 3-electrode system mode. Multiple 96-well-based glucose microbiosensors were tested individually or simultaneously in amperometric mode: for individual testing, contact between the three electrode tracks and a μAutolab potentiostat were made using gold alloy probes held in manual probe arms (Wentworth Labs Inc., Brookfield, CT, USA), whereas multiple simultaneous parallel measurements were made using a 5-channel PG580RM potentiostat (Uniscan Instruments Ltd., Buxton, UK) contacted via a single gold edge (zebra) connector to all five biosensors and their respective counter and reference electrodes; this was controlled using UiEChem software (Version 2.02) via a custom-made connector box (Uniscan Instruments Ltd.). In order to maintain well temperature at 37 °C, the strips were placed in a non-humidified incubator at 37 °C (culture medium experiments).

2.4. Culture well construction

Once the biosensors had been fabricated on the 5-sensor chip bases, wells were formed by attaching strips of 96-well bottomless 5-well strips (Greiner Bio-one) using 0.5 mm-thick press-to-seal 6.5-mm diameter hole silicone adhesive strips (Grace Bio-Labs, OR, USA).

2.5. Collagen coating of biosensor electrodes

Collagen was added to 0.02 N acetic acid, pH 2.65, to a concentration of 50, 100, 1000 or 2000 μg/ml and left on ice for 1 h to dissolve. Ten microlitre aliquots were then deposited onto the surface of 3×3 mm^2 glucose biosensor screen-printed macroelectrodes and their corresponding Ag/AgCl reference electrodes (Crouch et al., 2005b), or over the base of microwells containing screen-printed microbiosensors, Ag/AgCl reference and Pt counter electrodes. When these coatings were evaporated to dryness at room temperature (RT), and rinsed with phosphate buffer, SEM examination (not shown) revealed that detail of the underlying carbon electrode surface was visible at concentrations below

1000 μg/ml, but was covered by an opaque coating at the higher concentrations.

The performance of the biosensors was first assessed on macroelectrodes using standard amperometry in stirred solution over the glucose concentration range 1–10 mM. Initial investigation showed that for all collagen concentrations, compared with uncoated biosensors, there was a 5 to 15% reduction in the current response; this reduction appeared independent of coating concentration over the range 50–2000 μg/ml collagen. On this basis, a collagen solution concentration of 1 mg/ml was used for further investigations. In order to optimise the coating procedure for maximum reproducibility, several protocols were tested: biosensors were untreated, or were coated with 10 μl of acetate buffer only, or buffer containing collagen under three sets of conditions: (i) evaporation to dryness at 25 °C (this took up to 2 h), (ii) incubation for 2 h at room temperature (21 °C) in a moist box and (iii) incubation for 2 h at 37 °C in a moist box. Electrodes were rinsed with phosphate buffer following all treatments and were then tested for their amperometric responsiveness to glucose in phosphate buffer.

2.6. Cell culture

Human hepatocytes (HepG2 cell line) were cultured in high glucose DMEM/10% FBS/100 U ml^{-1} pen+100 μg ml^{-1} strep/1% non-essential amino acids/200 nM L-glutamine in 75 cm^2 flasks as described elsewhere (Pemberton et al., 2011). Confluent cells were trypsinised, washed with full medium, counted by haemocytometer, then resuspended in low glucose DMEM/5% FBS and placed into 96-well plates containing biosensor electrodes as described.

2.7. Electrochemical measurements

Single working biosensor electrodes on PVC or Si chips were contacted via gold edge-connectors and placed into a 10 ml bulk solution containing buffer or potassium ferrocyanide, together with a 0.5 mm diameter Pt wire counter electrode and a 2 mm × 8 mm screen-printed Ag/AgCl reference electrode PVC strip (Gwent Electronic Materials Ltd., code number: C61003P7). Cyclic voltammetry was performed in ferrocyanide solution from an initial applied potential of -0.2 V vs Ag/AgCl to a switching potential of +1.0 V. Amperometric measurements were made at a fixed applied potential in bulk 10 ml volumes of ferrocyanide, or in microwells containing 340 μl of buffer or culture medium. In order to perform calibration studies involving serial additions of glucose in microwells, half of the well volume was removed and replaced on each occasion with a more concentrated glucose solution.

2.8. Electron microscopy

Biosensor electrodes were rinsed 5X with phosphate buffered saline (PBS) and placed into fresh 96-well plate wells. Each well received 250 μl of a 4% glutaraldehyde (TAAB Laboratories, Aldermaston,UK) solution in PBS for 24 h at 4 °C to fix the samples. Samples were then rinsed 3X in PBS over a 72 h period, dehydrated using a graded ethanol series (20, 30, 50, 70, 80 and 100%), then transferred sequentially through mixtures of ethanol and hexamethyldisilazane (HMDS; Sigma-Aldrich) in ratios 2: 1, 1: 1 and 1: 2, respectively. After a final rinsing in HMDS, electrodes were mounted onto stubs and sputter-coated with gold. They were examined using a XL30 Environmental Scanning Electron Microscope (ESEM; Philips-FEI, Eindhoven, The Netherlands).

3. Results and discussion

The following sections describe the results of investigations into the compatibility of the solvent-free glucose biosensor ink with an underlying Pt substrate in the form of a Pt wire, followed by the results of attempts to make glucose microbiosensors based on the deposition of ink onto Pt electrodes in the form of (i) microdiscs and (ii) pads compatible with a 96-well format.

