Analytical Biochemistry 385 (2009) 334–341

Contents lists available at ScienceDirect

Analytical Biochemistry

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

Application of screen-printed microband biosensors to end-point measurements of glucose and cell numbers in HepG2 cell culture

R.M. Pemberton [a], J. Xu [b], R. Pittson [c], N. Biddle [c], G.A. Drago [d], S.K. Jackson [b], J.P. Hart [a,*]

[a] Centre for Analytical, Materials, and Sensors Science, Faculty of Health and Life Sciences, University of the West of England, Bristol BS16 1QY, UK
[b] Centre for Research in Biomedicine, Faculty of Health and Life Sciences, University of the West of England, Bristol BS16 1QY, UK
[c] Gwent Electronic Materials, Pontypool NP4 OHZ, UK
[d] Applied Enzyme Technology, Pontypool, Gwent NP4 OHZ, UK

ARTICLE INFO

Article history:
Received 3 October 2008
Available online 5 November 2008

Keywords:
Glucose biosensor
Microband electrode
Cell culture
HepG2 cells
Glucose metabolism

ABSTRACT

Microband glucose biosensors were produced by insulating and sectioning through a screen-printed, water-based carbon electrode containing cobalt phthalocyanine redox mediator and glucose oxidase enzyme. Under quiescent conditions at 37 °C, at an operating potential of +0.4 V, they produced an amperometric response to glucose in buffer solutions with a sensitivity of 26.4 nA/mM and a linear range of 0.45 to 9.0 mM. An optimal pH value of 8.5 was obtained under these conditions, and a value for activation energy of 40.55 kJ mol^{-1} was calculated. In culture medium (pH 7.3), a sensitivity of 13 nA/mM was obtained and the response was linear up to 5 mM with a detection limit of 0.5 mM. The working concentration was up to 20 mM glucose with a precision of 11.3% for replicate biosensors ($n = 4$). The microband biosensors were applied to determine end-point glucose concentrations in culture medium by monitoring steady-state current responses 400 s after transfer of the biosensors into different sample solutions. In conjunction with cultures of HepG2 (human Caucasian hepatocyte carcinoma) cells, current responses obtained in 24-h supernatants showed an inverse correlation ($R^2 = 0.98$) with cell number, indicating that the biosensors were applicable for monitoring glucose metabolism by cells and of quantifying cell number. Glucose concentrations determined using the biosensor assay were in good agreement, for concentrations up to 20 mM, with those determined spectrophotometrically ($R^2 = 0.99$). This method of end-point glucose determination was used to provide an estimated rate of glucose uptake for HepG2 cells of 7.9 nmol/(10^6 cells min) based on a 24-h period in culture.

The screen-printing process is particularly suitable for production of low-cost disposable biosensor devices [1,2] and has been employed in the manufacture of the amperometric test strips commonly used by diabetics to monitor their blood glucose levels [3]. Such biosensors are commonly screen-printed from a carbon ink that may also incorporate a redox mediator [4]. The presence of organic solvents and the need for heat curing of the printed ink at high temperature mean that the enzyme component must be added later in a separate fabrication step.

In a collaborative project between the University of the West of England and Gwent Electronic Materials (GEM),[1] a water-based carbon ink formulation (GEM product code C2030901R2), which incorporated cobalt phthalocyanine (CoPC) as an electrocatalyst together with the enzyme glucose oxidase (GOD), was recently developed. Glucose biosensors screen-printed from this ink, in a single fabrication step, maintained activity over an 18-month storage period and could be used amperometrically in stirred buffer solution to determine glucose concentrations in spiked bovine serum [5] and by chronoamperometry in human plasma samples [6].

We recently reported the fabrication and characterization of both tubular [7] and plain microband electrodes from carbon inks containing CoPC as redox mediator for the detection of hydrogen peroxide (H_2O_2). Plain microband biosensors, fabricated from the water-based carbon ink containing CoPC and also the GOD enzyme, were capable of producing steady-state amperometric currents in response to glucose at 25 °C under quiescent conditions [8]. These devices were applied successfully to the determination of glucose in serum, highlighting the potential for using microband biosensors in circumstances requiring glucose monitoring under quiescent solution conditions.

* Corresponding author. Fax: +44 117 3282904.
E-mail address: john.hart@uwe.ac.uk (J.P. Hart).

[1] *Abbreviations used:* GEM, Gwent Electronic Materials; CoPC, cobalt phthalocyanine; GOD, glucose oxidase; H_2O_2, hydrogen peroxide; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; dH_2O, deionized water; pen + strep, penicillin and streptomycin; PVC, polyvinyl chloride; LOD, lactate oxidase; CV, coefficient of variation; SD, standard deviation.

0003-2697/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2008.10.037

The role of glucose in metabolic energy production makes it a very important analyte not only in a clinical context for diabetic monitoring but also in food monitoring and biotechnology application areas. In addition to their use in the diagnosis and management of diabetes, potential application areas for glucose biosensors include the monitoring of cell culture growth and metabolism in bioreactor and cell culture systems. Various detection assay systems have been applied to making off-line glucose measurements in media from cell cultures; these have included colorimetric enzyme-linked immunosorbent assay (ELISA) systems [9] and Yellow Springs electrochemical glucose analyzers [10]. An adapted form of a commercial palm-size diabetic meter has also been reported as a means of monitoring glucose levels in cell culture supernatants [11,12].

The aim of the work described in the current article was to use microband biosensors screen-printed from the water-based, CoPC/GOD-containing carbon ink formulation as described above [8] to investigate the possibility of obtaining steady-state currents in response to glucose at 37 °C in quiescent buffer solutions and cell culture medium. The possibility of using these glucose microband biosensors to monitor changes in glucose concentration in cell culture was then investigated using the HepG2 (human Caucasian hepatocyte carcinoma) liver cell line. Cell number-dependent changes in glucose concentration were measured using end-point analysis.

