Analytical Biochemistry 396 (2010) 269–274

Contents lists available at ScienceDirect

Analytical Biochemistry

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

# A flow injection system, comprising a biosensor based on a screen-printed carbon electrode containing Meldola's Blue–Reinecke salt coated with glucose dehydrogenase, for the measurement of glucose

M. Piano [a], S. Serban [b], N. Biddle [b], R. Pittson [b], G.A. Drago [a], J.P. Hart [c,*]

[a] Applied Enzyme Technology, Monmouth House, Mamhilad Park, Pontypool NP4 6HZ, UK
[b] Gwent Electronic Materials, Monmouth House, Mamhilad Park, Pontypool NP4 6HZ, UK
[c] Centre for Research in Analysis, Materials, and Sensor Sciences, Faculty of Health and Life Sciences, University of the West of England, Frenchay Campus, Bristol BS16 1QY, UK

ARTICLE INFO

*Article history:*
Received 13 July 2009
Available online 18 September 2009

*Keywords:*
Flow cell
Screen-printed electrode
Meldola's Blue–Reinecke salt
Amperometric biosensor
Glucose
Serum

ABSTRACT

A biosensor for the measurement of glucose in serum has been developed, based on a screen-printed carbon electrode modified with Meldola's Blue–Reinecke salt, coated with the enzyme glucose dehydrogenase (from *Bacillus* sp.), and nicotinamide adenine dinucleotide coenzyme ($NAD^+$). A cellulose acetate layer was deposited on top of the device to act as a permselective membrane. The biosensor was incorporated into a commercially available, thin-layer, amperometric flow cell operated at a potential of only +0.05 V versus Ag/AgCl. The mobile phase consisted of 0.2 M phosphate buffer (pH 7.0) containing 0.1 M potassium chloride solution, and a flow rate of 0.8 ml $min^{-1}$ was used throughout the investigation. The biosensor response was linear over the range of 0.075–30 mM glucose, with the former representing the detection limit. The precision of the system was determined by carrying out 20 repeat injections of a 5-mM glucose standard, and the calculated coefficient of variation was 3.9%. It was demonstrated that this biosensor system could be applied to the direct measurement of glucose in serum without pretreatment. Therefore, this would allow high-throughput analysis, at low cost, for this clinically important analyte.


One of the most important measurements required in clinical analysis is the determination of blood glucose. Many biosensor strategies have been devised for this application, and most of these are based on the enzyme glucose oxidase [1]. However, an alternative approach would involve immobilization of glucose dehydrogenase (GDH)[1] and the cofactor $NAD^+$ (nicotinamide adenine dinucleotide coenzyme) onto a suitable transducer. This has the advantage of operating without the requirement of oxygen.

In addition, it has been reported that GDH–NAD has been recommended for blood glucose measurement owing to the lack of interference by other common sugars [2].

In previous studies, Hart and coworkers have developed amperometric biosensors, based on dehydrogenase enzymes, for a variety of analytes, including lactate [3,4], ethanol [5], and ammonium ions [6,7]. In these cases, the appropriate enzyme and cofactor were deposited on top of a screen-printed carbon electrode (SPCE) that had been modified with the electrocatalyst Meldola's Blue (MB). This electrocatalyst was selected because it allowed the detection of NADH (reduced form of NAD) at potentials close to 0 V [8]. The advantage of operating at such low potentials is that few naturally occurring compounds will undergo direct oxidation at the SPCE, significantly reducing interference. Therefore, we determined that an MB–SPCE would be an appropriate transducer for use in our proposed glucose biosensor.

The amperometric biosensors described earlier were operated in the batch mode and were intended for single use only. In this investigation, we decided to explore the possibility of developing an amperometric biosensor, based on GDH, for incorporation into a thin-layer flow cell for application in a flow injection analysis (FIA) system. Such systems are attractive because it is possible to perform high-throughput analyses of clinical samples with minimal operator effort and they are also readily automated. In addition, when the detector consists of a screen-printed biosensor that is used for multiple determination analyses, the manufacturing cost is low.