3.1. Pt wire biosensor

.The compatibility of the water-based carbon CoPC/GOx ink with a Pt substrate was investigated by dip-coating a 0.5 mm diameter Pt wire (5 mm exposed length) in the ink, allowing it to air-dry (1 h at room temperature) and then using it as a working glucose microbiosensor in a 3-electrode system; the amperometric response to glucose was tested in stirred buffer solution. The resulting amperogram and calibration plot (see Supplementary Data, Fig. 1) illustrate the extremely good performance of the resulting biosensor which gave a linear response (R^2=0.998) up to 3.5 mM glucose and a detection limit of less than 0.35 mM. The response of this biosensor was not fully stir-independent, but did display microelectrode-like behaviour since a steady-state current response was obtained when the stirrer was turned off. This rather large format electrode, though unsuitable for large-scale production or of suitable dimensions for cell culture microwell application, illustrated that the carbon CoPC/GOx ink could be deposited onto a Pt surface and function successfully as a glucose biosensor at an E_{app} of +0.4 V.

3.2. Microbiosensor based on Pt microdiscs

3.2.1. Fabrication of Pt microdisc base electrodes

In order to create Pt microelectrodes which could form base pads for deposition of the carbon-based working electrode glucose biosensor ink, the photolithographic etching method, based on SU8 as the insulation layer, was utilised to pattern a silicon chip (Fig. 1) bearing eight Pt microelectrode discs having a range of diameters. Cyclic voltammetric and amperometric investigations were performed in order to test the electrochemical behaviour of the fabricated microelectrodes.

Cyclic voltammetry of ferrocyanide solution at platinum microdisc electrodes of various diameters resulted in sigmoidal voltammograms, characteristic of microelectrode behaviour (see Supplementary Data, Fig. 2A). For electrode diameters up to 75 μm a single oxidation wave was evident at an E_p of around +0.3 V, followed by steady-state behaviour. In the CVs obtained at the larger diameter electrodes (100, 200, 300 and 500 μm), an increase in the oxidation current was evident at around +0.7 V, possibly due to contamination resulting from a partial breakdown of the UV-irradiated dielectric layer. However, this was not a concern for biosensor applications since it was well above the E_{app} that would be required to oxidise H_2O_2 at the CoPC-mediated biosensor electrodes. As anticipated for microdisc electrodes, where the diffusion current is directly proportional to the radius, ie. $I_{lim}=4nFD[C]_{bulk}r$ (Saito, 1968), a plot of current response at +0.3 V (ie. oxidation of ferrocyanide) against electrode radius (Supplementary Data, Fig. 2B) was linear (R^2=0.996) indicating that the Pt microelectrodes were predictable in their electrochemical performance.

Amperometry (results not shown) was also carried out at +0.4 V in the presence of ferrocyanide solution for each of the eight Pt microelectrodes on a fresh Si chip. Steady-state responses were obtained for electrodes of diameters down to 25 μm diameter. The 10 μm diameter electrodes gave noisy and less reliable responses and some failed to respond completely. The calibration plot resulting from microelectrode current responses showed good

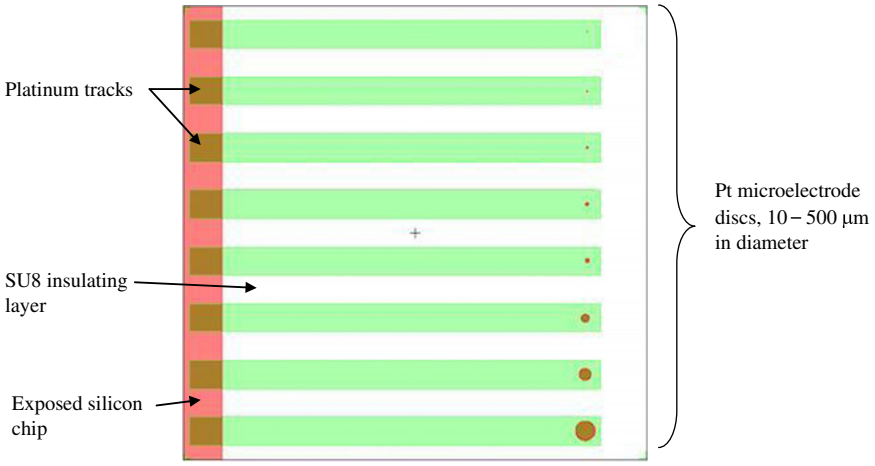

Fig. 1. Silicon chip bearing insulated tracks with exposed Pt microdiscs of diameter: 10, 25, 50, 75, 100, 200, 300 and 500 μm.

linearity (R^2=0.994) between 50 and 500 μm in diameter. These studies demonstrated that this fabrication method produced Pt pads displaying predictable electrochemical behaviour suitable for their intended use as the basis transducers for microbiosensors.

3.2.2. Deposition of ink on Pt microdiscs

In order to fabricate a microbiosensor, several approaches were investigated involving deposition of carbon CoPC/GOx ink onto the surface of the Pt microdiscs from a fine capillary tube or micro-pipette tip. The most successful approach involved loading a fine plastic 1–10 μl PL10 pipette tip (Barky Instruments International, Folkestone, UK) with ink by capillary uptake into a clamped tip; the Pt disc microdisc electrode chip was placed on a lab-jack and raised until contact was made with the end of the tip; this facilitated the transfer of a small drop of ink from the tip onto the appropriate electrode surface as the chip was lowered. This manual approach allowed deposition of around 1 μl of ink onto Pt microdiscs of diameters from 50 to 500 μm. These Si chip microbiosensors were then tested for their response to glucose by amperometry in stirred buffer solution at an E_{app} of +0.4 V. Initial experiments revealed that when the ink was allowed to dry for 2 h at RT and tested the same day or after overnight storage, responses were unreliable, noisy and detachment of ink from the Pt surface was observed after 5 min in solution. However, if biosensors were used fresh, or were stored immediately at 4 °C, reliable results were obtained. An amperogram obtained using an ink-coated 500 μm Pt microdisc biosensor is shown in Fig. 2A. The resulting calibration plot is shown (inset), together with plots for microdiscs of 50 and 100 μm diameter. A working range up to at least 12 mM was obtained for each biosensor and responses increased with increasing diameter (y=7x), suggesting that more contact between the Pt microdisc and active GOx enzyme/conductive carbon ink was influencing the current flux; it was also possible that the active surface area of carbon was slightly increased since the size of the deposited ink drop was not tightly controlled. When the stirrer was turned off, the behaviour of the current responses of the ink-disc biosensors, monitored over the ensuing 15 mins, varied in magnitude and rate of decay. The magnitudes of these currents after 15 mins could be ordered according to Pt microdisc diameter (Fig. 2B) and showed the greatest increase between 50 and 100 μm diameter, with smaller relative increases between 100 and 500 μm. The rates of current decrease of these currents, when calculated for each biosensor as a measure of their steady-state nature, showed that there was a clear influence of Pt microdisc diameter: the smaller diameter electrodes (50 and 100 μm) maintained the nearest