Materials and methods

Chemicals and reagents

Fetal bovine serum (FBS), hepatocyte medium, high-glucose (4.5 g/L)-supplemented Dulbecco's modified Eagle's medium (DMEM), penicillin and streptomycin sulfate, and all other chemicals were of analytical reagent grade and purchased from Sigma–Aldrich. L-Glutamine was obtained from GibcoBRL. Phosphate buffer (0.05 M) was prepared from 0.5 M stock solutions of sodium dihydrogen orthophosphate and disodium hydrogen orthophosphate, which were mixed to obtain the desired pH and then diluted with deionized water (dH_2O, Purite R200 system, Purite, Oxon, UK). β-D-Glucose was dissolved in water and allowed to mutarotate at room temperature overnight before being used to prepare standard solutions.

HepG2 cell culture

The HepG2 cell line (obtained from the European Collection of Animal Cell Cultures [ECACC]) was cultured as a monolayer in 75-cm^2 flasks in a 5% CO_2-in-air atmosphere at 37 °C at an initial density of 10^5 cells/cm^2. Cells were counted using a hemocytometer. The medium was high-glucose DMEM containing 10% FBS, 1% nonessential amino acids, 2 mM L-glutamine, and 100 U/ml penicillin and 100 μg/ml streptomycin (pen + strep). When confluent, the cells were detached by trypsinization, centrifuged at 1000*g*, and resuspended in high-glucose DMEM with added 5% FBS, L-glutamine, and pen + strep. For the single-cell assays described, the resulting glucose concentration in the full DMEM was approximately 22.5 mM. Cells were counted and diluted initially to a density of 10^6 cells/ml; twofold dilutions were made in the same medium to give lower densities, as stated below. Cells were plated out in 3-ml volumes into 12-well tissue culture plates.

Biosensor fabrication

Microband biosensor working electrodes were fabricated as described previously [8] from a water-based carbon ink formulation containing carbon, binder, CoPC, and GOD (entire formulation is GEM product code C2041124D3) [6]. The enzyme loading in the ink was 628 U GOD per gram of carbon, as described previously [6]. No further enzyme optimization was performed for the current studies. Briefly, 3 × 3-mm square working electrodes (thickness 20 μm) were screen-printed onto polyvinyl chloride (PVC), covered with insulating tape, and then sectioned using a razor blade to expose a 3 mm × 20-μm working microband edge. The procedure is illustrated in Fig. 1. Reference electrodes were screen-printed separately onto 0.5-mm-thick heat-shrunk PVC using an Ag/AgCl ink (GEM code C61003P7) and were used in the form of 2-mm-wide strips.

Apparatus

Experiments were performed using a water-jacketed glass electrochemical cell or a 12-well tissue culture plate (Greiner Bio-One). Microband biosensors, reference electrodes, and Pt wire counter electrodes were connected to separate leads (for water-jacketed cell experiments) or were combined in homemade three-electrode sensor heads (push-fitting into 12-well plate wells for cell culture experiments). These were connected to a computer-controlled μAutolab type II potentiostat (Windsor Scientific, Slough, UK). Water jacket temperature was maintained at 37 °C using a circulating water bath (Haake D3, Haake, Saddle Brook, NJ, USA). Tissue culture plates were placed into a shallow water bath at 37 °C. Solution pH levels were monitored using a pH meter (Fisherbrand Hydrus 400) and a glass pH electrode (Russell).

Cyclic voltammetry

Microband glucose biosensors, or as controls microband lactate biosensors [13] fabricated using the enzyme lactate oxidase (LOD) rather than GOD, were used in a three-electrode system. Electrodes were placed in a 10-ml volume of 0.05 M phosphate buffer (pH 7.3) containing 150 mM NaCl at 37 °C. Specified concentrations of glucose or H_2O_2 were added to the electrolyte and, using a fresh microband electrode each time, cyclic voltammograms were obtained by scanning at 20 mV/s in the range of 0 to +1.0 V.

Calibration studies by amperometry in electrochemical cell

Calibration plots for glucose were obtained using a 10-ml solution of 0.05 M phosphate buffer (pH 8.0) containing 150 mM NaCl, or hepatocyte medium, into which a biosensor was immersed. An appropriate operating potential was applied to the working electrode, and the system was left for the current response to reach a steady state (~500 s). Using a micropipette, a series of small additions (70–500 μl) of a standard glucose solution (pH 8.0) was then made, awaiting steady-state currents between additions. Calibration plots were constructed by plotting each current response against the final cell glucose concentration. Mean current responses were calculated from calibrations performed on replicate biosensors.

Effect of temperature

A microband biosensor was immersed in a quiescent pH 8.0 buffer solution (in a water-jacketed cell) containing 150 mM NaCl and 5 mM glucose at 20 °C. An operating potential of +0.4 V was applied. Once a steady-state current had been reached (500 s), the temperature of the solution, recorded using a digital Fluke 51 thermocouple (John Fluke, Seattle, WA, USA), was increased gradually up to 37 °C. During this time, the steady-state current was monitored and recorded.