As mentioned earlier, we determined that an MB–SPCE with the biocomponents immobilized on the surface would be advantageous for our proposed glucose biosensor. In addition, in previous studies involving amperometry in stirred solution [3,5], the biosensor components were successfully retained at the electrode surface by use of a cellulose acetate membrane; consequently, we in-

* Corresponding author. Fax: +44 0117 328 2904.
*E-mail address:* john.hart@uwe.ac.uk (J.P. Hart).

[1] *Abbreviations used:* GDH, glucose dehydrogenase; $NAD^+$, nicotinamide adenine dinucleotide coenzyme; SPCE, screen-printed carbon electrode; MB, Meldola's Blue; NADH, reduced form of NAD; FIA, flow injection analysis; MBRS, Meldola's Blue–Reinecke salt; RS, Reinecke salt; AET, Applied Enzyme Technology; SCE, saturated calomel electrode.

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doi:10.1016/j.ab.2009.09.028

tended to investigate this approach in the current investigation. It should also be emphasized that we were interested in developing a flow system for continuous use on a daily basis, and we wanted to ensure that the water-soluble electrocatalyst MB did not leach from the transducer. It was reported by Schumann and coworkers [9] that a Meldola's Blue–Reinecke salt (MBRS) complex can be easily prepared and that this complex has low solubility in aqueous solutions. Therefore, we decided to investigate the use of this complex in our new amperometric glucose biosensor.

This article initially describes the fabrication and characterization of an MBRS–SPCE for NADH detection using cyclic voltammetry and amperometric detection within a flow injection system. Then we describe the preparation and characterization of a glucose biosensor based on the MBRS–SPCE. The application of this biosensor within an FIA system for the direct measurement of glucose in calf serum is discussed.

## Materials and methods

### *Chemicals and reagents*

GDH (*Bacillus* sp.) was purchased from Genzyme (EC 1.1.1.47) and had a specific activity of 76.9 U/mg. Newborn calf serum from bovine calves was obtained from Sigma–Aldrich (UK). All other chemicals were of analytical reagent grade and also purchased from Sigma–Aldrich.

$NaH_2PO_4$ (0.2 M) and $Na_2HPO_4$ (0.2 M) containing KCl (0.1 M) were mixed together to prepare the buffer solutions for all electrochemical investigations. The β-D-glucose standard solutions were prepared in phosphate buffer (pH 7.0) at a concentration of 1 M and allowed to mutarotate overnight at room temperature.

The MBRS complex was prepared by a modification of the method described by Schumann and coworkers [9]. Briefly, a solution containing 0.1 M Reinecke salt (RS) and 0.075 M MB was prepared by dissolving the appropriate weights of the two compounds in deionized water by heating for 2 h at 60 °C with constant stirring. The resulting precipitate was filtered and then dried in an oven at 50 °C, and the dried complex was then crushed to a fine powder using a pestle and mortar. This MBRS powder was then used in the preparation of the appropriate modified SPCEs and for the fabrication of the glucose biosensors.

### *Apparatus and biosensors*

Cyclic voltammetric measurements, for characterization of the screen-printed electrodes, were performed using a μAutolab Type II computer-controlled potentiostat (Windsor Scientific, UK).

FIA was performed with a homemade peristaltic pump connected to a Rheodyne (AnaChem, UK) four-way injection valve fitted with a 65-μl loop. This was connected to a thin-layer flow cell (Uniscan, UK) containing a screen-printed glucose biosensor (Applied Enzyme Technology [AET], UK).

All screen printing was done with a DEK 1202 semiautomatic printer (DEK Weymouth, UK). The MBRS–SPCEs (GEM [UK] base electrode code: BE2060227R2) were printed on an alumina substrate (from Coorstek–Fife–UK, laser cut at Laser Cutting–Ceramics, UK) for incorporation into the flow cell. Some electrodes were fabricated by screen printing carbon inks containing 2% MBRS (GEM code: C2070418D4), 2% MB (GEM code: C2030319P5), or just 2% RS (GEM code: C2060111R2) onto a plastic substrate (Valox). All of these were prepared by modification of a base carbon ink (GEM code: C2030319D4) using a design described previously [3], and in all cases an Ag/AgCl reference/counter electrode (GEM ink code: C61003P7) was screen-printed alongside the working electrode. These screen-printed electrode strips were investigated for their cyclic voltammetric behavior with NADH.