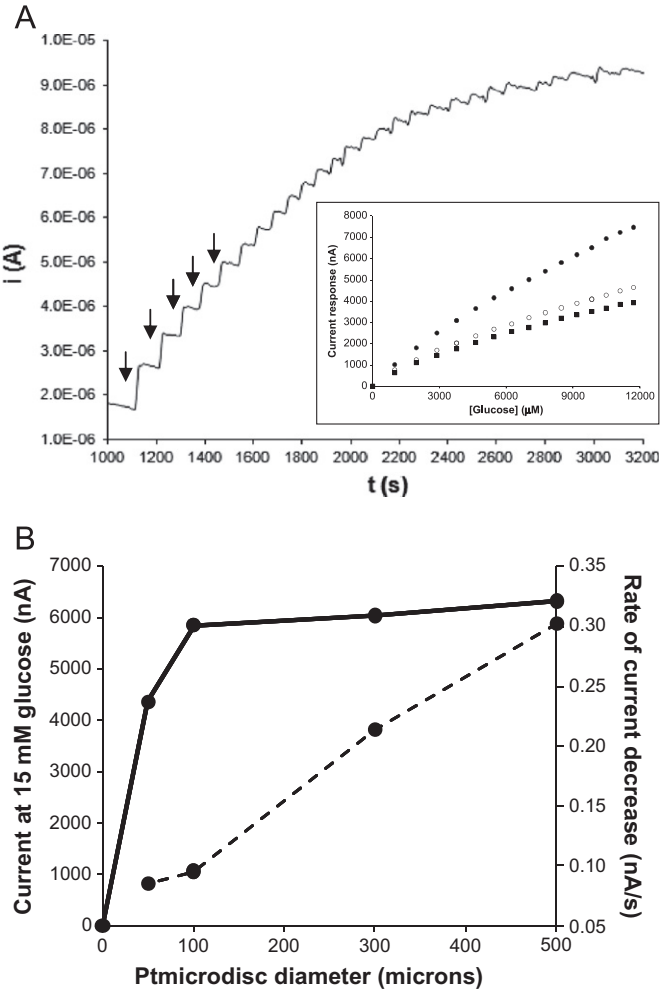

Fig. 2. (A) Amperogram obtained for 200 μl (arrows show first 5) additions of 50 mM glucose to a carbon CoPC/GOD ink-coated Pt microdisc biosensor (500 μm diam) in 10 ml stirred 0.05 M Na+/K+ phosphate buffer, pH 7.5 at 25 °C; E_{app}=+0.4 V; inset shows calibration plots for biosensors of microdisc diameter 500 (●), 100 (○) and 50 (■) μm; (B) Plot of amperometric current (solid line) and rate of current decrease (dashed line) versus microdisc diameter for biosensors in unstirred glucose (15 mM) solution.

steady-state responses, which then became less steady-state as diameters were increased to 300 and 500 μm (Fig. 2B). These studies demonstrate the compatibility of the water-based

biosensor ink with an underlying Pt base electrode and show that it is possible to fabricate a biosensor that demonstrates microelectrode behaviour. It appears to be possible to obtain reasonable control over the microelectrode behaviour, and therefore the analytical applicability of the resulting biosensor, by controlling the size of the Pt substrate and resulting ink deposit. However, at this stage, we decided to investigate an alternative approach to depositing the biosensor ink in a more controlled manner.

3.3. Screen-printed 96-well format microbiosensors

3.3.1. Screen-printing of ink onto Pt pads

The goal of obtaining good repeatability and automation of the process of microbiosensor fabrication led us to consider the use of screen-printing for the glucose working electrode. The screen-printing process is suitable for deposition of suitably formulated carbon printing inks at a resolution of about 50 μm. By design of a

