CRANFIELD UNIVERSITY

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Biomimetic Sensors for HbA1c

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Biomimetic Sensors for HbA1c

SUPERVISED BY

PROF. SERGEY PILETSKY
PROF. ANTHONY TURNER

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for the degree of Doctor of Philosophy

Abstract

Diabetes mellitus is a growing health problem worldwide. Suitable long-term control and management of this disease are enabled by determination of glycated haemoglobin (HbA1c) in blood. The results are given as %HbA1c of total haemoglobin. Presently available tests vary in cost and convenience and there is an identified need to introduce improved equipment for self-monitoring. This dissertation focuses on fast and straightforward detection of glycated haemoglobin (HbA1c) using cyclic voltammetry and chronoamperometry. Haemoglobin was determined by monitoring its reaction with potassium ferricyanide on screen printed electrodes at an oxidative potential +500 mV. A working electrode was modified with carbon nanotubes to enhance electron transfer. A calibration curve was linear in a range from 0.83 to 83 mg/mL. Another innovative approach to detecting haemoglobin using its enzymatic activity was also developed. Detection of haemoglobin was performed with hydroquinone and hydrogen peroxide on screen printed electrodes at a potential -400 mV in a Flow Injection Analysis system (FIA). It enabled fast detection and amplification of electrochemical signals. Haemoglobin was detected in a range from 0.05 to 2 mg/mL. Also interferences caused by oxidation of substances such as ascorbic acid, uric acid, and acetaminophen present in blood were minimised at a reduction potential. Further work included the development of selective receptors for glycated haemoglobin (HbA1c). Aminophenyl boronic acid was polymerised on screen printed electrodes and on microplates in the presence of haemoglobin (imprinted polymer) or without protein (non-imprinted polymer). Improved binding of haemoglobin was obtained on an imprinted polymer as compared to a non-imprinted polymer which indicates the imprinting effect. Determination of glycated haemoglobin (HbA1c), in blood and control samples was also combined with chromatographic separation of total glycated haemoglobin and its further electrochemical detection. Results verified against spectrophotometric detection showed a good correlation with $R^2 = 0.9945$.

Acknowledgements

I would like to thank Prof. Sergey Piletsky and Prof. Tony Turner for their valuable help and supervision of the project. I wish to thank Pelikan Technologies Inc. for sponsoring my PhD. Also I would like to thank colleagues from Cranfield University for their help and friendliness.

Aim and objectives of the project

The goal of the project was to design a sensor for a fast and straightforward determination of glycated haemoglobin (HbA1c).

The first task of the project was to detect haemoglobin using electrochemical methods. Further work was focussed on the recognition of glycated haemoglobin (HbA1c) separately and in its mixture with different types of haemoglobin. The studies included research of molecularly imprinted polymers, boronic acid derivatives and their integration with an electrochemical sensor.

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Abbreviations

A: surface area of the electrode (mm^2)

ABTS: 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

APBA: 3-Aminophenylboronic acid monohydrate

CMCNa: carboxylmethyl cellulose sodium salt

CV: cyclic voltammetry

CE: counter electrode

DCPIP: dichlorophenol indophenol

DDAB: didodecyldimethylamonium bromide

E: electrode potential (V)

EC: electrochemical cell

E_p: peak potential (V)

E_{pa}: anodic peak potential (V)

E_{pc}: cathodic peak potential (V)

F: Faraday's constant (96485 Coulomb/mol)

FcBA: ferroceneboronic acid

FcCOOH: ferrocenecarboxylic acid

FIA: flow injection analysis

GHb: total glycated haemoglobin

*gp*51: bovine leukemia virus glycoprotein

Hb: haemoglobin

HbA0: non-glycated haemoglobin

HbA1c: glycated haemoglobin

$HbFe^{2+}$: haemoglobin isolated from human blood, mainly reduced form of Hb

$HbFe^{3+}$: haemoglobin, mainly oxidized form of Hb

HbS: abnormal haemoglobin in sickle-cell anaemia

HbC: abnormal haemoglobin caused by substitution of lysine for glutamic acid in β-chain

HbD: abnormal haemoglobin caused by genetic disorder

HbE: abnormal haemoglobin caused by substitution of lysine for glutamic acid in β-chain