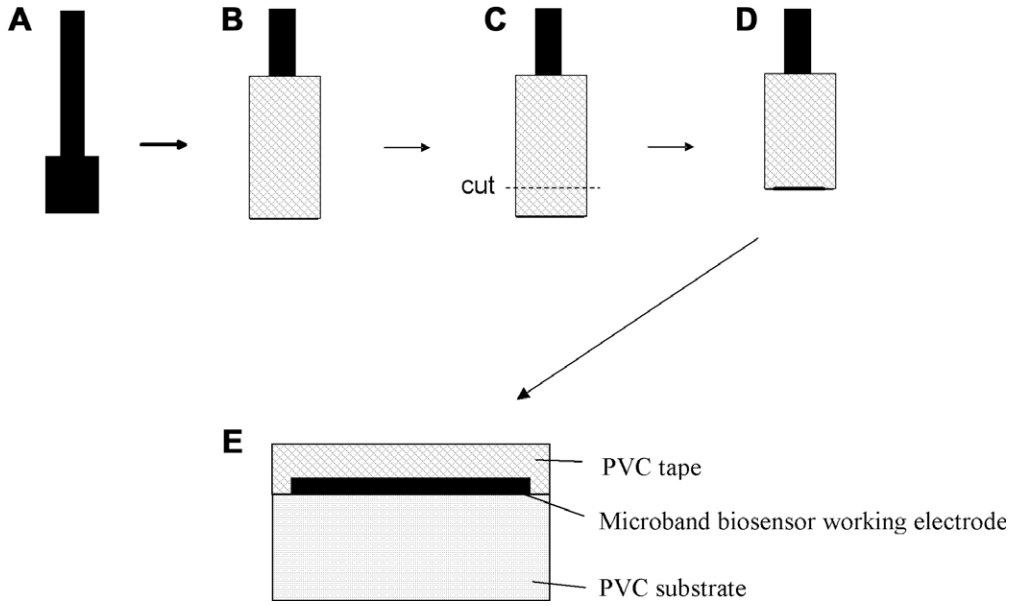

Fig. 1. Diagram illustrating fabrication of microband working electrodes. (A) Screen-printed, water-based carbon/CoPC/GOD ink on PVC comprising 3 × 3-mm square planar carbon area plus conducting track. (B) PVC tape covers working area. (C) Transverse razor blade cut through center of working area. (D) Track plus exposed electrode edge. (E) Expanded view of edge revealing microband biosensor working electrode.

Effect of pH

The dependence of the microband biosensor response on pH was investigated using a series of 0.05 M phosphate buffer/150 mM NaCl solutions (pH range of 5–10) containing 20 mM glucose. Volumes (3 ml) of each solution were placed into separate tissue culture plate wells, and the plate temperature was maintained at 37 °C. A sensor head, holding a microband biosensor, reference electrode, and counter electrode, was inserted into the first well (pH 8.0) so that an approximate depth of 4 mm of each electrode was immersed in the solution. An operating potential of +0.4 V was applied, and 1500 s was allowed for the current to reach the steady state. The sensor head was then transferred into each of the other wells in turn, and steady-state responses were recorded each time. A plot of current versus pH was constructed.

Calibration studies by dipping microbiosensors into wells containing known glucose concentrations

Aliquots (3 ml) of hepatocyte medium, containing different glucose concentrations over the range of 0 to 20 mM, were added to separate wells of a 12-well tissue culture plate. A sensor head, holding microband biosensor, reference electrode, and counter electrodes, was inserted into the first well, and a steady-state current at +0.4 V was attained (see previous section). The sensor head was then transferred progressively (constant applied potential of +0.4 V) into wells containing higher or lower concentrations of glucose, as indicated, and steady-state currents were awaited prior to each transfer (approximately every 3000 s). The resulting steady-state current values were recorded. The mean current differences obtained between zero glucose and any given millimolar concentration were calculated for four replicate biosensors and plotted versus glucose concentration.

Glucose determination in 24-h supernatants from HepG2 cell cultures

Individual 12-well tissue culture plate wells were seeded with decreasing (twofold) numbers of HepG2 cells in 3-ml volumes of high-glucose DMEM/5% FBS/pen + strep. The resulting cell densities, therefore, were 1, 0.5, 0.25, 0.125, 0.625, and 0×10^6/ml. The plate was incubated for 24 h in a 5% CO_2 atmosphere at 37 °C, after which time the culture medium was harvested from the wells and stored frozen at −20 °C until analysis.

Electrochemical analysis, using glucose biosensors, was performed on the thawed culture medium samples that were placed in a fresh tissue culture plate at 37 °C. Microband biosensors were transferred from well to well with a constant applied potential of +0.4 V, as described in the previous section. The resulting steady-state currents were plotted against HepG2 cell density. The glucose content of the samples was also determined spectrophotometrically.

Spectrophotometric glucose assay

The glucose concentration in buffer and culture medium samples was determined using a spectrophotometric method based on that of Trinder [14]. Then 1-ml volumes of assay reagent, prepared as described previously [6] from phenol, 4-aminoantipyrine, GOD, and peroxidase in phosphate buffer, were added to cuvettes containing 200-μl volumes of samples diluted 10-fold in dH_2O. Cuvettes were incubated for 1 h at 37 °C, and then absorbances at 505 nm were read against a reagent blank. Absorbances due to tissue culture components were corrected by adding 1 ml of dH_2O instead of the reagent. Unknown concentrations were determined using a calibration plot constructed for glucose standard solutions over the range of 0 to 25 mM.

Results

For all experiments, an applied potential of +0.4 V versus Ag/AgCl was selected for amperometric investigations into the performance of the microband biosensors. This potential was based on voltammetric experiments reported elsewhere [8] and represents the potential at which the CoPC-mediated electrocatalytic oxidation of H_2O_2, produced by the action of GOD on glucose [15], occurs under the conditions used in the current study.

Amperometry in quiescent buffer solutions

Influence of temperature

Whereas previously reported calibration plots, using microband biosensors and undertaken at a temperature of 25 °C, were obtained using a stir-to-mix step followed by a quiescent measurement step [8], the experiments reported here were conducted under completely quiescent conditions. Thus, additions of glucose were made directly into the electrochemical cell, and sufficient time was allowed for diffusion of the glucose to the electrode to occur before the current response was observed. Initial studies were performed in solutions of phosphate buffer (pH 8.0) containing 150 mM NaCl. Calibration studies performed at 25 °C under these conditions (data not shown) demonstrated that it was not realistic to measure glucose concentrations below 1 mM unless the solution was stirred to mix the glucose into the bulk volume and, thus, bring it to the electrode surface. In contrast, when operating at 37 °C, it was possible to obtain responses in completely quiescent solutions, indicating a more rapid diffusion of glucose to the electrodes and/or enhanced enzyme catalytic activity at the higher temperature. Thus, calibration plots were obtained in quiescent buffer showing a zero intercept and with a linear region between 450 μM and approximately 9 mM. The mean sensitivity of the glucose response for replicate biosensors in these preliminary studies was 26.4 nA/mM. The coefficient of variation (CV) of the sensitivity for triplicate biosensors from different electrode batches ranged from 5.9% to 26.5%.

