Sensors/biosensors, based on screen-printing technology for biomedical applications

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This article describes three recent developments from our laboratory, in which screen-printed carbon electrodes have been modified in order to develop sensors/biosensors for analytes of biomedical importance. The analytical applications described are (i) progesterone, (ii) glucose and (iii) haemoglobin determination. For each application, the modification procedure for the base transducer and the sensor/biosensor performance characteristics are described. The potential biomedical application areas for these devices are discussed.

In recent years there has been considerable interest in the development of electrochemical sensors and biosensors based on screen-printing technology. The reason for such interest is that the devices can be mass-produced in a wide range of geometries at low cost, particularly when carbon is used as the working electrode; therefore, they can be considered disposable. This is of particular importance when dealing with biological fluids, where contamination could lead to infection of the biosensor operator.

Hart and co-workers have previously reported on the development of various disposable screen-printed biosensors for compounds of biomedical importance. These devices used a carbon ink, known as C10903DI4 and supplied by Gwent Electronic Materials Ltd. (GEM), which has been shown to possess superior electrochemical characteristics. A biosensor for lactate was developed by employing a screen-printed carbon electrode (SPCE) comprising the above ink which had been doped with the electrocatalyst Meldola’s Blue (MB). The configuration of a typical SPCE is shown in Fig. 1. The enzyme lactate dehydrogenase was coated on top of the transducer together with NAD⁺; the signal resulted from the enzymatic conversion of lactate to pyruvate, which produces NADH, and this latter compound undergoes electrocatalytic oxidation at low potentials (0V). The device could be used either in stirred solution or in the chronoamperometric mode. A glucose biosensor has also been developed using the same primary ink to which had been added the electrocatalyst cobalt phthalocyanine (CoPC); glucose oxidase was immobilised onto the sensor surface using a simple drop coating procedure. In this case the signal arose from the enzymatic conversion of glucose to gluconolactone and hydrogen peroxide; the latter compound undergoes electrocatalytic oxidation at the CoPC-SPCE. Both of these approaches can be considered generic. Indeed, the CoPC-SPCE has been adapted for the detection of uric acid and cholesterol; the MB-SPCE has been modified to measure AST and NH₄⁺ (ref. 10).

Further biosensors have been developed using antibody-based systems and electrodes containing the primary C10903DI4 ink. The analyte was progesterone and the SPCE was coated with a monoclonal anti-progesterone antibody. The method involved a competitive assay using alkaline phosphate-labelled progesterone. Detection limits down to 5 ng ml⁻¹ were possible using the enzyme.

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Fig. 1—Diagram of disposable screen-printed electrode.
substrate naphthyl phosphate; the product, 1-naphthol, was detected at +0.3 V by chronoamperometry. In addition to fixed-potential methods with screenprinted devices, we have used stripping voltammetry to detect both Cu²⁺ (ref. 12) and Pb²⁺ (ref. 13); the former was present in a serum sample.

In the present paper we report on some new approaches to the measurement of the biomedically-important compounds glucose, progesterone and haemoglobin, using sensor technology based on the C10903D14 printing ink.

1. Antibody-based biosensor for progesterone

Increasingly sensitive and selective devices are continually being sought to monitor the levels of small analytes such as steroid hormones in biological fluids. Immunosensors provide a means of introducing the required selectivity in the form of specific monoclonal antibodies (mAbs). The use of an electrochemical method rather than a spectrophotometric measurement step provides the opportunity to record data with improved accuracy, reproducibility and automation in complex media, such as serum or milk, which may interfere with spectroscopic measurements, particularly in an automated, repetitive scenario. Automated immunoassay analysers are favoured in hospital laboratories for screening large numbers of clinical samples.

In the agricultural arena, an important potential application area for the electrochemical immunosensor exists to monitor progesterone levels in the milk of dairy cattle. This would enable the farmer to predict the onset of oestrus in cows, and would consequently be of considerable economic benefit to the dairy industry. Several studies towards this goal have been published using sensors based on SPCEs. These prototype devices were prepared by passive immobilisation, onto the SPCE surface, of anti-progesterone mAb via a capture layer of rabbit IgG (rIgG). The assay format for this application was a competitive immunoassay, using an alkaline phosphatase (ALP)-labelled progesterone conjugate. The enzyme substrate, 1-naphthyl phosphate, proved most effective in producing a sensor capable of monitoring progesterone concentrations down to the required detection limit of 5 ng mL⁻¹ (refs 16,17).