HbF: fetal haemoglobin

HQ: hydroquinone

i_{pa}: anodic peak current (Amps)

i_{pc}: cathodic peak current (Amps)

j_{pa}: anodic current density ($\mu A/cm^2$)

j_{pc}: cathodic current density ($\mu A/cm^2$)

MB: Methylene Blue

MED: mediator

n: number of electrons exchanged within electrode reaction

NIP: screen printed electrodes (working electrode) covered with non-imprinted polymer

MIP: screen printed electrodes (working electrode) covered with imprinted polymer, template - $HbFe^{3+}$

MIP-0: screen printed electrodes (working electrode) or microplate covered with imprinted polymer, template – HbA0

MIP-1c: screen printed electrodes (working electrode) or microplate covered with imprinted polymer, template – HbA1c

PB: phosphate buffer

PBS: phosphate buffer saline tablet

PFi: potassium ferricyanide

PFo: potassium ferrocyanide

SPE-C: screen printed electrodes produced at Cranfield University

SPE CNT-D: screen printed electrodes modified with carbon nanotubes from DropSens Ltd.

SPE-D: screen printed electrodes purchased from DropSens Ltd.

SPE-G: screen printed electrodes provided by GEM Ltd.

SPE-P: screen printed electrodes provided by Pelikan Technologies Inc.

SPE-S: screen printed electrodes provided by Microarray Ltd.

R: universal gas constant

RE: reference electrode

T: temperature (^oC)

t: time (s)

v: potential sweep rate (V/s)

WE: working electrode

SECTION I: LITERATURE REVIEW

Chapter 1: Haemoglobin properties and determination of glycated haemoglobin in diabetes

1.1 Introduction

Diabetes mellitus is a disease characterised by an excess of glucose in blood and tissues as a result of insulin deficiency or/and insulin resistance. Type I diabetes (15% of all diabetes cases) and the most common Type 2 diabetes (85% of all cases) are caused by interplay of genetic and environmental factors. Most cases of diabetes are caused by obesity and physical inactivity and are on rise due to population ageing and urbanization. It can lead to vascular disease, such as stroke, blindness, renal failure, heart attack and circulatory insufficiency (Tuch *et al*., 2000; Watkins, 2003). Worldwide projections suggest that the total number of people with diabetes will rise up to 366 million in the year 2030. Health care costs due to diabetes are estimated to amount to about 14% of the total health care budget (Wild *et al.,* 2004). The disease formulation is very complex and depends on many factors and this complexity partly explains why there is still no cure available. Correct treatment depends on frequent estimation of glucose in blood and the periodic determination of glycated haemoglobin (HbA1c). Glucose measurement is important for setting insulin dosage, food intake and exercise. Most glucose meters measure glucose in a blood sample collected from a finger or arm using a lancet. Usually glucose measurements are performed 2-4 times per day, however higher frequency may be required. Unfortunately, it is possible to miss reoccurring high or low blood sugar levels. Therefore, attempts to develop an "artificial pancreas" have been undertaken. Sensors measuring glucose *in vivo* have been introduced onto the market. Medtronic offer a continuous glucose monitoring system (CGMS) alongside an insulin pump. Another suggested advantage of this approach is painless measurement of glucose and insulin delivery. However, it is necessary to implant the sensor under the skin of the abdomen. The use of this device may vary the detected level of HbA1c when compared with finger-sticks (Tuch *et al*., 2000; http://www.minimed.com access 07.05.2010). Measurement of HbA1c is the optimal

integrated indicator of glycemic control in diabetic patients and important for long-term control. Glucose passes easily into erythrocytes and therefore the rate of formation of glycated haemoglobin is directly proportional to the concentration of glucose in blood. Because the lifetime of red blood cells is around 120 days, evaluation of glycated haemoglobin reflects an average glucose concentration during this period (Hoffbrand *et al*. 2001). The American Diabetes Association recommends that physicians measure HbA1c at least twice a year in diabetic patients with stable glycemic control. Where control is unstable or if therapy has been changed, it is proposed to test more frequently (http://www.diabetes.org/ada access 07.05.2010).