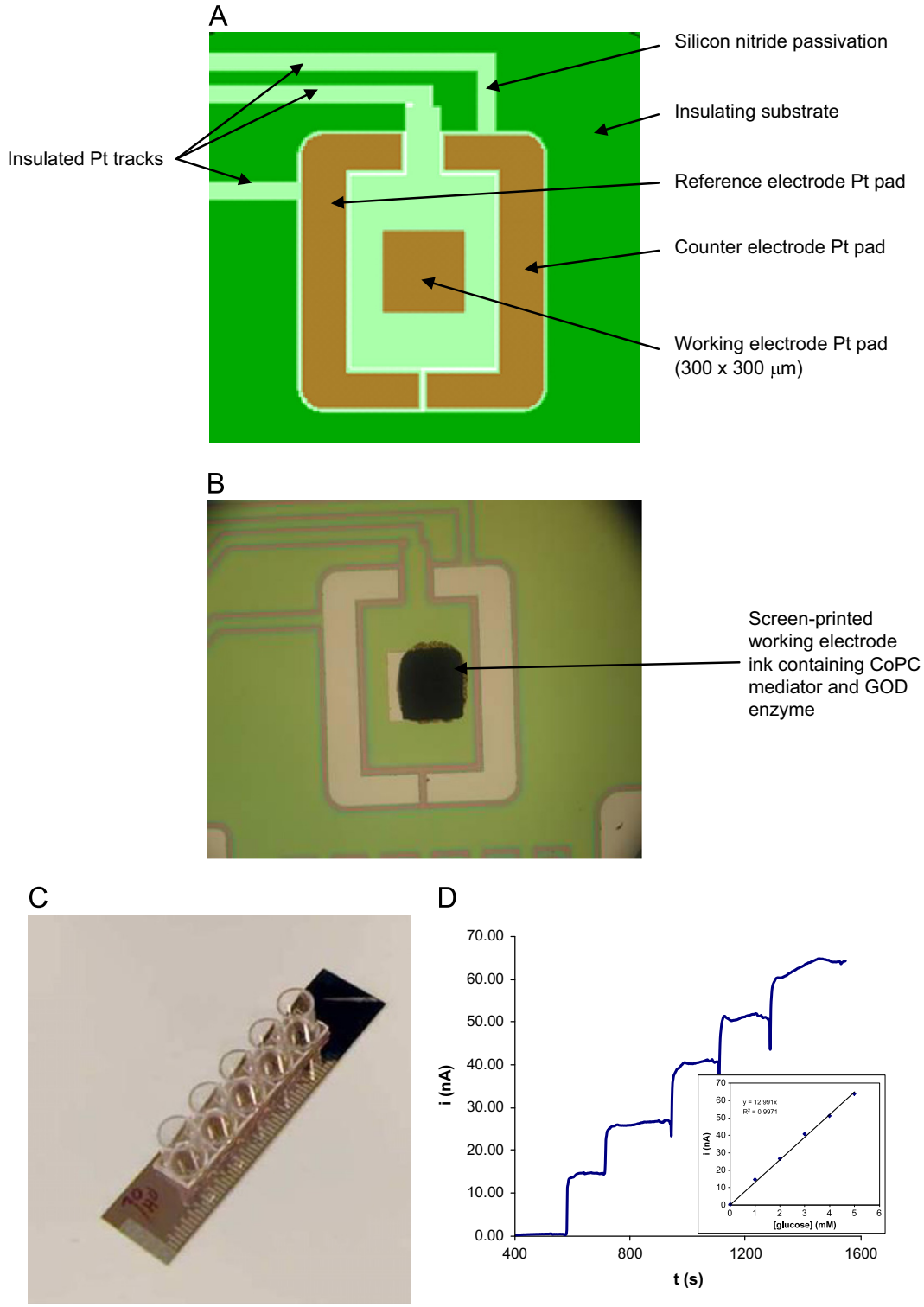

Fig. 3. (A) Diagram of SU8-dielectric patterned tracks and pads for 3-electrode system glucose microbiosensor (B) Photograph of complete glucose biosensor comprising microstructured tracks, Pt pads and screen-printed square of working electrode ink. (C) Photograph of 5-well glucose microbiosensor silicon chip. (D) Representative amperogram obtained upon incremental 1 mM glucose additions to a microbiosensor in 340 μl of 50 mM Na+/K+ phosphate buffer, pH 7.2 at 25 °C. $E_{app} = +0.4$ V.

suitable screen and attention to the issue of print alignment onto a microfabricated silicon substrate, the aim was to lay down small deposits of water-based carbon ink containing GOx and CoPC onto pre-formed Pt electrode pads, and to therefore fabricate working microelectrode structures.

Initial investigations into the achievable resolution of the screen-printing process using the water-based carbon ink were conducted using 12×12 mm^2 Pt-coated Si chips. A screen was designed to print the ink in squares of two different dimensions: 150×150 μm^2 and 450×450 μm^2. It was immediately apparent that the smaller 150 μm squares were poorly defined and not reproducible; however, the larger ink squares appeared visually to be reasonably well-defined and consistent in size, the final printed edge length being around 350 μm. These ink squares were examined for biosensor activity by insulating the remainder of the Pt area with PVC insulating tape; glucose was added to either buffer solution or culture medium and responses were measured amperometrically in quiescent solution using separate counter and reference electrodes. Steady-state responses were obtained in both media, indicating that the deposited ink was allowing radial-diffusion of the analyte to the working electrode and that the biosensors were behaving as microelectrodes. The results showed that responses were linear up to 3 mM, with a dynamic range up to 5 mM glucose.

The design for the silicon chip microstructure bearing tracks and Pt pads for a single 3-electrode glucose biosensor is shown in Fig. 3A. Exposed Pt areas for use as working, counter and reference electrodes are illustrated. Based on the above results and the influence of Pt base area on biosensor performance, together with the anticipated resolution and deliverable ink volume onto a small Pt pad, the choice of a 300 μm × 300 μm area Pt base was made, upon which the carbon CoPC/GOx ink was to be screen-printed. Five such microstructures were fabricated onto a single rectangular silicon chip, thus forming the base for a 5 microwell strip.

The Ag/AgCl reference electrodes were fabricated first by electroplating. The working electrode biosensor ink squares were then screen-printed onto the central Pt pads. A photograph of a printed working electrode is shown in Fig. 3B. Five biosensors were printed onto a chip base which would form the base for five microwells. The alignment of the ink square over the Pt pad proved challenging and an offset was often obtained (Fig. 3B), leaving up to 25% of the Pt pad area exposed. However, this offset would not alter the performance of the resulting biosensors, since the analytical signal resulted from mediated oxidation of H_2O_2 via the CoPC in the ink at the applied potential of +0.4 V which was too low to drive direct oxidation of H_2O_2 at the exposed Pt. The printed biosensor bases were converted into microwells by attaching bottomless well strips (Section 2.3). The resulting 5-well glucose biosensor strips (Fig. 3C) were examined for their amperometric response to glucose additions, initially by being interrogated individually using three probe arms to connect the three terminal pads to the appropriate potentiostat leads. Remarkably good steady-state responses were obtained for glucose concentrations up to 5 mM under quiescent conditions (Fig. 3D). The CV for the current response to a 1 mM concentration of glucose was around 6% for $n=5$ sensors.