An alternative enzyme, horseradish peroxidase (HRP), is frequently used in enzyme immunoassays. One enzyme substrate 3,3',5,5'-tetramethylbenzidine (TMB) has been used in conjunction with HRP in various assay systems. The product may be determined either colorimetrically or electrochemically. Volpe et al. performed an ELISA in 96-well plates, based on an HRP-TMB system, and used spectrophotometric or amperometric detection methods to determine the product formed. By using an operating potential of +100 mV versus Ag/AgCl, they were able to reduce the product electrochemically and detect it using FIA at a glassy carbon electrode. The electrochemical results reproduced the spectrophotometric calibration data well. The performance of the progesterone immunosensor, using antibodies immobilised on the surface of an SPCE, has been investigated, this time in conjunction with horseradish peroxidase-progesterone (HRP-prog) conjugate and using TMB as substrate. In the present approach, rather than measure the reaction product by electrochemical reduction, the option has been used to measure the current response as a result of electrochemical oxidation at a higher positive applied potential. The assay scheme is shown in Fig. 2.

Experimental

Reagents and buffers—Buffer solutions were prepared in water drawn from a Purite RO200 purification system (Purite Ltd., Oxon, UK). Carbonate buffer (CB) was prepared using 0.15 M Na₂CO₃ and 0.035 M NaHCO₃ final pH = 9.6. Diethanolamine-HCl buffer (DB) was prepared using 0.1 M diethanolamine and adjusted to pH 7.2 with HCl. Citrate-phosphate buffer (CPB) contained 0.15 M citric acid, 0.15 M disodium hydrogen phosphate and 0.1 M KCl. Hydrogen peroxide (H₂O₂) was purchased from Sigma-Aldrich as a 30% w/w aqueous stock solution. HRP and TMB were purchased from Sigma-Aldrich Company Ltd., Poole, Dorset, UK. Polyclonal rabbit anti-sheep IgG (rIgG) was purchased from Sigma-Aldrich. Monoclonal anti-progesterone antibody (mAb), raised in sheep, was kindly provided in the form of culture supernatant by Dr. J. Foulkes, Ridgeway Science Ltd., Alvington, Gloucestershire, UK. This antibody originates from Drs. D.J.Groves and B.A.Morris, Department of Biochemistry, University of Surrey. HRP-prog, unlabelled progesterone and Tween 20 were purchased from Sigma-Aldrich. Ninety-six-well immunoassay plates were purchased from Dynatech Immulon, Fahrenheit Lab Supplies, Milton Keynes, UK.

Sensor preparation—Sensors were prepared as described previously17, by adsorbing rIgG passively in
CB, followed by mAb in DB, onto the 3 mm x 3 mm square surface of working electrodes which had been screen-printed onto PVC using code C10903D14 carbon ink supplied by GEM (Mamhilad, Gwent). Sensors were prepared in batches of up to thirty and were stored overnight in a moist box at 4°C before use.

**Cyclic voltammetry**—Cyclic voltammetric examination of TMB-plus-H$_2$O$_2$ in the presence or absence of HRP enzyme was conducted using bare C10903D14 working SPCEs in a three electrode system using a saturated calomel reference electrode and a platinum wire counter electrode. A cell volume of 20 ml CPB containing $10^{-2}$ M TMB and $10^{-3}$ M H$_2$O$_2$ served as a blank and was scanned from 0 V to +800 mV and back at 50 mV s$^{-1}$. The effect of HRP was evaluated after repeat scan of a fresh solution, following incubation in the presence of 65 units of enzyme for 15 min.

**Assay protocol**—Standard progesterone solutions, prepared in DB, were mixed with the HRP-progesterone conjugate (1 µg ml$^{-1}$) in a 3:5 v/v ratio. Eight-microlitre volumes of these mixtures were applied to the working electrode surface of biosensors and incubated at RT for 30 min. Sensors were washed twice with DB containing 0.05% Tween 20, followed by a single wash with CPB.