Tests which are currently used for HbA1c determination vary in cost, convenience and performance. Market is dominated by laboratory testing, blood samples have to be collected and tested in a hospital laboratory. More convenient is Point of Care (POC) technology. It is possible to collect a blood sample at home and send to the laboratory or HbA1c test could be performed and interpreted in a physician's office. POC devices such as Bayer DCA 2000, Cholestech GDX, Metrika and Axis-Shield Nycocard HbA1c are available on the market. However, results may be imprecise due to difficulties in operating those devices, for example GDX and Nycocard devices require to perform several manual steps to estimate content of HbA1c (John *et al.*, 2006). There is still a case for introducing equipment for self-control. Therefore, production of an affordable HbA1c sensor or its integration with a glucometer, suitable for home use would be highly desirable for the market. This can improve a person's life and decrease institution health care cost.

1.2 Haemoglobin structure and properties

The major component of normal adult blood is haemoglobin type A. This molecule is a tetramer which is composed of two α and two β protein subunits which are made of 141 and 146 amino acid residues, respectively. All proteins are noncovalently bound by salt bridges, hydrogen bonds and hydrophobic interaction and form quaternary structure. Interactions between α and β subunits are responsible for allosteric properties of

haemoglobin (α1β1, α2β2). Each subunit is associated with one non-protein heme group which is shown in Figure 2 (Stryer, 2006).

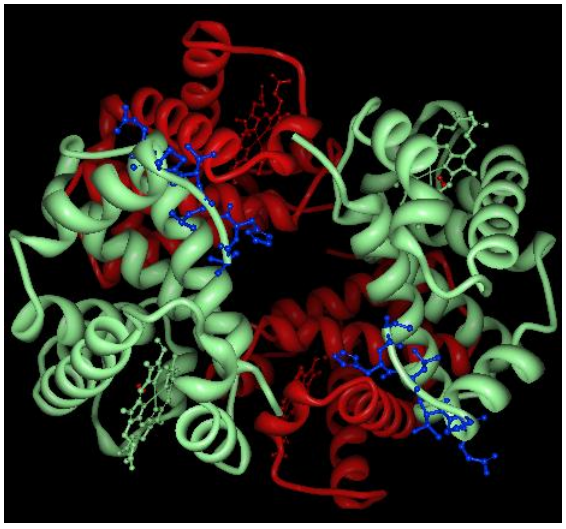

Figure 1. 3-dimensional structure of HbA. The four subunits are shown in red and green with heme groups, hexapeptide on β-chain in blue (www.rcsb.org./pdb/explore.do?structureId=1GZX access 08/05/2010)

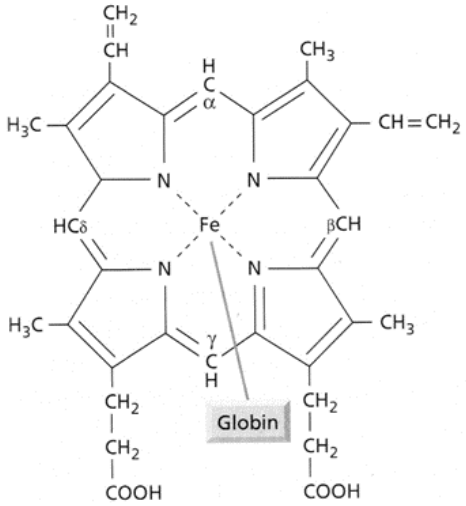

Figure 2. The structure of haem group (Hoffbrand *et al*., 2001)

The heme group consists of a protoporphyrin and a central iron ion, normally in the ferrous (Fe^{2+}) oxidation state which can form two additional coordination bonds. The iron atom is bound to four pyrrole nitrogen atoms and also to the protein via a histidine residue, precisely by its imidazole ring (Stryer, 2006). The total molecular weight of haemoglobin is about 68 kDa (Hoffbrand *et al*., 2001).

Different types of normal and abnormal haemoglobin are listed in the Table 1. Normal haemoglobin values are: 13.5 – 17.5 g/dL and 11.5 – 15.5 g/dL for males and females adults, respectively. Hb content is in a range 11 – 12 g/dL during pregnancy, 15 – 21 g/dL for newborn children and 11 – 13.5 g/dL for children (Hoffbrand *et al*., 2001).