3.4. Collagen coating of biosensors

3.4.1. Optimisation of coating conditions

The results showed that the response to glucose was affected by the type of collagen treatment. Control biosensors treated with buffer alone showed linearity up to 2 mM glucose; dynamic range up to 7 mM. When collagen was dried on, the calibration plot was a similar shape, but less sensitive, resulting in a plateau current diminished by 17% compared to untreated controls. Compared to the dried-on collagen, the coating protocols conducted under moist conditions resulted in better sensitivity and reproducibility of the biosensors: % CV values at 5 mM glucose for dried on, RT moist and 37 °C moist treatments were 12.5, 5.7 and 7.0, respectively. On the basis of the sensitivity and these precision results, moist conditions at RT were adopted for collagen coating on microbiosensors, using a concentration of 1 mg/ml, for all subsequent studies.

3.4.2. Performance of coated microbiosensors

Screen-printed glucose microbiosensors (3-electrode system on the base of the wells) in 96-well format 5-well strips were left untreated, or were coated with 1 mg/ml collagen for 2 h at RT under moist conditions. The amperometric response of the biosensors to glucose was then tested in culture medium (glucose-free medium/5% FCS). The untreated wells responded rapidly to added glucose (Fig. 4A). After each glucose addition, the current settled to a steady-state: this was evidence that the microbiosensor response was radial diffusion-limited. The resulting calibration plot was linear up to 2 mM and had a working range up to 12 mM (Fig. 4B). When the collagen-coated microbiosensors were tested, there was little change in the speed of response and steady-state responses were observed (Fig. 4A). The resulting calibration plot (Fig. 4B) showed similar sensitivity to the uncoated biosensor over the linear range up to 2 mM glucose. At higher glucose concentrations however, both sensitivity and

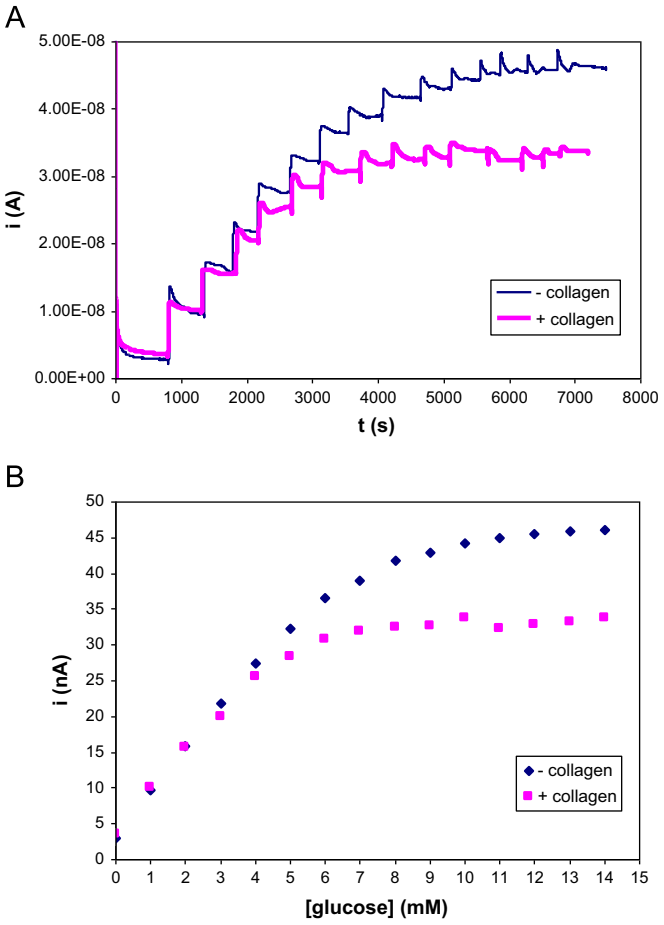

Fig. 4. (A) Amperograms and (B) calibration plots obtained following additions of incremental (1 mM) concentrations of glucose to collagen coated and uncoated microbiosensors in hepatocyte medium (340 μl in microwells; unstirred). $E_{app}=+0.4$ V; temperature = 21 °C.

working range were reduced compared to the uncoated biosensor response, the latter now extending only up to 7 mM. It seems likely that this effect may be due to the collagen diffusion barrier limiting the flux of glucose to the underlying biosensor ink. This behaviour clearly demonstrates that 96 well-format collagen-coated microbiosensors are functional in responding to glucose in culture medium over a useable working range.

3.4.3. SEM examination—cell adhesion to biosensor surface

Untreated and collagen-coated screen-printed carbon electrodes (SPCEs) were placed into 96-well plate wells in the presence of 7.5×10^5 HepG2 cells in full medium and incubated in a 5% CO_2 incubator at 37 °C for 4 h. The SPCEs were then rinsed in PBS and prepared for SEM examination at high (2024 ×) or low (253 ×) magnification. The resulting micrographs (Fig. 5) show that cells