Biosensors were prepared by depositing onto the MBRS–SPCE 3 μl of a solution containing 10 U of GDH, 225 μg of $NAD^+$, 152 μg of stabilizer, and 15 μg of glutaraldehyde.

A membrane was deposited on top of the dried biosensor by depositing 15 μl of 2% cellulose acetate in acetone and allowing time to dry (30 min at room temperature).

### *Procedures*

Cyclic voltammetry was performed with 10-ml aliquots of NADH prepared at a concentration of 1 mM dissolved in 0.2 M phosphate buffer (pH 7.0) and 0.1 M KCl. The cyclic voltammetric behavior of this solution was examined with three different modified screen-printed electrodes, namely MB–SPCEs, MBRS–SPCEs, and RS–SPCEs. The voltammetric conditions were as follows: initial potential, −0.60 V; final potential, +0.80 V (vs. Ag/AgCl); scan rate, 50 mV $s^{-1}$.

All flow injection studies were performed with the MBRS–SPCEs housed in a commercial thin-layer amperometric cell. The mobile phase was 0.2 M phosphate buffer (pH 7.0) and 0.1 M KCl, and the flow rate was set at 0.8 ml $min^{-1}$. All injections of standards (prepared in the mobile phase) and neat serum samples were made through a 65-μl loop.

Using this system, a hydrodynamic study was performed to deduce the optimum potential to be used for the detection of NADH. The initial and final potentials were set at −0.4 and +0.4 V, respectively, versus Ag/AgCl.

A calibration study for NADH was performed by setting the potential at +0.05 V and injecting 65-μl aliquots of standard solutions over the range of 0.01–1.0 mM, with each concentration being injected in triplicate.

To prolong the lifetime of the proposed glucose biosensor, a study was performed to ascertain the suitability of various enzyme stabilizers being incorporated into the biosensor formulation. The following proprietary stabilizers (STKED formulation from AET) were investigated: Q2030317P47, Q2030317P48, Q2030317P49, Q2030317P50, and Q2030317P52.

The biosensor formulations were examined regularly for enzyme activity over a period of 240 days at 37 °C (15% humidity).

A calibration study for glucose, using the optimum biosensor composition, was performed with standard solutions over the range of 0.5–50 mM. All standard solutions were injected in triplicate through a 65-μl loop, and the operating potential was +0.05 V.

The optimized biosensor system was evaluated by carrying out glucose determination with commercially available calf serum. For these determinations, untreated serum was injected directly into the FIA system through a 65-μl loop, and this procedure was repeated seven times. The unknown glucose concentration was determined by comparing the resulting FIA peaks with a calibration plot constructed from known standard solutions.

## Results and discussion

### *Principle of biosensor operation*

The operation of the amperometric glucose biosensor is based on the sequence of reactions shown in Fig. 1. Initially, glucose undergoes enzymatic oxidation in the presence of GDH and $NAD^+$ to produce gluconolactone and NADH. Next, the reduced form of the cofactor chemically reduces the oxidized form of MB, which then undergoes electrochemical oxidation at the SPCE. The latter two reactions represent the electrocatalytic oxidation of NADH that generates the analytical response. It should be noted that,

the greater the concentration of glucose, the greater the turnover of NADH and the higher the response current.

*Voltammetric studies of NADH at different modified SPCEs and calibration by FIA*

Initial cyclic voltammetric studies were carried out to compare the redox behavior of NADH with the three different types of modified SPCEs. Both MB–SPCEs and MBRS–SPCEs exhibited well-defined anodic peaks at a potential of −0.074 V versus Ag/AgCl (Fig. 2, curves a and b, respectively). From this behavior, we deduced that an electrocatalytic response occurred for NADH with an MBRS–SPCE in a similar manner to that described earlier using an MB–SPCE (Fig. 1). Curve c in Fig. 2 shows a cyclic voltammogram for NADH obtained with the RS–SPCE; the anodic peak occurred at a potential of +0.508 V versus Ag/AgCl. It is clear that no lowering of the overpotential occurred in this case, indicating that the RS itself does not play a role in the electrocatalytic oxidation of NADH. These results indicated that it should be feasible to detect NADH, at a low operating potential, in studies where an MBRS–SPCE was employed within the FIA detector. It was also anticipated that this device would have the advantage of better operational stability over a prolonged analysis time.