Table 1. Types of haemoglobin

<table>
<tr><td colspan="7">Normal haemoglobin
(content in blood %)</td></tr>
<tr><td rowspan="3"></td><td colspan="4">HbA
(96-98%)</td><td>HbA2
(2.5%)</td><td>HbF
(0.5-0.8%)</td></tr>
<tr><td colspan="3">HbA1</td><td colspan="2">HbA0 (major)</td><td colspan="2" rowspan="3"></td></tr>
<tr><td>HbA1a</td><td>HbA1b</td><td>HbA1c</td><td>non-glycated</td><td>glycated
(2-4%)</td></tr>
<tr><td>remarks</td><td colspan="2">react with other carbohydrated than glucose</td><td>glycated at N-terminal Val (β chain)</td><td></td><td>glycated at ε-N-terminal Lys (α-chain)</td></tr>
<tr><td rowspan="2">structure</td><td colspan="4">α2 β2</td><td>α2δ2</td><td>α2γ2</td></tr>
<tr><td colspan="6">Hb is a tetramer. Two α globin chains are encoded by α globin genes (α1 and α2) located on the chromosome 16, while γ, δ, β chains are encoded by their respective genes ($^{G}\gamma$, $^{A}\gamma$, δ, β) located on the chromosome 11. Polypeptides γ, δ and β show structural differences.</td></tr>
<tr><td colspan="7">Abnormal Hb</td></tr>
<tr><td></td><td>HbS</td><td>HbE</td><td>HbC</td><td>HbD</td><td>HbH</td><td colspan="2"></td></tr>
</table>

Haemoglobin can undergo reversible and also irreversible modifications. This molecule is well known for its function of oxygen and carbon dioxide transport. It also acts as a buffer regulating the pH level of blood. A sixth position can be reversibly occupied by

oxygen. One haemoglobin molecule can bind four oxygen molecules cooperatively. This event causes changes in quaternary structure, namely α1β1 and α2β2 dimers move with respect to one another. This site can be also occupied by other small ligands, such as CO. Methaemoglobin (MetHb) is able to bind strongly ions, such as CN^- and N^{3-}. It is known that SH and NO blocking reagents react with cysteine residues at a position 93 on the β-chain subunit (Scheller *et al*., 2005). Haemoglobin can also interact with another protein and direct (heterogeneous) electron transfer between these molecules is possible. Its also has catalytic activity, catalysing a dehalogenation reaction and reduction of hydrogen peroxide (Imai, 1999; Winterbourn, 1985).

Haemoglobin has about 44 possible glycation sites which can react with glucose or derivatives and form different glycated haemoglobins. The most abundant type is HbA1c. 60 % of glycated haemoglobins are always a result of post-translational, non-enzymatic reaction of glucose and N-terminal valine of the β-chain. The reaction proceeds in two steps. Firstly, aldimine compound (Schiff base) is formed in a fast, reversible reaction. After that, ketoamine is formed due to internal, Amadori rearrangement. The last process is slow and irreversible (Peacock, 1984; Miedema, 2005). The resulting molecule does not have the ability to transport oxygen (Hoffbrand *et al*., 2001).

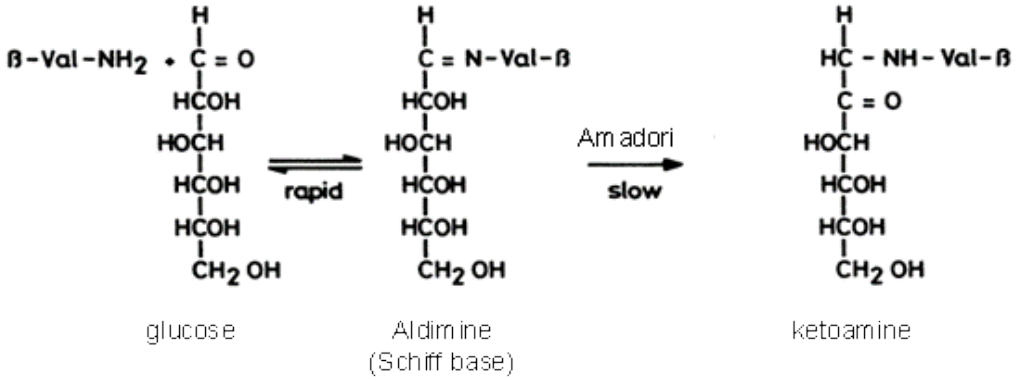

Figure 3. The formation of HbA1c (Peacock, 1984).

A useful property to distinguish HbA_0 from HbA1c is their isoelectric points, which is 6.972 and 6.935, respectively, and their different structure (Hempe *et al.* 1997).