A comparison of the mean sensitivities of response to glucose at 25 and 37 °C gave values of 10 and 26.4 nA/mM, respectively. This increase in reaction rate with increasing temperature is consistent with the Q_{10} factor observed for enzyme reactions having typical activation energies (15–70 kJ M^{-1}), where a two- to threefold increase in reaction rate is observed for every 10 °C rise in temperature [16]. Indeed, a current versus temperature plot, obtained using a single microband biosensor in a 0.05-M phosphate buffer solution (pH 8.0) containing 5 mM glucose was linear over the range of 20 to 37 °C (R^2 = 0.986), with a slope of 12.5 nA/°C. This was equivalent to approximately a doubling of the current response for a 10 °C rise in temperature.

The value of the energy of activation (E^*) was determined by plotting log rate (V) against 1000/T [17] for a microband biosensor in the presence of saturating (25 mM) glucose (Fig. 2). The equation for this straight-line plot was $\log V = -2.1185\ (1000/T) + 9.0525$. The slope (−2.1185) was equal to $-E^*/19.14$. Thus, an estimated value for E^* of 40.55 kJ mol^{-1} was obtained for the GOD-catalyzed conversion of glucose to gluconolactone and H_2O_2. On this basis, the majority of the biosensor reaction rate would appear to be under kinetic (enzyme) control rather than being diffusion limited. The effect of temperature on diffusion currents is considerably less, with expected increases of 1% to 2%/°C rise in temperature.

Influence of pH

The influence of pH on the response of the microband glucose biosensors was investigated using a single biosensor. The resulting pH plot (Fig. 3) shows that the sensitivity of the biosensor response increased with increasing pH levels from 5.0 to a maximum at 8.5; at higher pH levels, the sensitivity then decreased. As expected, this plot shows a pH profile similar to that obtained previously at 25 °C using sensitivity values obtained from entire calibration plots obtained by amperometry using separate biosensors for each pH point [8]. The optimal pH value of approximately 8.5 obtained here is slightly lower than that (~9.2) obtained at 25 °C with separate biosensors. The difference may reflect the time taken for the pH environment within the ink microband to equilibrate depending on the method used for measurement. In practical terms, the optimal pH for operation of the devices at 37 °C would appear to be pH 8.5. Although this behavior is of interest, further studies involving culture medium with and without cells would require a pH of 7.3.

Cyclic voltammetry

Before commencing amperometric studies in culture medium, cyclic voltammetry was used to confirm the role of the GOD enzyme in the response of microband glucose biosensors to glucose at pH 7.3. Cyclic voltammetry was performed at glucose microband biosensors (containing GOD enzyme) and also at microband biosensors containing LOD instead of GOD. The resulting cyclic voltammograms (Fig. 4) demonstrated the catalytic oxidation response at +0.5 V for glucose at GOD-containing electrodes (trace b) characteristic of the CoPC-mediated electrocatalytic oxidation of H_2O_2 [15]. No response to glucose was observed at electrodes containing the (inert for glucose) LOD enzyme (trace e), confirming that there was no direct oxidation of glucose at the CoPC or carbon

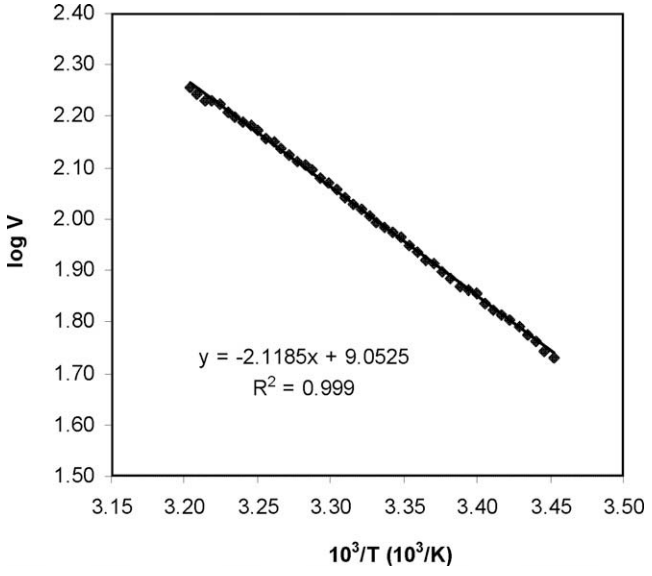

Fig. 2. Plot of log current response (rate, V) versus 1000/temperature for a microband glucose biosensor in quiescent 0.05 M phosphate buffer/150 mM NaCl solution (pH 8.0) containing 25 mM glucose. E_{app} = +0.4 V versus Ag/AgCl.

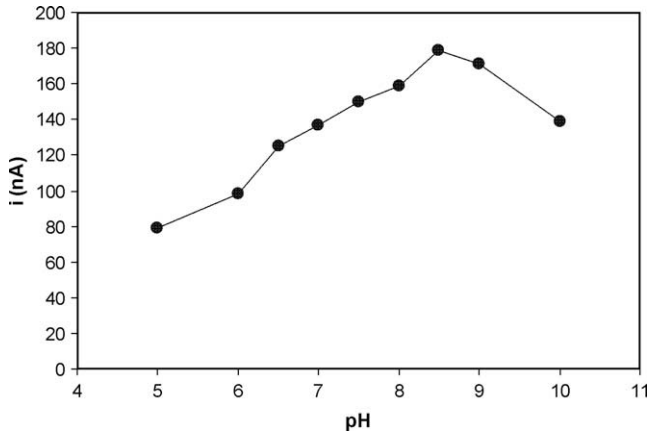

Fig. 3. Plot of steady-state current versus pH for a microband glucose biosensor in 0.05 M phosphate buffer/150 mM NaCl solutions containing 20 mM glucose at various pH levels over the range of 5.0 to 10.0. E_{app} = +0.4 V versus Ag/AgCl.