Next, the hydrodynamic voltammetric behavior of NADH was examined using a flow injection system incorporating an MBRS–SPCE housed in the thin-layer flow cell. Fig. 3 shows the resulting plot of peak current versus applied potential. The $E_{1/2}$ value occurred at a similar potential to the $Ep_a$ obtained for NADH using cyclic voltammetry, confirming that the MBRS–SPCEs could indeed be operated at low potentials under hydrodynamic conditions. For operation as a flow injection detector, we selected a potential of +0.05 V versus Ag/AgCl.

To establish that a reliable analytical response could be achieved for the reduced cofactor, under flow injection conditions using an MBRS–SPCE, a calibration study was carried out over the range 0.01–1.0 mM NADH concentration, with triplicate injections of each concentration being made via a 65-μl loop. Fig. 4 shows the resulting flow injection responses from which the calibration plot was constructed (see inset). The plot was found to be linear from 0.01 to 1 mM NADH, the graph obeyed the equation $i_p$ (μA) = 2.46 × [NADH] (mM) + 0.03, and the $R^2$ value was 0.99.

From all of the above electrochemical studies involving NADH, we concluded that our new MBRS–SPCEs should indeed have application as the base transducer for construction of an amperometric glucose biosensor.

*Stabilization of the proposed biosensor and its characterization with standard glucose solutions and commercially available serum*

To be of commercial use, biosensors that can be stored for many months without deterioration are required. Consequently, a batch of biosensor formulations were stored at 37 °C (15% humidity), and after selected time intervals representative formulations were examined for their activity toward glucose.

The biosensor components contained one of the five stabilizers mentioned earlier; one further formulation did not contain a stabilizer and acted as the control. The relative stabilities of these compositions are shown in Fig. 5. Although all of the stabilizers