1.3 Haemoglobin reduction / oxidation in human organism

The iron atom participates in redox reactions and may be either in the Fe^{2+} or Fe^{3+} state. The process of oxygen binding is associated with the partial electron transfer from the ferrous ion to the oxygen. Oxygenated haemoglobin contains a structure of ferric ion (Fe^{3+}) and superoxide anion (O^{2-}). Some ligands are able to stabilize the ferric form for example CN^- and lead to a MetHb formation which is unable to transport oxygen. MetHb is a by-product of respiration but is efficiently reduced by enzymes, thus less than 2% of haemoglobin exists in the form of MetHb (Stryer, 2006).

1.4 Influence of blood composition on Hb determination

Blood consists of a fluid medium, plasma (55% of blood volume) and different types of cells such as erythrocytes containing haemoglobin, leukocytes and thrombocytes. Blood plasma is mainly composed of water (90%), but also contains many vital proteins including fibrinogen, globulins and human serum albumin and inorganic electrolytes. Blood serves as transport medium for glucose, lipids, amino acids, hormones, vitamins, enzymes, antibodies, metabolic end products (urea, ammonia, bilirubin, urobilinogen), carbon dioxide and oxygen (Stryer, 2006).

1.4.1 Interferences during molecular recognition and electrochemical detection

Electrochemical sensors can suffer from interferences caused by substances, such as ascorbate, urate and acetaminophen, which may be oxidised instead of haemoglobin. However, the concentration of species mentioned above is less than 10% of the Hb concentration (Chen *et al*., 2003; Turner *et al*., 1999). Measurements performed at reduction potentials may also minimize interferences. There is a possibility to reduce $HbFe^{3+}$ (Shang *et al.,* 2003) or HbO_2 and thus avoid interferences caused by the oxidation of other electroactive species which are present in blood. Chen *et al.* (1998) proposed the following mechanism:

$$HbFe^{2+} + O_2 \rightleftharpoons HbFe^{2+}O_2 \rightleftharpoons HbFe^{2+} + H_2O_2$$

The reduction peak was estimated at potential -220 mV vs SCE on a bare glassy carbon electrode (GCE) containing 100 μM HbO_2 in a buffer solution (Chen *et al.*, 1998). Due to this reaction it could be possible to distinguish HbA1c from HbA0 since HbA1c does not bind the oxygen (Stryer, 2006). However, this solution seems to be impractical due to necessity to monitor precisely concentration of oxygen. Other potential interferents, such as human serum albumin can affect the Hb detection process. This molecule can interfere with the recognition step since the molecular mass (67 kDa) is similar to Hb and concentration of albumin is high, in a range 36 to 47 g/L in venous serum (Christopher *et al.*, 1999). Research performed by Bossi *et al*. (2001) showed that compounds, such as serum albumin have influence on the HbA_0 binding process. They found that the higher the addition of competitor, in that case bovine serum albumin (BSA), the lower is the absorption of HbA0 (Bossi *et al*., 2001). In other research, it was reported that it is possible to distinguish Hb from BSA due to different size and shape of these two proteins (Xia *et al*., 2005). Different studies utilising MIP to detect glycoprotein *gp*51 (bovine leukemia virus protein) showed a small influence of BSA towards the affinity of imprinted polypyrrole (Romanaviciene and Ramanavicious, 2004). Also abnormal haemoglobins and derivatives may interfere and produce a different signal in charge-dependent methods. In separation carbamylated and acetylated haemoglobin may co-migrate with HbA1c and HbA2 may be eluted with HbC (Hempe *et al*, 1997).

1.5 Reference method for determination of glycated haemoglobin (%HbA1c)

The A1C test is given as a percentage of haemoglobin that is glycated at N-terminal valine of β chain to the total content of haemoglobin. It is a useful index of glycaemic control and reflects the management of diabetes for the preceding 120 days. HbA1c values between 4-6% are considered as normal. People with diabetes are advised to keep their level of HbA1c below 7%, but the exact figure can be different for each method. Also, target value of HbA1c depends on health condition and age of diabetics. A target range may indicate acceptable values for a person with diabetes and this may

differ from the normal range seen in the non-diabetic population (Watkins, 1998; Silink and Mbanya., 2007).