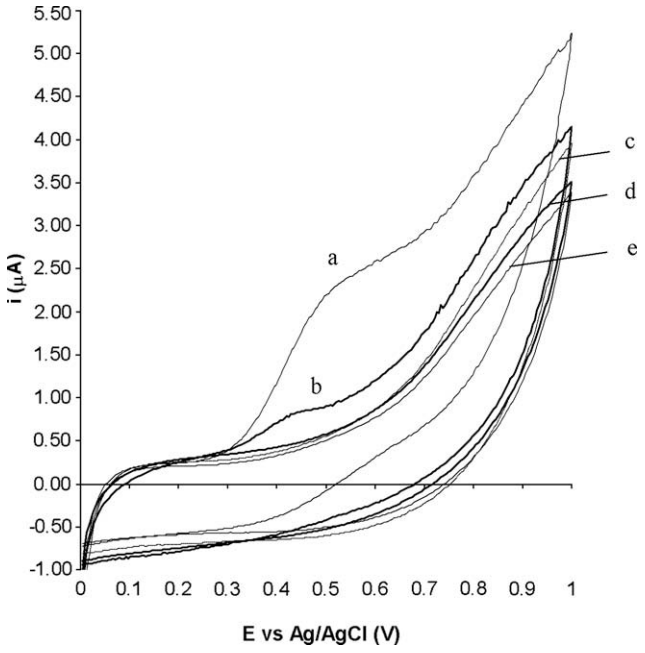

Fig. 4. Cyclic voltammograms obtained at lactate microband (a, c, e) and glucose microband (b, d) electrodes. Electrolyte solutions: 0.05 M phosphate buffer (pH 7.3) containing 150 mM NaCl alone (c, d) or in the presence of 10 mM glucose (b, e) or 10 mM H_2O_2 (a).

in the electrodes. The electrocatalytic response to H_2O_2 in solution at these LOD-containing microbands is shown in trace a; the same response occurs at GOD-containing microbands (not shown). No responses were obtained for either GOD- or LOD-containing CoPC microbands in buffer alone (traces c and d).

Amperometry in quiescent culture medium

Calibration by multiple spiking

Having established that microband biosensors could respond to glucose in unstirred buffer solutions at 37 °C, it was of interest to begin the current investigation by examining the amperometric response of microband biosensors in quiescent culture medium so as to progress toward using these devices for monitoring glucose in cell cultures. Fig. 5A shows a typical amperogram obtained following the addition of glucose into hepatocyte culture medium at 37 °C. The pH of this medium was 7.3. From the time of glucose addition, there was a rapid rise in current over the first 50 s, and it then took approximately 400 s for the current response to reach a steady state. The corresponding calibration plot is shown in Fig. 5B. The plot showed zero intercept, and the response was linear up to 5 mM, with a working range up to approximately 20 mM. The mean slope for $n = 4$ replicate biosensors was 12.8 nA/mM, with a precision of 11.3% CV. There was no significant difference between the sensitivities of response obtained in the absence and presence of 10% serum in the culture medium.

It was important to establish whether the sensitivity of the biosensor responses would be affected by the size of glucose aliquots added in terms of either volume or moles of glucose added. Three replicate calibrations were performed using additions of 150-, 250-, and 500-μl volumes of a 100 mM glucose stock solution into a 10-ml volume of culture medium (pH 7.3); these resulted in additions of 15, 25, and 50 μmol, respectively. The resulting plots (Fig. 6) showed a similar sensitivity (mean 13 nA/mM) and linear range (up to ~13 mM), thereby demonstrating that the microband biosensor performance, in terms of the solution conditions for these parameters, was independent of the size of the addition made.

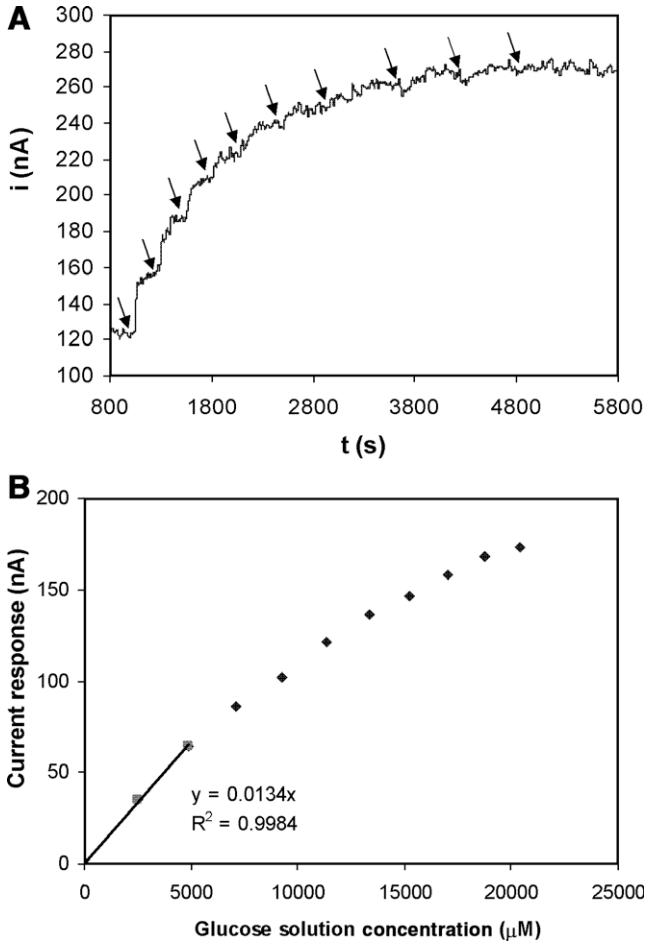

Fig. 5. (A) Typical amperogram obtained in quiescent hepatocyte medium using microband biosensors. Arrows indicate 250-μl additions of 100 mM glucose standard into a 10-ml cell volume. (B) Corresponding calibration plot.