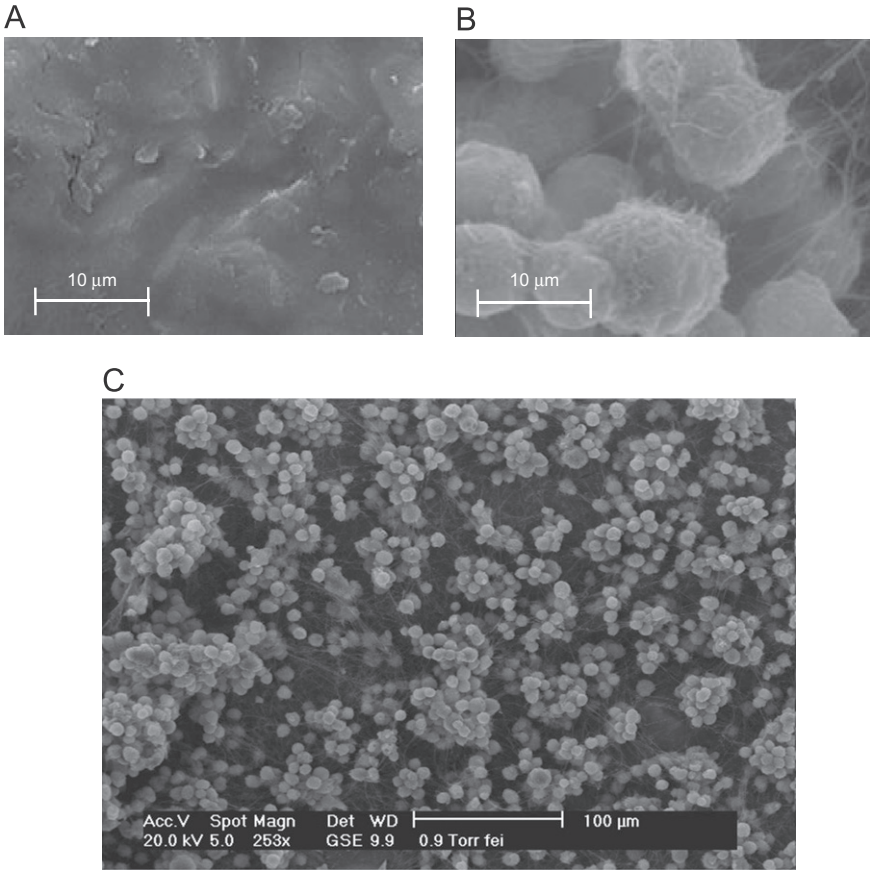

Fig. 5. Scanning Electron Micrographs of (A) untreated and (B) collagen-coated glucose biosensor working electrode surfaces after addition of HepG2 cells for 4 h at 37 °C in culture medium at high magnification (C) Lower magnification showing collagen-coated electrode plus cells.

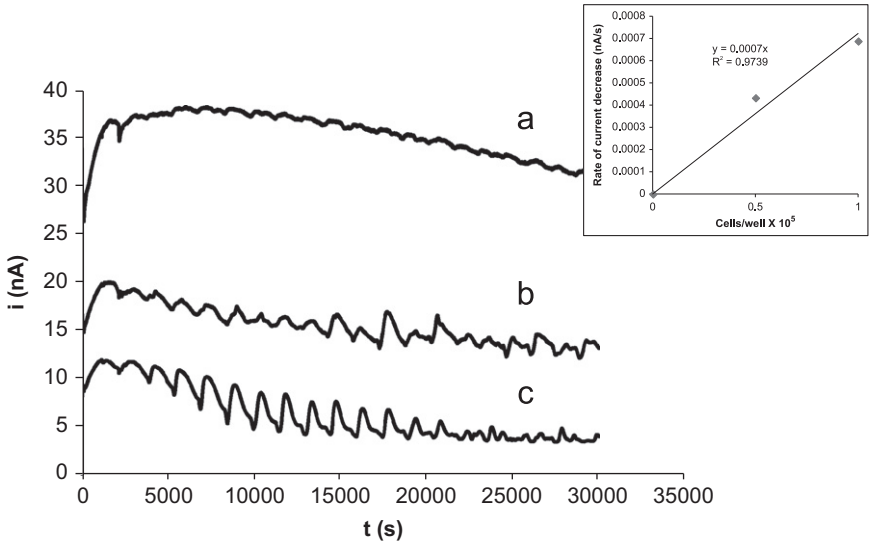

Fig. 6. Amperograms obtained for collagen-coated glucose microbiosensors in microwells containing 340 µl of low glucose full DMEM in the presence of (a) 0, (b) 0.5 and (c) 1×10^5 HepG2 cells. $E_{app} = +0.4$ V; Temp = 37 °C; 5% CO_2 dry incubator. Inset shows plot of rate of current decrease versus cell number/well.

Table 1
Working principle, design and performance characteristics of glucose microbiosensors cited in recently published papers.