Methods for separation of glycated haemoglobins are based on differences in their electric charge, molecular structure or immunological reactivity. Current measurements of HbA1c are based on three main assay principles, namely cation exchange chromatography, affinity chromatography and immunoassays. Affinity chromatography has minimal interferences resulting from hemoglobinopathies, carbamylated Hb and sample degradation problems as compared to the ion-exchange, electrophoresis or immunoassay methods. HPLC technique gives high precision and permits fast separation of HbA1c from HbA0 and the other minor components. It is very important to choose right reference method for all studies (Gallagher *et al*., 2009; http://www.ngsp.org/docs/methods.pdf access 05/05/2010).

In the past there has been a concern regarding a lack of standardisation. Measurements of glycated haemoglobin from different laboratories have not been easily compared. Presently many laboratories provide test that correlates well with the Diabetes Control and Complications Trial studies (DCCT, 1993). New methods are verified with National Glycohemoglobin Standardization Program (NGSP) which uses a reference method developed by Diabetes Control and Complications Trial, the BioRad Diamat (HPLC cation exchange chromatography). International Federation of Clinical Chemistry (IFCC) which established an international standardization program recommends two reference methods, HPLC-MS or HPLC-CE. Measurement is based on determination of the glycated terminal hexapeptide, cleaved from the haemoglobin molecule (Miedema, 2005; Gallagher *et al*. 2009). Another reference method can be compared with IFCC method. Complete list of methods and laboratories validated with NGSP standards can be found on NGSP website (http://www.ngsp.org/certified.asp, accessed 05.05.2010).

Chapter 2: Biosensor / sensor technology

Chemical sensors consist of a sensitive layer reacting with analyte and a transducer, which converts the binding event into a quantifiable output signal. The recognition element has a significant influence on selectivity, while the transducer mainly determines sensitivity. Biological materials such as enzymes, antibodies, tissue fragment and cells are frequently used as a recognition element in biosensors. However the application of biomaterials may be limited because of low stability and laborious, time-consuming and expensive production (Jankiewicz, 2001).

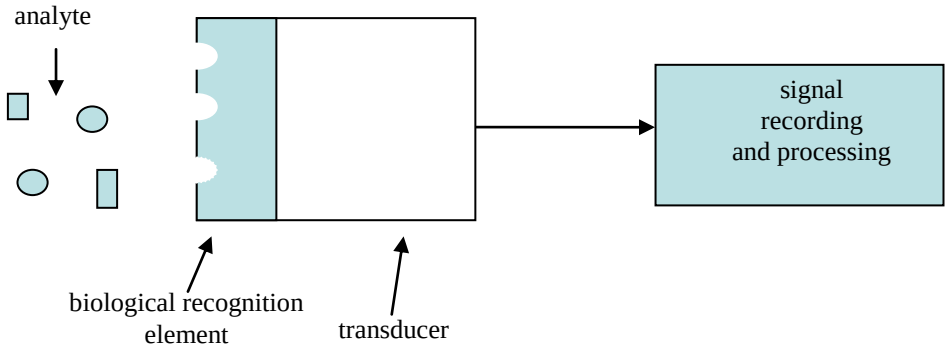

Figure 4. Scheme of biosensor (modified from Jankiewicz, 2001).

2.1 Transducer

The most frequently used transducers are electrochemical (for example potentiometric, amperometric, conductometric), piezoelectric (QCM) and optical (for example SPR, fluorescent, evanescent field devices). The most common in biosensors are amperometric transducers. Their construction is relatively uncomplicated which provides experimental simplicity and cost efficiency necessary in commercialisation (Law *et al.*, 2002). Amperometric, QCM and SPR transducers have already been tested for haemoglobin detection and examples will be presented in the review.

2.1.1 Electrochemical techniques

Potentiometric and voltammetric techniques are often used as diagnostic tools. Voltammetric techniques include: cyclic voltammetry (CV), linear sweep voltammetry (LSV), normal pulse voltammetry (NPV), differential pulse voltammetry (DPV), square wave voltammetry (SWV), anodic stripping voltammetry (ASV), cathodic stripping voltammetry (CSV), adsorptive stripping voltammetry (AdSV), polarographic techniques, chronoamperometry and chronocoulometry.

Electrochemical methods provide a direct relation between the electrical signal measured and the concentration of species in solution. In most cases equipment is not sophisticated and precise, fast measurement can be provided (Higson, 2003; Bilitewski and Turner, 2000). The majority of commercial biosensors are based on the electrochemical transducers, where the electrochemical cell consist of a working electrode (WE), reference electrode (RE) and counter electrode (CE). Electrodes commonly used are the silver/silver chloride for the reference electrode, carbon as a working electrode and carbon or platinum as a counter electrode. Current is measured following application of a potential difference between the working electrode and reference electrode and results from the oxidation or reduction of analyte on the working electrode.