For the colorimetric assay, sensors were air-dried, then each was placed into the well of a 96-well immunoassay plate. Each well then received 150 µl of CPB, pH 5.0, containing $10^{-2}$ M TMB and $10^{-3}$ M H$_2$O$_2$. After incubation at room temperature (RT) for 50 min, sensors were removed from the wells and the absorbance of the solutions was read at 650 nm. For the electrochemical assay, sensors were placed into the chamber of a prototype thin-layer cell having a depth of 1 mm and internal volume of approximately 40 µl. A three-electrode system was employed, using an Ag/AgCl reference and a stainless steel counter electrode. This was connected to a Metrohm E611 VA Detector (Metrohm, Herisau, Switzerland). Substrate solution, comprising CPB, pH 5.0 containing $10^{-4}$ M TMB, $10^{-3}$ M H$_2$O$_2$ and 0.1 M KCl was injected into the cell. After 5 min incubation at RT, a potential of +450 mV was applied to the working electrode. The chronoamperometric response was recorded over the subsequent 60 seconds using an ADC-100 converter (Pico Technology Ltd., Cambridge, UK) connected to a Pentium computer running the PicoScope Virtual Instrument software for Windows.

**Results and Discussion**

The cyclic voltammetric response of TMB substrate is shown in Fig. 3, before and after addition.

![Fig. 2—Reaction scheme for competitive electrochemical immunoassay using HRP and TMB.](image-url)
of HRP enzyme in the presence of H$_2$O$_2$. In the absence of enzyme, the TMB/peroxide solution showed two distinct redox couples. These gave oxidation peaks at $E_p=+260$ mV and $E_p=+490$ mV respectively, versus a saturated calomel electrode. This indicated that TMB was predominantly in the reduced form before addition of HRP. The addition of HRP (Fig. 3) resulted in disappearance of the first of these peaks and a decrease in size and shift of the second peak to a more positive potential. This indicated that the HRP had oxidised the TMB to form an intermediate product, by converting the first redox couple to the oxidised form. Consequently, at an operating potential of up to about +450 mV, the oxidation signal from the TMB would be negligible following incubation with HRP. Figure 4a shows the result of the colorimetric immunoassay for progesterone, performed on the SPCE surface, and using TMB substrate solution. The plot shows decreasing absorbance with increasing progesterone concentration over the range 0-500 ng ml$^{-1}$. The steepest region of the plot occurs between 0 and 25 ng ml$^{-1}$. This result indicates that free progesterone can successfully compete with the enzyme-labelled progesterone over this concentration range.

For the chronoamperometric assay, an operating potential of +450 mV versus Ag/AgCl was chosen, being sufficiently positive to include the first of the two TMB oxidation peaks. Figure 4b shows a calibration plot obtained following the electrochemical assay. Current response increased with increasing progesterone concentration over the range 0-5 ng ml$^{-1}$ and appeared to reach a maximum at around 25 ng ml$^{-1}$. The coefficient of variation for a mean of replicate sensors ranged from 6.8% (n=3) at 50 ng ml$^{-1}$ to 19.1% (n=5) at 0 ng ml$^{-1}$. TMB has been used as a substrate for the HRP enzyme in numerous ELISA-type enzyme assays. Detection of the oxidation product of the enzyme reaction is commonly carried out colorimetrically, since it absorbs at wavelengths of 450 and 650 nm. TMB and its product are also electrochemically active compounds and TMB has been used as a substrate in electrochemical immunoassay systems. In these cases, immunoassays were performed in ELISA format. Electrochemical measurement steps were then carried out on the supernatants, either directly in the wells using a small gold working electrode$^{19}$, or following their removal to a flow-injection apparatus incorporating a glassy-carbon working electrode$^{18,20}$. The present study is novel in using TMB in conjunction with an immunoassay, all performed on the surface of the working electrode. In this respect, the format is self-contained and lends itself to single-use disposable biosensor production. By carrying out chronoamperometry at an operating potential which
will oxidise TMB, this approach measures the presence of the original TMB substrate i.e., that which remains unreacted. Consequently, the calibration plot obtained for the competitive assay has a positive slope, with a sensitive current response. This type of approach for progesterone detection, and its adaptation for detection of additional or alternative hormones, may have application in areas related to fertility, such as screening, diagnosis and monitoring.