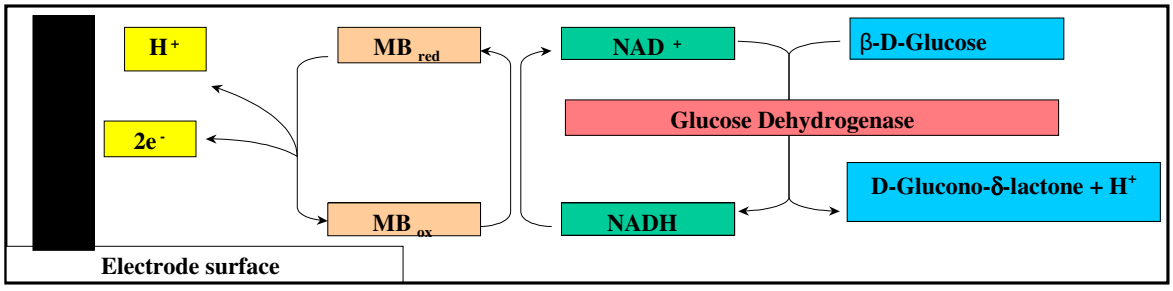


**Fig. 1.** Sequence of reactions involved in the operation of the glucose biosensor.

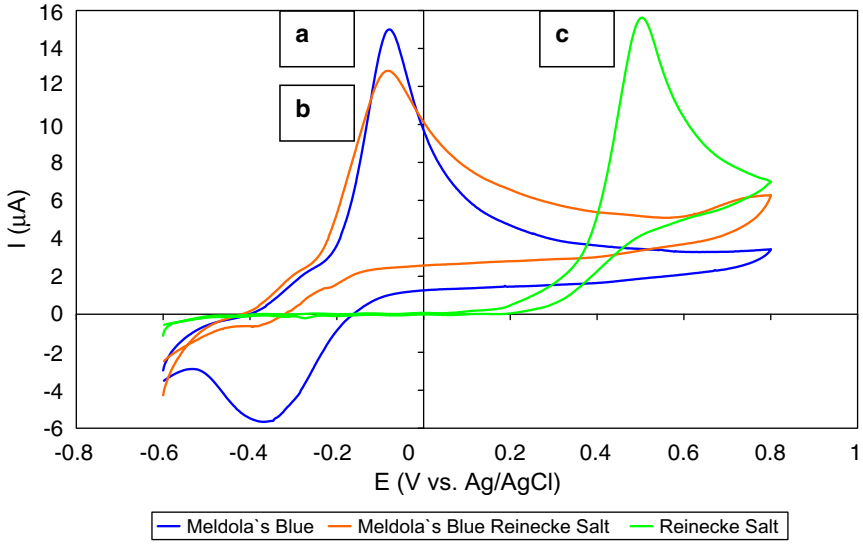


**Fig. 2.** Cyclic voltammetry of MB–SPCEs (a), MBRS–SPCEs (b), and RS–SPCEs (c) with 2.8 mM NADH in 0.2 M phosphate buffer (pH 7.0) and 0.1 M KCl. Scan rate: 50 mV/s.

examined conveyed some degree of stability over a period of 240 days at 37 °C, Q2030317P48 conveyed greatest stability; therefore, this was selected for the construction of all further biosensors discussed in the current investigation. It should be men-

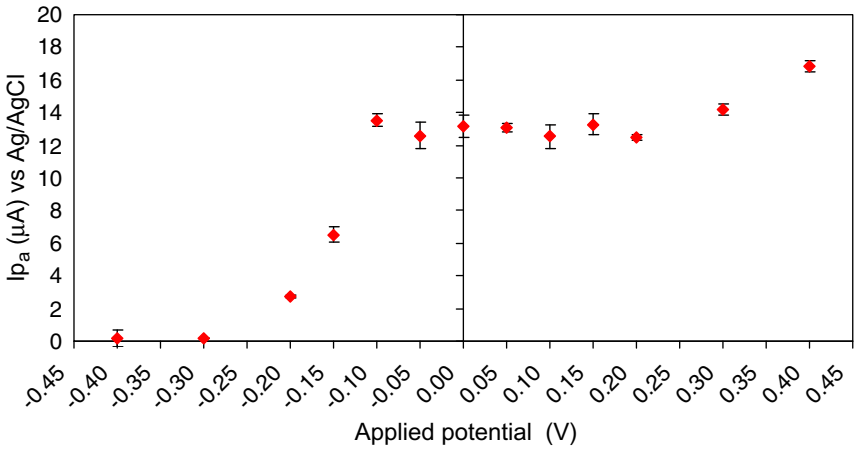


**Fig. 3.** Hydrodynamic voltammogram for MBRS–SPCEs obtained in 0.2 M phosphate buffer (pH 7.0) and 0.1 M KCl containing 3 mM NADH. Triplicate injections of 65 μl of NADH for each potential were applied.

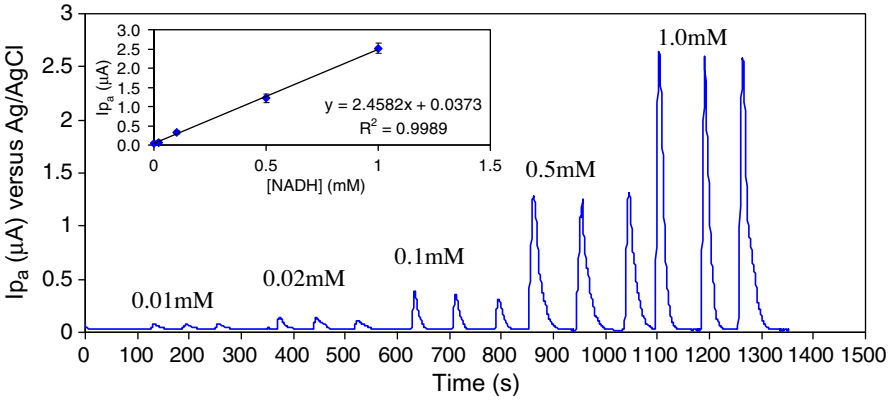


**Fig. 4.** Calibration study showing the individual FIA peaks at different NADH concentrations on single MBRS–SPCEs. Flow rate: 0.8 ml/min; carrier: 0.2 M phosphate buffer (pH 7.0) and 0.1 M KCl; potential applied: 50 mV. The inset shows the resulting calibration plot.