Example	Reaction principle using GOx	Design	Working electrode dimensions	Medium used	Measurement technique; quiescent (Q) or stirred (S)	Operating potential	Sensitivity	Linear range (mM)	Limit of detection (μM)	Reference
1	Direct measurement of O_2 consumption by enzyme	Polyacrylamide-immobilised enzyme on Clarke-type Pt microwire	15–40 μm diameter bulb	Phosphate buffer, pH 7.4	Amperometry (S and Q)	−0.75 V vs Ag/AgCl	22 pA/mM (thick membrane) 73 pA/mM (thin membrane)	0–12	2	Peteu et al. (1996)
2	Direct measurement of H_2O_2 production by enzyme	GOx on Pt wire with polyphenol membrane	0.534 mm^2 area	PBS, pH 7.4	Amperometry (S)	+0.65 V vs Ag/AgCl	5.5 nA/mM	25	ND	Chen et al. (2002)
3	Direct measurement of H_2O_2 production by enzyme	Electropolymerised membranes (including GOx and catalase) precursor on Pt base	0.4×1 mm^2	PBS and bovine serum	Amperometry (Q)	+0.5 V vs Ag/AgCl	0.8–4 nA/mM	0–40	ND	Jobst et al. (1996)
4	Direct measurement of H_2O_2 via carbon nanotube/Pt black	Pt/Ir m'electrode coated with Pt black/MWCNT/Nafion/GOx	2 μm diameter	PBS and culture medium	Amperometry (S)	+0.5 V vs Ag/AgCl	0.531 nA/mM	0.01–17.5	10	Shi et al. (2011) McLamore et al. (2011)
5	Direct measurement of H_2O_2 production by enzyme	Au-coated screen-printed carbon electrode; GOx in solution; flow system	0.3×2.5 mm^2	Phosphate buffer, pH 8.5	Amperometry (S)	+1.0 V vs Ag/AgCl	5.1 nA/mM	0.6	30	Wang et al. (2007)
6	Electrocatalytic measurement of H_2O_2 via CoPC	Screen-printed GOx and mediator in WB carbon ink	350×350 μm^2	Culture medium+serum	Amperometry (Q)	+0.4 V vs Ag/AgCl	7 nA/mM	0–2	<200	This work (2012)
7	Electrocatalytic measurement of H_2O_2 via prussian blue-modified Pt nanoparticles	Lab-on-a-tube capillary sampling device: rolled Kapton film+PtNPs/PB/GOx in chitosan	<2.5 mm	PBS/human serum	Chronoamperometry (40 s) (Q)	−0.1 V vs Ag/AgCl	76 nA/mM	0–30	300	Li et al. (2010)
8	Electrocatalytic measurement of H_2O_2 via prussian blue	PB-modified carbon fibre electrode/Nafion/GOx	10 μm diameter	PBS	Amperometry (Q)	0.0 V vs SCE	0.7 nA/mM	0–2	8	Salazar et al. (2010)
9	Electrocatalytic measurement of H_2O_2 via MNO_2	Carbon fibre microelectrode in glass capillary+MNO_2/GOx/Nafion	10 μm diameter	PBS, pH 7.5	Amperometry (Q)	+0.58 V vs Ag/AgCl	1 nA/mM	1.5–15	0.8	Hocevar et al. (2004)
10	Electrocatalytic measurement of H_2O_2 via Ru	Carbon fibre microelectrode with deposited Ru/PD/GOx/Nafion	30 μm diameter	PBS	Amperometry (Q)	+0.4 V vs Ag/AgCl	10 nA/mM	0–4	2	Schuvailo et al. (2006)
11	Electrocatalytic measurement of GOx oxidation state via Os^{II}/Os^{III}	MEMS fabricated Au/Os polymer coating/GOx in PEG-DA hydrogel	500 μm diameter	PBS, pH 7.2	SWV (Q) Batch-type measurement	−0.2 V to +0.8 V vs Ag/AgCl	0.5 nA/mM	0–20	ND (<10)	Mugweru et al. (2006)
12	Electron shuttling from enzyme to electrode via ferrocene	MEMS fabricated Pt electrode coated with polypyrrole, ferrocene and GOx	100 μm $\times 300$ μm	PBS/kidney perfusate	Amperometry (S and Q)	+0.3 V vs Pt	20 nA/mM	0–25	100	Pesantez et al. (2012)
13	Potentiometric detection of ionic enzyme products	GOx coated ZnO nanowires on Ag wire microelectrode	250–300 nm diameter nano-wires	PBS, pH 7.4	Potentiometry (Q)	Ag/AgCl reference electrode	35 mV/0.5–10,000 μm	0.0005–10	0.5	Usman Ali et al. (2010)

only adhered to collagen-coated electrodes (B) and not to untreated electrodes (A) and had therefore been rinsed off untreated electrodes before fixation. The cells did not form a confluent monolayer layer at the density tested, but appeared to form into a 3-dimensional matrix with areas of cell-free collagen also being visible (C).

3.5. Microbiosensor responses in the presence of metabolising cells.

In order to test the operation of the 96-well format microfabricated microbiosensors in conjunction with cells, different numbers of HepG2 cells (0, 0.5 and 1×10^5) were placed into collagen-coated microbiosensor wells (5-well strip) in glucose-containing culture medium (DMEM containing 5 mM glucose), in a volume of 340 µl. The biosensor chip was connected to the 5-channel potentiostat, the wells were loosely covered with parafilm to reduce evaporation, and amperometric responses were recorded over the ensuing 6 h period (Fig. 6). In all wells, there was an equilibration period during the first 1000 s (15 min) after the potential of +0.4 V was applied, during which time the current response rose and reached a pseudo-steady-state level for each microbiosensor. A plot of these equilibrated current responses against cell number shows an inverse linear relationship (R^2=0.966), indicating that these currents represented the responses of the microbiosensors to glucose in the bulk medium, prior to depletion of glucose by metabolic activity. The decreasing current values reflect the decrease in electrode area due to coverage by increasing numbers of cells.

After 15 min, currents begin to decrease as a result of metabolism of glucose by the cells. This decrease in current is considerably more pronounced for the highest cell density and becomes constant at 25,000 s. This suggests that glucose has been completely metabolised. The resulting rates of current decrease are proportionate to cell number (R^2=0.974; Fig. 6, inset). It should be noted that a slight drift is also seen with the control (Fig. 6a) which could be explained by a slight change in pH: this may be due to loss of CO_2 from the culture medium.

4. Conclusions

Three different approaches were used to investigate the principle of fabricating glucose microbiosensors from a solvent-free carbon ink containing the redox mediator and enzyme components necessary to generate a selective electrochemical analytical signal. The ink was successfully deployed using a dip-coating, capillary-deposition, or screen-printing fabrication step onto an underlying Pt electrode. A comparison of the biosensor performance using these three approaches revealed similar linearities of response, up to around 4 mM, whereas the sensitivities of response varied, being 800, 896 and 7–13 nA/mM, for Pt wire-, capillary-deposited microdisc- and screen-printed-microbiosensors, respectively. The reduced sensitivity for the screen-printed microbiosensor reflected its small working electrode active area of around 0.12 mm^2 (350×350 μm^2), in addition to the fact that the responses were obtained under quiescent solution conditions, since the responses showed microelectrode behaviour but were not fully stir-independent.