2. Glucose Biosensor based on GDH and Meldola’s Blue

The measurement of glucose in blood is important in the diagnosis and management of diabetes, a disease that is increasing in prevalence worldwide. The majority of research and development towards an amperometric glucose biosensor for use in blood has used electrodes modified with the enzyme glucose oxidase, in combination with the electrocatalytic mediator ferrocene. Individual electrode strips are attached to a small device that applies the appropriate operating potential (+400 mV) and converts the current response into a glucose concentration, which is displayed digitally. Such devices can readily be used for personal blood glucose monitoring.

Although these devices show good levels of performance, their operating potential is relatively high for maximum selectivity and avoidance of interferent signals. As a result, attention has focussed on possible use of the NAD⁺-dependent glucose dehydrogenase enzyme (GDH) as an alternative to glucose oxidase. GDH utilises its cofactor NAD⁺ in order to facilitate oxidation of glucose to D-glucono-δ-lactone. The consequent reduction of NAD⁺ to NADH can be measured electrochemically in the presence of the electrocatalyst, Meldola’s Blue (MB). MB can be incorporated into the matrix of the working electrode and the measurement can be performed at an operating potential as low as ~0.05 V versus Ag/AgCl. The low operating potential reduces the likelihood of interference due to electroactive species present in the chosen sample matrix.

The following section describes some of the steps involved in the development of a disposable amperometric glucose biosensor, based on GDH and MB. The reaction scheme for the GDH-based biosensor is shown in Fig.5.

**Experimental**

**Chemicals and reagents**—All chemicals were of analytical reagent grade. GDH (EC 1.1.1.47) was obtained from either calf liver or *Bacillus megaterium* and supplied by Sigma or ICN pharmaceuticals (Basingstoke, Hants, U.K.), respectively. This enzyme was received as a lyophilised powder and was reconstituted in phosphate buffer of appropriate ionic strength and pH. The buffer used was 0.1 M potassium phosphate, prepared by making a 0.1 M solution of KH₂PO₄, then adjusting to the appropriate pH by the drop-wise addition of 1 M KOH. All solutions were prepared using water de-ionised by a Purite RO200 HP system. Whole blood samples were obtained from Southmead Hospital, Bristol, UK.

**Apparatus**—The electrochemical techniques were performed using an EG & G Model 264A polarographic analyser/striping voltammetry (EG & G, Princeton, U.S.A.), connected to a Linseis LY17300 plotter (Recorderlab, Sutton, Surrey, U.K.). All determinations were carried out in a glass voltammetric cell (D.A. Jones, Downend, Bristol, U.K.). During hydrodynamic measurements, a 14 mm diameter circular stirring disk (BDH) was rotated in the cell at a constant rate using a Gallenkamp magnetic stirrer.

The screen-printed electrodes were connected to the potentiostat by using two separate coaxial leads terminated with gold edge connectors.

**Biosensor construction**—The carbon ink (C10309D14, incorporating the electrocatalyst MB at 2 % w/w) and Ag/AgCl ink were supplied by GEM and screen-printed at GEM onto 0.5 mm thick PVC substrate. These electrodes were termed MB-SPCEs. The electrode configuration is shown in Fig.1. Rather than using PVC, a layer of Tippex correction fluid (Camberley, Surrey, U.K.) served as a dielectric layer to define the 9 mm² area of the working electrode. To construct glucose biosensors, MB-SPCEs were modified by drop-coating both GDH and NAD⁺ onto the surface of the working electrode. The biosensors were fabricated in two different fashions according to the source of the GDH.

Initial studies used GDH from calf liver (crude extract, low cost). A 15 µl volume of this enzyme, in
0.1 M phosphate buffer, was dropped onto the working electrode in three 5 μl steps, giving a total of 0.1875 units per sensor. This solution was highly viscous due to high protein concentration. GDH loading was followed by 10 μl of NAD⁺ of appropriate concentration, which was then allowed to dry. Finally, a polycarbonate membrane (0.4 mM pore size, Whatman, Maidstone, Kent, U.K.) was applied to the surface of the working electrode using superglue (UHU, Iseworth, Middlesex, U.K.).

The enzyme loading and subsequent studies were carried out using GDH from Bacillus megaterium (purified, higher cost). This form of GDH gave a low viscosity solution and could be drop-coated onto the electrodes in one step. As this type of the enzyme was used for loading studies, the volume drop-coated was dependent on the loading required. The volumes ranged from 1 μl (0.25 units) to 10 μl (2.5 units). Once the enzyme had dried, 10 μl of NAD⁺ of appropriate concentration was drop-coated onto the electrode and allowed time to dry before the application of the polycarbonate membrane.