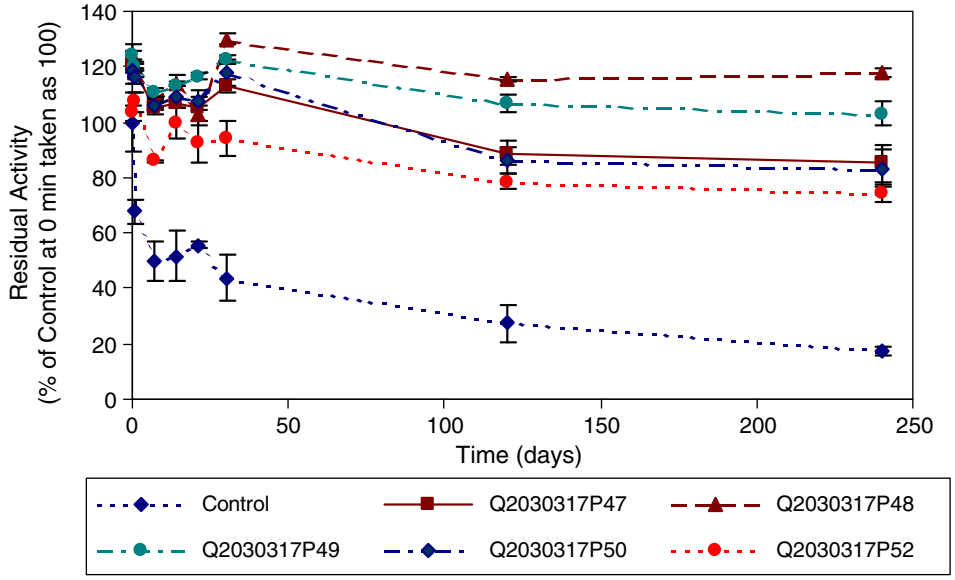


**Fig. 5.** Eight-month stability trial of GDH from *Bacillus* sp. enzyme stored using STKED AET formulations and with no stabilizer as control. Shown is a plot of percentage residual activity with respect to control. Biocomponents were incubated at 37 °C (15% humidity). All samples were run in triplicate and tested over a period of 240 days.

tioned, of course, that at normal temperatures for examples at 25 °C our biosensor would be stable for a period of 355 days. An initial dip in activity occurs but is fully restored after 20 days (Fig. 5). It is not clear why this occurs, but this phenomenon has been reported previously [10].

A calibration study on glucose was performed using standard solutions prepared in phosphate buffer (pH 7.0), with triplicate injections of each concentration being made via a 65-μl loop. Fig. 6 shows typical flow injection responses obtained for a variety of glucose concentrations. The resulting calibration plot (Fig. 6 inset) was linear over the range from 0.075 to 30 mM glucose, and the sensitivity determined from this plot was calculated to be 0.051 μA $mM^{-1}$. The precision was examined by making 20 repeat injections of 5 mM glucose (Fig. 7), and the calculated coefficient was determined to be 3.9%.

To evaluate our new biosensor for possible clinical applications, we performed replicate determinations on commercially available calf serum. The serum samples were simply injected into the flow system via a 65-μl loop without any previous treatment. Table 1 shows the calculated glucose concentrations that were found by referring the FIA peak currents to the calibration plot. Bearing in mind that the method required no sample pretreatment, it might be expected that for less complex matrices (e.g., fruit juices, food) glucose would be determined with somewhat better precision.

**Table 1**
Multiple injections of 65 μl of untreated calf serum using a single biosensor.

| Sample number | Calculated glucose concentration |
|---|---|
| 1 | 5.63 mM |
| 2 | 4.60 mM |
| 3 | 4.44 mM |
| 4 | 5.45 mM |
| 5 | 5.54 mM |
| 6 | 5.73 mM |
| 7 | 5.28 mM |
| Mean | 5.24 mM |
| Standard deviation | 0.51 mM |
| Coefficient of variation | 9.80% |
| Supplier's specified concentration | 5.828 mM |

*Note.* Flow rate: 0.8 ml/min. For $n = 7$ injections, 5.24 mM glucose was detected with a coefficient of variation of 9.80%, close to the manufactured specification of 5.82 mM glucose.