Cyclic voltammetry is a very useful method for fast characterisation of redox reactions occurring on the electrode surface. This method can be also used to determine a concentration of reduced or oxidized species. Also a diffusion coefficient of electroactive substances can be determined. Voltage is changed linearly with time and oscillates between two switching potentials. Redox system is characterised by position of oxidation (E_{pa}) and reduction peak (E_{pc}), their separation (ΔE_p), midpotential (Em), and current density (j). Reversible reaction can be characterised under diffusion mass transport control by parameters such as: peak separation 0.059 V for one electron reaction and 0.0295 V for two electron reaction. Furthermore peaks position do not alter with a different scan rate, a ratio of anodic and cathodic peak current is equal to 1 ($i_{pa}/i_{pc}=1$). Peak current is proportional to square root of scan rate

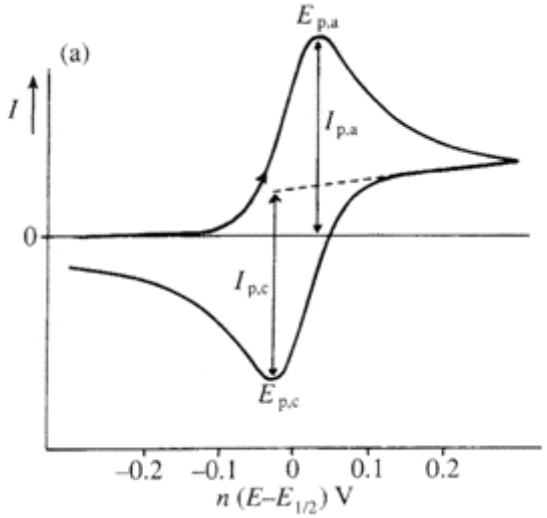

Figure 5. Cyclic voltammogram of reversible system (Brett and Brett, 1998)

Scan rate has a significant influence on shape of CV and is particuraly important for studies different coupled chemical reactions. For example, an electrochemical reaction may be reversible or not at different scan rate. Chemical reaction may not be observable at high scan rate. Furthermore scan rate should be considered particularly when an analyte concentration is very low since it has an influence on a charging current. Influence of background current is increased with higher scan rate. It is associated with faradaic and also with non-faradaic current, namely charging current between electrode and electrolyte (Eggins, 2002).

Chronoamperometry is very useful to evaluate an analyte concentration. Measurements are performed at a controlled potential where an electroactive substance undergoes a reduction or oxidation reaction. If a process is under diffusion control without an effect of convection and migration, and the electrode is relatively large and planar, the current is governed by the Cottrell equation:

$$i=nFAD^{1/2}C_{Ox}/\pi^{1/2}t^{1/2}$$

where, i is the current, n the number of electrons transferred per molecule, A is the electrode surface area, D is the diffusion coefficient, C_{Ox} is the substrate concentration, t is the time.

Reactants decrease near the electrode with time and create a diffusion layer. The current decreases with a square root of time. The current density strongly depends on mass transfer at a high overpotential and is independent of mass transfer at a low overpotential. The rate of the process is determined by the electron transfer at a low overpotential.

Experiments can be carried under conditions where a mass transfer is mainly caused by diffusion. A solution is not stirred and an electrode is not moved since it is important not to perturb the system. To avoid natural convection it is necessary to reduce timescale of experiment. However data can be distorted by an influence of charging current which is high at the beginning of measurement. The second type of experiments involves a controllable form of convection. It can be achieved by application of the rotating disc electrode. The same effect can be obtained in a flow system. A test solution flows through a channel in which there is placed a planar electrode. Two different configurations, such as thin-layer and wall-jet flow cells are commonly used. The migration is reduced by addition of an inert electrolyte (Pletcher, 1991; Wang, 2006). Figure 6 shows a current density versus time under different type of control.