The addition of the polycarbonate membrane enabled the biosensor to be used in bulk solution, by retaining the enzyme and co-factor in the vicinity of the working electrode, but also by preventing the leaching of MB from the carbon ink.

Electrochemical procedures—Cyclic voltammetry was used to investigate the interfering effects of ascorbic acid on the biosensor by using unmodified and MB-modified electrodes, both with and without polycarbonate membranes. Analyses were performed in a 10 ml buffer volume after a ten-minute period of degassing with nitrogen. The starting potential was -0.4 V and the switching potential was +0.4 V. Studies on initial response kinetics, NAD⁺ and GDH loadings, calibration and blood samples were undertaken using amperometry in stirred solution in a 6ml volume of 0.1 M potassium phosphate buffer. Once a baseline current had been achieved, either glucose or whole blood was introduced to the cell. The applied potential was 0 V vs. Ag/AgCl. All measurements were carried out at room temperature.

Results and Discussion

Initial studies into the effect of pH on biosensor response kinetics, indicated that pH 8.0 gave maximum values for Vmax and Km. For best precision, a sub-optimal pH value of 7.6 was selected for further study, based on the shape of the Vmax and Km-versus-pH plots (not shown). The loading of NAD⁺ was optimised by fabricating biosensors using a fixed loading of GDH (0.19 units/sensor), and varying the NAD⁺ loading from 120 to 300 μg/sensor. The highest sensitivity of response was obtained with 220 μg/sensor, whereas the Km of GDH showed very little variation over this range.

In order to determine the optimal loading of GDH, and working at pH 7.6 with 220 μg NAD⁺/sensor, concentration plots were obtained over the range 0-50 mM glucose, using biosensors fabricated with enzyme loading over the range 0.25-2.5 units/sensor. Maximum sensitivity, in terms of current response (at low glucose concentration), was obtained at a loading of around 0.5-0.75 units/sensor (Fig. 6). At higher loading, of 1 unit and above, a loss of sensitivity was evident. The physiological reference range for glucose lies between 2.5 and 7.3 mM. The best linearity over this range was obtained using an enzyme loading of 1 unit/sensor (Fig. 6).

Biosensors fabricated and operated under optimised conditions (ie., 0.75 units GDH, 220 μg NAD⁺, pH 7.6) were used to perform a calibration plot, in order to establish the working linear range for the glucose biosensor. The biosensor response was linear over the concentration range 2x10⁻⁴ to 1x10⁻³ M glucose (Fig. 7). The limit of detection (3xbackground current) was 8x10⁻⁵ M.

Results obtained from the operation of the biosensor on patient blood samples could be divided into two groups. The Group A population (four samples) gave a reasonable correlation between the biosensor and hospital (colorimetric) methods. However, the Group B population (8 samples) showed a positive bias towards the biosensor method. Cyclic voltammetric investigation confirmed that L-ascorbic acid will undergo electrocatalytic oxidation at a MB-SPCE. Investigations using amperometry in stirred solution containing candidate interferents at 10⁻³ M, demonstrated that L-ascorbic acid enhanced the current response by 180-fold, compared to enhancements of less than 5-fold by any of the alternative species (included paracetamol, L-cysteine HCl, reduced glutathione). From this preliminary study, it can be hypothesised that the presence of elevated Vitamin C levels in Group B samples was the likely cause of the observed bias in biosensor performance.

This study has demonstrated the feasibility of developing a biosensor based on GDH and MB to measure glucose. The observed linear range and detection limits are consistent with the required
3. Electrochemistry of haemoglobin at a carbon electrode based on a screen-printing ink and its voltammetric determination in biological samples

The role of haemoglobin (Hb) has been extensively studied, functioning in the pH control and the rapid exchange and storage of gases in the mammalian blood system. The accurate determination of Hb is also important in a number of clinical conditions, such as haematuria and haemoglobinuria. Commonly used methods to undertake this determination are based on the absorbance of cyanometglobin at 540 nm (ref. 26). However, this necessitates the use of KCN, with its obvious associated problems of toxicity. Other methods have been developed, but also suffer from similar toxicity problems, such as o-tolidine and benzidine.