## Conclusions

This article has demonstrated the possibility of using screen-printing technology to fabricate MBRS-based sensors that produce well-defined electrocatalytic oxidation signals in the presence of NADH (Fig. 2). These devices were successfully converted to reusable glucose biosensors by depositing GDH, $NAD^+$, glutaraldehyde,

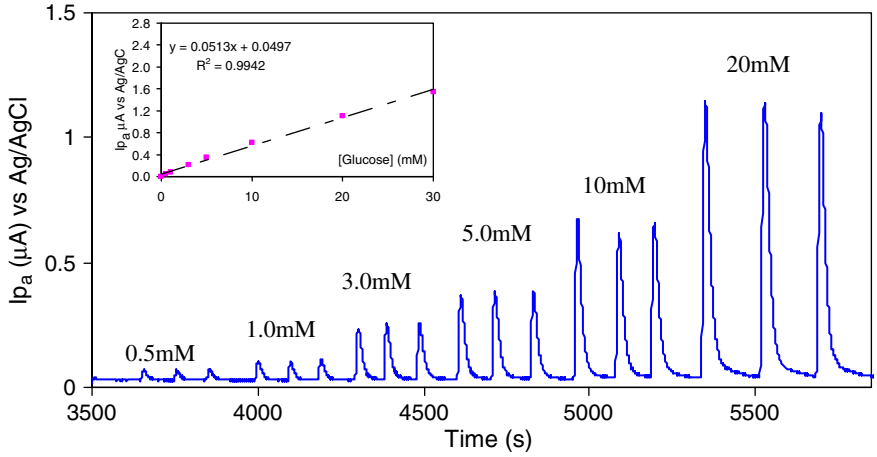


**Fig. 6.** Glucose calibration study using a flow cell containing MBRS–SPCEs. Flow rate: 0.8 ml/min; carrier: 0.2 M phosphate buffer (pH 7.0) and 0.1 M KCl; potential applied: 50 mV. Triplicate injections of 65 μl for each glucose concentration were tested. The inset shows the resulting calibration plot.

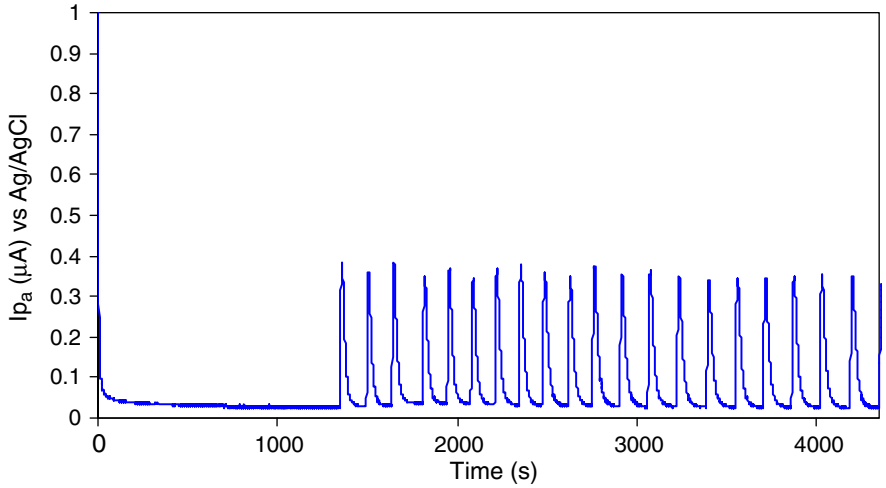


**Fig. 7.** Repeat injections of 5 mM glucose using the proposed biosensor.

and stabilizer onto the surface of the sensors, and these were coated with a cellulose acetate permselective membrane. These prototype biosensors were found to be stable for at least 240 days at a temperature of 37 °C. Calibration studies were performed using these devices in a flow injection system, and it was found that they gave a linear response to glucose over the range 0.075–30 mM. The coefficient of variation determined with 5 mM glucose was calculated to be 3.8% ($n = 20$). It was shown that the new biosensor system could be used for the direct analysis of glucose in commercially available serum without pretreatment with a reasonable coefficient of variation. It is feasible that other dehydrogenase enzymes may be incorporated with the MBRS–SPCE to produce amperometric biosensors for other clinically important compounds.

## Acknowledgments

One of us (M.P.) thanks AET and GEM for funding and Uniscan for use of the flow cell.

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