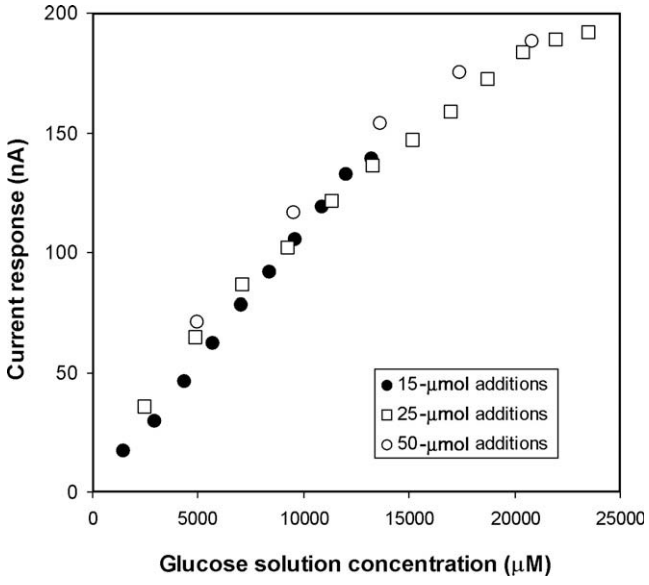

Fig. 6. Calibration plots for glucose at microband biosensors obtained by amperometry in quiescent hepatocyte medium using different volume/molar glucose increments. Three-electrode system; E_{app} = +0.4 V.

All of the current responses shown in Figs. 5 and 6 were measured from a steady-state baseline; the estimated detection limit for the

method, calculated from the baseline current + 3 SD (standard deviations), was 0.5 mM glucose.

These results demonstrated that, at 37 °C in unstirred solutions, glucose microband biosensors are capable of responding to changes in glucose concentration within 5 min and of measuring glucose in culture medium over a range of 0.5 to 20 mM.

Calibration by transferring a microband between glucose solutions

Another approach to using microband biosensors to determine glucose concentrations in medium was in batch or end-point mode, thereby measuring glucose concentrations at fixed times. By using a single biosensor, maintained at its applied potential and transferred from well to well, multiple separate sample solutions could be analyzed. Fig. 7 shows the amperograms resulting from representative experiments where single microband biosensors, maintained at an applied potential of +0.4 V, were transferred successively between solutions of culture medium containing high-to-low (Fig. 7A) or low-to-high (Fig. 7B) glucose concentrations. The resulting steady-state current responses were recorded in each case. The resulting plot of mean currents against glucose concentration (Fig. 7C) gave a working range of 0 to 20 mM, with the most linear portion being from 1 to 5 mM. The current response for 20 mM glucose was 370 nA. Using this method, the detection limit was estimated to be 1 mM glucose.

Application to cell culture

Cell-dependent glucose depletion end-point determinations

The ability to use microband biosensors for batch mode glucose determinations in cell culture was applied to 24-h supernatants obtained from cell cultures containing different numbers of individual HepG2 cells. A single microband biosensor was transferred from well to well, and the resulting steady-state currents were recorded. Triplicate measurements were made using three separate biosensors. The resulting (steady-state) current responses obtained using the glucose biosensor were inversely proportional to cell number (Fig. 8), with a correlation coefficient of $R^2 = 0.98$ (inset).

The corresponding glucose concentrations were determined from the calibration plot (Fig. 7C), and these were compared with the respective readings obtained for the same culture supernatants when tested using the colorimetric glucose assay. The resulting plot (Fig. 9) showed good agreement between the two assay methods for glucose concentrations up to 20 mM ($R^2 = 0.99$). At higher concentrations (which were >22.5 mM due to slight evaporation from culture wells over the 24-h period, leading to a reduction in volume from 3.00 to 2.76 ml), the biosensor gave a value of 30 mM for the equivalent spectrophotometric determination of 21.7 mM. The biosensor, therefore, showed a positive bias at higher concentrations. This effect was not associated with release of an electroactive material (other than glucose) from cells because the high glucose concentrations occur in the absence of cells. So far as possible, any drift in pH during the course of the experiments was corrected, although it is possible that some experimental error occurred at this point in the data processing.

Estimation of glucose uptake

At the start of the experiments, each culture well contained 67.5 μmol of glucose. By multiplying the determined concentrations by the well volumes, the values for total micromoles of glucose remaining were calculated for each of the culture wells. The