Electrochemistry can offer an alternative approach, allowing for a possible assay to be developed without the use of these toxic reagents. However, electrochemical investigations of Hb have been hampered by the slow electron transfer rates exhibited by the molecule at solid electrodes. Various efforts have been made to enhance electron transfer rates, by a number of different authors. The slow electron transfer is thought to be a result of the large three-dimensional shape of Hb, which leads to the inaccessibility of the haem centres to the influence of the electrode. The situation is further hampered by problems of electrode passivation, resulting from protein adsorption.

In preceding reports, we have described the development of a range of electrochemical sensors and biosensors, based on the ink formulation designated C10903D14, for various other organometallic haem-like compounds, namely cobalt phthalocyanine, iron phthalocyanine, and cytochrome c. Accordingly, we considered that the same ink formulation would be suitable for the direct electron transfer processes encompassed in Hb. The following investigation is divided into three main sections. We firstly investigate the electrochemical response of Hb at our SPCEs and then optimise the conditions necessary for the determination of Hb. Finally, we appraise the sensor performance by attempting the quantification of Hb in a real blood sample.

**Experimental**

C10903D14 carbon printing ink and C61003D7 Ag/AgCl ink were supplied by GEM. Measurements were conducted using both plain and modified carbon working electrodes. In the latter case, the working electrode was a carbon printing ink impregnated electrode (CPIE). The CPIE was prepared by depositing 500 μl of a 50:50 mixture of C10903D14 ink/cyclohexanone onto a cellulose filter paper (Whatman qualitative, Whatman, UK), which was then dried at 60°C for 3 hours. A 4 mm-diameter disk...
was then cut out and arranged over the SPCE and used as the working electrode. All chemicals were of analytical reagent grade and all solutions were prepared in deionised water obtained from a Purite RO200-Stillplus HP System, (Purite Oxon, UK). Cyclohexaneone and ethyltrimethylammonium bromide (CTAB) were obtained from Aldrich, (Loughborough, UK). Lyophilised bovine haemoglobin was obtained from Sigma (Loughborough, UK). All other chemicals were obtained from BDH. Sodium dihydrogen orthophosphate (1.0 M), disodium orthophosphate (1.0 M) and trisodium orthophosphate (1.0 M) were used to prepare supporting electrolytes with different pH values. This was achieved by titrating the two appropriate salt solutions together until the desired pH was obtained. Sterile defibrinated horse blood was obtained from TCS Biosciences Ltd (Claydon, Buckingham, UK).

Voltammetric procedures—Cyclic voltammetry was performed with either an EG & G Princeton Applied Research Model 264A Polarographic Analyzer/Stripping Voltammetry in conjunction with a HP7047A x-y recorder, or an Autolab Pstat10 potentiostat interfaced to a PC for data acquisition, via the General Purpose Electrochemical System Software Package (GPES) version 3.4 (Autolab. Windsor Scientific Limited, Slough, Berkshire, UK). Hb solutions for voltammetric analysis were made by weighing the appropriate mass into a clean 10 ml glass vial, containing 0.10 g of CTAB. 6.0 ml of the appropriate buffer solution was then added and the resulting solution thoroughly mixed. The cyclic voltammogram was then recorded at the appropriate scan rate, with a starting potential of -0.3 V and a switching potential of 0.8 V, versus the screen-printed Ag/AgCl reference electrode. All the measurements were carried out on quiescent solutions.

Results and Discussion

Electrochemical response of Hb at plain unmodified SPCEs—Figure 8 shows the results of the cyclic voltammetric investigations. There was no detectable electrochemical response for Hb at the plain unmodified SPCEs (Fig. 8a). Modification of the working electrode has proved successful in our previous studies of metalloproteins. However, the CPE demonstrated only a small degree of direct electron transfer to Hb (Fig. 8b). Consequently, we investigated the possible use of the SPCE and the CPE in conjunction with an electron transfer promoter, such as CTAB. In the presence of CTAB, the SPCE showed no electrochemical response for Hb (not shown). However, in conjunction with the CPE a quasi-reversible response was obtained for Hb (Fig. 8c), and hence further investigations were performed to optimise this parameter. The electrochemical response for Hb was seen to increase rapidly for CTAB concentrations from 0 to 16.6 mg ml⁻¹, then become constant. At higher concentrations the solubility of CTAB became problematic; consequently further studies were performed at a concentration of 16.6 mg ml⁻¹ CTAB.