The great majority of published reports on microbiosensors for glucose detection utilise GOx as the enzyme of choice and operate amperometrically (representative published examples are listed in Table 1). Those utilising a direct electrochemical measurement (examples 1–5) measure O_2 consumption at a (Clarke-type) Pt microwire (example 1), or enzyme-produced H_2O_2 at Pt (examples 2 and 3) or after its modification with carbon nanotubes (example 4). The only report of direct H_2O_2 measurement at screen-printed glucose microbiosensors (example 5) utilised Au modification of carbon, onto which GOx was immobilised; this format was used in a flow system (Wang et al., 2007). Besides the present work which utilises CoPC, glucose microbiosensors using electrocatalysts to measure H_2O_2 have included Prussian Blue at Pt (example 7) and carbon fibre (example 8), MNO_2 at carbon fibre (example 9), Ru at carbon (example 10), Os at Au (example 11) and ferrocene at Pt (example 12). Example 13 describes a potentiometric device which comprises ZnO nanowires on a Ag microelectrode. Amongst the examples cited in Table 1, the present work is unique in describing a screen-printable microelectrode device based on an electrocatalytic mechanism incorporating all of the reagents into the ink. Consequently, this biosensor has a major advantage over the other fabrication methods cited. In terms of comparative performance, the sensitivity of the device (7 nA/mM) compares favourably with most of the cited examples and the linear range of up to 2 mM is fit-for-purpose in this case, but could be extended by addition of a perm-selective membrane.

In terms of the overall goal of developing a microbiosensor suitable for use in a 96-well microplate format, the screen-printing approach formed the basis of a procedure with the potential to be automated for large-scale manufacture. Beyond the scope of the funding provided for this project, some further iterations of screen design and alignment, using slightly smaller Pt pad dimensions (eg. 100×100 µm) would enable the alignment of the screen-printed ink onto multiple Pt pads to be optimised.

The amperometric microbiosensor responses obtained in culture medium using collagen-coated microwells have demonstrated that continuous monitoring of glucose is feasible using these micro-devices. The issue of pH drift will be addressed in further studies using medium containing bicarbonate-independent buffering so as to maintain pH in the absence of a 5% CO_2 atmosphere.

We have demonstrated that our microbiosensor in conjunction with HepG2 cells provides a means of measuring both cell density and metabolism. In line with previous data obtained using microband biosensors (Pemberton et al., 2011), further work will be undertaken to test the microbiosensors for their ability to monitor changes in cell metabolism as a result of exposure to selected toxins. Towards this goal, the glucose microbiosensors are being incorporated, together with additional "housekeeping" (bio)sensors, including those for pH, O_2, temperature and lactate, into an integrated microwell system for cell well-being. The results of these studies will be described in a forthcoming report. The final integrated system could have application for toxicity testing in the pharmaceutical industry.

Acknowledgements

R.M.P. was funded by a Technology Strategy Board Micro & Nanotechnology project. The authors are grateful to Leah Jones (A.E.T., Ltd.) for help with the screen-printing. They also wish to acknowledge the expert assistance of Dr. D. Patton with the SEM work.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2012.11.032.

References

Chen, X., Matsumoto, N., Hu, Y., Wilson, G.S., 2002. Analytical Chemistry 74, 368–372.

Crouch, E., Cowell, D.C., Hoskins, S., Pittson, R., Hart, J.P., 2005a. Analytical Biochemistry 347, 17–23.
Crouch, E., Cowell, D.C., Hoskins, S., Pittson, R.W., Hart, J.P., 2005b. Biosensors and Bioelectronics 21, 712–718.
Dungchai, W., Chailapakul, O., Henry, C.S., 2009. Analytical Chemistry 81, 5821–5826.
Hocevar, S.B., Ogorevc, B., Schachl, K., Kalcher, K., 2004. Electroanalysis 16, 1711–1716.
Jobst, G., Moser, I., Varahram, M., Svasek, P., Aschauer, E., Trajanoski, Z., Wach, P., Kotanko, P., Skrabal, F., Urban, G., 1996. Analytical Chemistry 68, 3173–3179.
Li, C., Shutter, L.A., Wu, P.-M., Ahn, C.H., Narayan, R.K., 2010. Lab on a Chip 10, 1476–1479.
McLamore, E.S., Shi, J., Jaroch, D., Claussen, J.C., Uchida, A., Jiang, Y., Zhang, W., Donkin, S.S., Banks, M.K., Buhman, K.K., Teegarden, D., Rickus, J.L., Porterfield, D.M., 2011. Biosensors and Bioelectronics 26, 2237–2245.
Mugweru, A., Clark, B.L., Pishko, M.V., 2006. Electroanalysis 19, 453–458.
Nie, Z., Nijhuis, C.A., Gong, J., Chen, X., Kumachev, A., Martinez, A.W., Narovlyansky, M., Whitesides, G.M., 2010. Lab on a Chip 10, 477–483.
Pemberton, R.M., Pittson, R., Biddle, N., Hart, J.P., 2009a. Biosensors and Bioelectronics 24, 1246–1252.
Pemberton, R.M., Xu, J., Pittson, R., Biddle, N., Drago, G.A., Jackson, S.K., Hart, J.P., 2009b. Analytical Biochemistry 385, 334–341.
Pemberton, R.M., Xu, J., Pittson, R., Drago, G.A., Griffiths, J., Jackson, S.K., Hart, J.P., 2011. Biosensors and Bioelectronics 26, 2448–2453.
Pesantez, D.E., Castracane, J., Gadre, A.P., 2012. Electrochimica Acta 65, 196–203.
Peteu, S.F., Emerson, D., Worden, R.M., 1996. Biosensors and Bioelectronics 11, 1059–1071.
Saito, Y., 1968. Review of Polarography 15, 177–187.
Salazar, P., Martin, M., Roche, R., Gonzalez-Mora, J.L., O'Neill, R.D., 2010. Biosensors and Bioelectronics 26, 748–753.
Schuvailo, O.M., Soldatkin, O.O., Lefebvre, A., Cespuglio, R., Soldatkin, A.P., 2006. Analytica Chimica Acta 573, 110–116.
Shi, J., McLamore, E.S., Jaroch, D., Claussen, J.C., Mirmira, R.G., Rickus, J.L., Porterfield, D.M., 2011. Analytical Biochemistry 411, 185–193.
Usman Ali, S.M., Nur, O., Willander, M., Danielsson, B., 2010. Sensors and Actuators B 145, 869–874.
Wang, J., Chatrathi, M.P., Collins, G.E., 2007. Analytica Chimica Acta 585, 11–16.