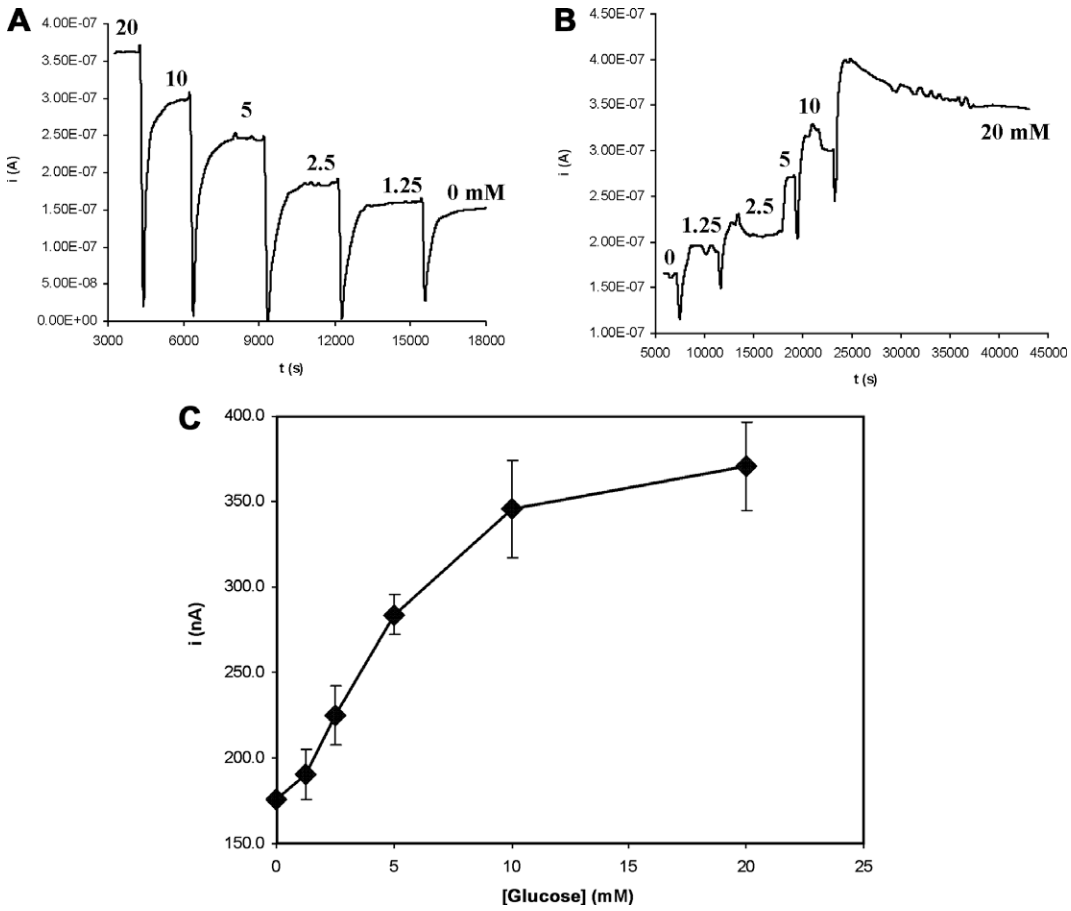

Fig. 7. Amperograms obtained for glucose microband biosensors after transfer into hepatocyte medium containing decreasing (A) or increasing (B) concentrations of glucose. (C) Corresponding calibration plot showing mean Δi values obtained for repeat ($n = 4$) biosensors. Error bars represent ± 1 SD.

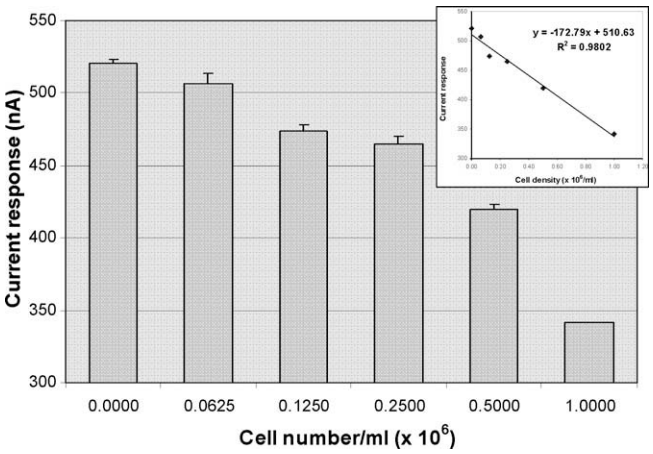

Fig. 8. Bar graph showing current responses versus HepG2 cell density for supernatants collected after 24 h in culture. Error bars represent ± 1 SD for triplicate biosensors. The inset shows the correlation plot.

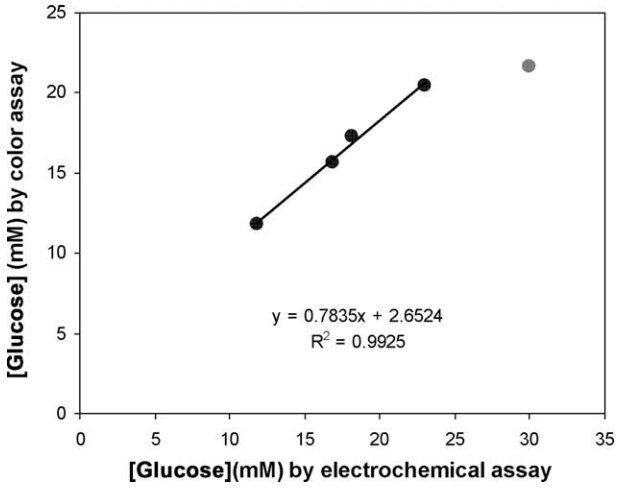

Fig. 9. Estimates of glucose concentrations in 24-h culture supernatants: Correlation plot between electrochemical microband biosensor and spectrophotometric assay.

resulting values for the spectrophotometric and biosensor assays are shown in Table 1. The spectrophotometrically derived values indicate that, allowing for experimental error, the initial 22.5-mM glucose concentration was maintained in the absence of cells for the duration of the experiments. Results show that 0.5 million cells/ml had consumed 24.1 µmol over 24 h (43.4 remaining), whereas 1 million cells/ml had consumed 34.9 µmol over 24 h. These results, at the end of a 24-h period, gave an estimated glucose uptake of 8.1 nmol/(10^6 cells min) by the spectrophotometric assay; the corresponding value by electrochemical biosensor was 7.9 nmol/(10^6 cells min). Recently published experiments, in which HepG2 cells were cultured in 6-well plates with shaking in the absence of glutamine and serum [18], gave an estimated rate of glucose uptake of 16.3 nmol/(10^6 cells min) after sampling hourly over 3 h and analyzing medium for glucose by gas chromatography–mass spectrometry. The lower average estimate of glucose uptake in the current study, for 24 h compared with 3 h, is consistent with the knowledge that cultured cells metabolize rapidly during the first few hours of culture as they proliferate to confluence. The current estimate is of the correct order of magnitude and supports the notion that microband glucose biosensors hold promise for monitoring applications involving cell quantification and metabolism studies and that they can operate successfully in culture medium.

Table 1
Spectrophotometric and biosensor molar estimates of glucose remaining in (3-ml) culture wells after 24 h in the presence of varying numbers of HepG2 cells

HepG2 cell number × 10^6/well	Micromoles glucose remaining (spectrophotometric assay)	Micromoles glucose remaining (biosensor assay)	% Accuracy of biosensor estimate
3.000	32.6	33.2	101.8
1.500	43.4	46.7	107.6
0.750	47.8	50.0	104.6
0.375	56.7	63.6	112.2
0 188	65 1	83 0	127 5
0.000	65.1	83.0	127.5

Note. Values are means from triplicate wells.