We believe that the cyclic voltammetric behaviour of Hb seen in our present study (Fig. 8c) resulted from the ferric (Fe³⁺) met haemoglobin, present before the application of the scan, being reduced to the ferrous form by the application of -0.3 V for 30 s (Eq. 1). This was then oxidised to Fe⁴⁺ (Eq. 2) on the forward anodic scan, resulting in the peak at la (Fig. 8c). This is then reduced to Fe⁵⁺ at peak lc (Eq. 3) on the return cathodic scan. The more positive peak seen at lla was concluded to be the result of the oxidation of bromide⁻⁻, present in the CTAB.

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\begin{align*}
\text{Fe}^{3+} + e^- & \rightarrow \text{Fe}^{2+} \quad \ldots \quad (1) \\
\text{Fe}^{2+} & \rightarrow \text{Fe}^{3+} + e^- \quad \ldots \quad (2) \\
\text{Fe}^{4+} + e^- & \rightarrow \text{Fe}^{2+} \quad \ldots \quad (3)
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Effect of pH on haemoglobin voltammetric response—The electrochemical behaviour of Hb was studied over the pH range 3.7 to 12.2, in 0.1 M phosphate buffer solution. The maximum response occurred at pH 5.6 and at this pH a reversible redox couple was observed. At pH values other than this, the system was seen to be irreversible. The scan rate (v) was varied between 10 and 100 mVs⁻¹ (n = 4) for each pH investigated. For all pH values investigated the iₒ was linearly related to vⁿ, showing that the system was diffusion-controlled. Next, the effect of the phosphate buffer concentration was examined on the voltammetric response of a sensor using 3.2 mg ml⁻¹ Hb in the presence of 16.6 mg ml⁻¹ CTAB. A plot of the response of iₒ vs. buffer concentration demonstrated a maximum at around 0.5 M. A buffer strength of 0.5 M was therefore selected for further investigations.

Calibration studies—Under the optimised conditions, the voltammetric response for the cathodic signal was seen to follow a linear response, up to 8 mg ml⁻¹, with a slope of 35.6 μA/mg ml⁻¹ and R² value of 0.9968. The anodic signal was linear up to 4.0 mg ml⁻¹, with a slope of 50.0 μA/mg ml⁻¹.

Analytical application—The proposed CPIE sensors were evaluated by carrying out Hb determinations on a defibrinated blood sample. A 6.0 ml aliquot of a Hb-free 0.5 M pH 5.7 phosphate buffer solution, containing 16.6 mg ml⁻¹ of CTAB, was pipetted directly into the electrochemical cell. A blank voltammogram was then recorded using the optimised electrochemical conditions. A 30 μl aliquot of the blood sample under investigation was then pipetted directly into the same solution. This was then thoroughly mixed by manual stirring, prior to recording the voltammogram under quiescent conditions. The concentration of Hb was determined using the method of multiple standard additions, with each addition the equivalent of 0.5 mg ml⁻¹ Hb. A mean recovery of 93.3%, with an associated coefficient of variation of 4.9%, was obtained for a blood sample fortified with 52.9 mg ml⁻¹ Hb (Table 1).

The data demonstrate that the proposed method holds promise for the determination of Hb in such blood samples, giving an overall assay time of about 30 s per sample.

The above results suggest the possibility that our CPIEs may find applications with other related metalloproteins. In further work we will investigate the use of other waveforms, such as square wave and differential pulse voltammetry, to improve the detection limit of the assay, and further investigate the surface characteristics of the CPIE. In parallel studies, we also intend to investigate the possibility of employing the CPIE in conjunction with Hb as the basis of biosensors for other analytes.

Conclusions

The three analytical applications described in the present article have demonstrated the potential for developing sensor/biosensors based on screen-printed carbon electrodes for biomedical use. The C10309D14 ink supplied by Gwent Electronic Materials Ltd. has proved to be suitable, as either the primary ink or in a modified form, for use in the working electrode. It may be feasible to convert these prototype concepts into commercial sensors with further development. It should be added that the C10309D14 ink is indeed the basis of several biosensors currently undergoing commercialisation.

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