Discussion

In this article, electrochemical microband biosensors for glucose, fabricated using screen-printing technology, have been characterized for operation at 37 °C and in medium suitable for mammalian cell culture. The applications described rely on the ability of the biosensors to give steady-state amperometric current responses in quiescent solution [8]. Glucose determination, by dipping biosensors into solutions containing finite concentrations, takes advantage of the biosensors' ability to operate continuously at constant applied voltage even when transferred from one sample solution to another. This application lends itself to the analysis of a set of sample solutions and standards, and it provides internal consistency by using a biosensor that retains sensitivity over a prolonged period of several hours. When used in this way, the end-point determination of glucose concentration within supernatants from mammalian monolayer cell cultures gave a good correlation with cell number and, by implication, metabolic activity during the culture period. This information provides validation for the biosensor operation (robustness, accuracy, and sensitivity) and could have application for end-point or batch mode determinations of glucose. There is potential to use these biosensors to quantify numbers of actively metabolizing cells under various defined conditions, perhaps in bioreactor/fermentation applications.

There is currently a caveat that the concentration of glucose does not exceed 20 mM so that the calibration of the biosensors is predictable. However, because most media for cell culture applications contain glucose supplements within the concentration range of 2.5 to approximately 20 mM, this would not appear to be a problem. So far as we are aware, this is the first report of the application of screen-printed microband biosensors for use in cell culture. Further investigations in which these microband biosensors may be used with other cell types and in other media are under way. The novelty of fabrication for these sensors lies in the incorporation of the biologically active constituent (the enzyme) within the ink, thereby conferring the potential for single-step fabrication and the possibility of adaptation for other analytes by substituting GOD for other oxidase enzymes. The possibility of using microband biosensors for prolonged real-time glucose monitoring is also currently being investigated and, if successful, will provide key information on rates of glucose uptake or release under a variety of culture conditions.

Acknowledgments

R.M.P. was funded by a Technology Strategy Board Micro and Nanotechnology project. The authors thank Leah Jones (Applied Enzyme Technology) for help with screen printing.

References

[1] P.I. Hilditch, M.J. Green, Disposable electrochemical biosensors, Analyst 116 (1991) 1217–1220.

[2] J.D. Newman, S.J. Setford, Enzymatic biosensors, Mol. Biotechnol. 32 (2006) 249–268.

[3] D.R. Mathews, E. Bown, A. Watson, R.R. Holman, J. Steemson, S. Hughes, D. Scott, Pen-sized digital 30-second blood glucose meter, Lancet 1 (1987) 778–779.

[4] J.P. Hart, A. Crew, E. Crouch, K. Honeychurch, R.M. Pemberton, Recent developments in screen-printed carbon electrode sensors/biosensors for electrochemical analysis, in: S. Alegret, A. Merkoci (Eds.), Comprehensive Analytical Chemistry, vol. 49, Electrochemical Sensor Analysis, Elsevier, Amsterdam, 2007, pp. 495–556.

[5] E. Crouch, D.C. Cowell, S. Hoskins, R.W. Pittson, J.P. Hart, A novel, disposable, screen-printed amperometric biosensor for glucose in serum fabricated using a water-based carbon ink, Biosens. Bioelectron. 21 (2005) 712–718.

[6] E. Crouch, D.C. Cowell, S. Hoskins, R. Pittson, J.P. Hart, Amperometric, screen-printed, glucose biosensor for analysis of human plasma samples using a biocomposite water-based carbon ink incorporating glucose oxidase, Anal. Biochem. 347 (2005) 17–23.

[7] F.J. Rawson, W.M. Purcell, J. Xu, D.C. Cowell, P.R. Fielden, N. Biddle, J.P. Hart, Fabrication and characterisation of novel screen-printed tubular microband electrodes and their application to the measurement of hydrogen peroxide, Electrochim. Acta 52 (2007) 7248–7253.

[8] R.M. Pemberton, R. Pittson, N. Biddle, J.P. Hart, Fabrication of microband glucose biosensors using a screen-printing water-based carbon ink and their application in serum analysis, Biosens. Bioelectron., in press.

[9] K.A. Frauwirth, J.L. Riley, M.H. Harris, R.V. Parry, J.C. Rathmell, D.R. Plas, R.L. Elstrom, C.H. June, C.B. Thompson, The CD28 signalling pathway regulates glucose metabolism, Immunity 16 (2002) 769–777.

[10] Y. Sidorenko, A. Wahl, M. Dauner, Y. Genzel, U. Reichl, Comparison of metabolic flux distributions for MDCK cell growth in glutamine- and pyruvate-containing media, Biotechnol. Prog. 24 (2008) 311–320.

[11] I.K. Wang, K.M. Chang, L. Ho, Glucose measurement for cell culture: GlucCell, a modified blood glucose monitor, Genet. Eng. Biotechnol. News 26 (10) (2006) 40–43.

[12] Dunn Labortechnik, GlucCell glucose monitoring system. Available from: <http://www.dunnlab.de>.

[13] F.J. Rawson, W.M. Purcell, J. Xu, R.M. Pemberton, P.R. Fielden, N. Biddle, J.P. Hart, A microband lactate biosensor fabricated using a water-based screen-printed carbon ink, Talanta, in press.

[14] T. Trinder, Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor, Ann. Clin. Biochem. 6 (1969) 24–27.

[15] M.A.T. Gilmartin, J.P. Hart, D.T. Patton, Prototype, solid-phase glucose biosensor, Analyst 120 (1995) 1973–1981.

[16] P.W. Hochachka, G.N. Somero, Biochemical Adaptation, Princeton University Press, Princeton, NJ, 1984.

[17] J.G. Morris, The kinetics of enzyme-catalysed reactions, in: E.J.W. Barrington, A.J. Willis (Eds.), A Biologist's Physical Chemistry, second ed, Edward Arnold, London, 1974, pp. 312–317.

[18] U. Hofmann, K. Maier, A. Niebel, G. Vacun, M. Reuss, K. Mauch, Identification of metabolic fluxes in hepatic cells from transient ^{13}C-labeling experiments: I. Experimental observations, Biotechnol. Bioeng. 100 (2008) 344–354.