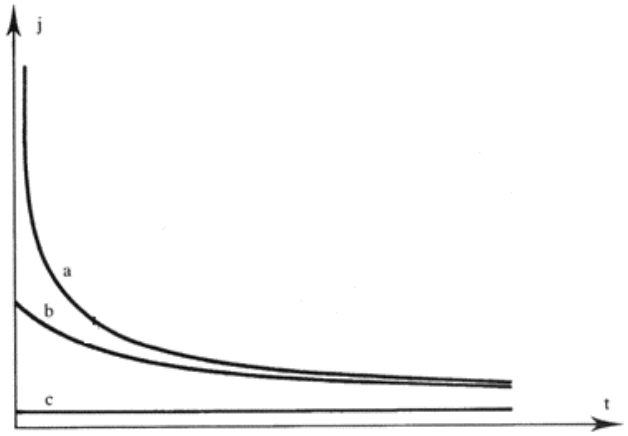

Figure 6. Current density versus time at a) diffusion control b) mixed control and c) electron transfer control (Pletcher, 1991).

Potentiometric sensors rely on monitoring a potential at zero current, which is expressed by the Nernst equation:

$$E=E°+2{,}303\ RT/nF\ \log_{10}([O_x]/[R])$$

where, E° is the standard redox potential, R is the gas constant (8.314 J/molK), T is the temperature, n is the number of electrons exchanged in reaction, F is the Faraday constant (96500 C/mol), Ox and R are the concentration of oxidised and reduced form of reagent (Eggins, 2002).

The important types of electrochemical sensors are ion-selective electrodes with example of ion-selective field effect transistors (ISFET). ISFETs are devices in which a transistor amplifier is used as a miniature transducer. They are used for measurement of potentiometric signals, which are produced on the gate (metal) of the FET. The gate is coated with a chemically sensing surface or an ion-selective membrane (Wang, 2006).

In many cases it is necessary to modify the electrode since compounds such as proteins cause a passivation of electron flow after being immobilised directly on the electrode surface (Brett *et al.,* 1999; Hart *et al.,* 2003). In recent years there has been interest in using different modifications of electrode surfaces employed to achieve better electron transfer and catalytic activity of Hb and other compounds.

2.1.2 SPR and QCM

Detection of binding process can be also realised using surface plasmon resonance (SPR) technique. Surface plasmons are formed in the boundary of a solid (for example metal). Electrons behave like a quasi-free electron gas. Exterior electrical fields in the boundary produce quanta of oscillations of surface charges with high-frequency electromagnetic fields. These plasmons can be excited by light or by electron beams. SPR is one of the most often exploited reflection technique. In one basic configuration, a quartz prism is covered on one surface by thin layer of metal for example silver or gold. A ligand film, able to bind an analyte, is coated on the metal surface. SPR reflectivity measurements can be used to detect proteins by the changes in the refractive index due to adsorption of the target molecule to the metal surface. This technique has been widely exploited in affinity biosensors (Law *et al.,* 2000). SPR technique was used to detect haemoglobin in blood sample by Yuk *et al.* (2007). They studied interaction between haptoglobin (Hp) and haemoglobin (Hb). Solution of mercaptoundecanoic acid

and mercaptohexanol were used to modify gold arrays. Significant interaction was observed when haptoglobin or antibodies were used as ligands against Hb (Yuk *et al.*, 2007).

A quartz crystal microbalance (QCM) measures mass by measuring the change in frequency of a piezoelectric quartz crystal when it is disturbed by the addition of an analyte. Its behaviour is explained by the Sauerbrey equation:

$$\Delta f = -2.3 \times 10^6 f^2 \Delta m/A$$

where, f is the overall resonant frequency, Δm is the mass of the adsorbed materials, A is the surface of a sensing region

It is useful to determine adhesion of proteins using frequency measurements with a high precision (Jankiewicz, 2001). Biosensors based on QCM transducer allow label-free determination of kinetic parameters of affinity interactions. This type of transducers was successfully used to estimate HbA1c. A self-assembled monolayer of aminophenylboronic acid (APBA) was directly deposited on the activated surface of a QCM transducer. The sensor was found to have a linear response in the range from 0.5 to 2.0 mg/mL. Thus it could be useful in HbA1c analysis since the values are close to physiological levels (Pribyl and Skladal, 2004). Further research using a combination of the piezoelectric sensor for HbA1c and a flow-through photometric sensor for total haemoglobin determination also showed good results. The detection ranges of total and glycated haemoglobin (HbA1c) were 0.05–2.0 mg/mL (R.S.D. 3%) and 0.01–0.090 mg/mL (R.S.D 12%), respectively. The calibration was made for a standard solution, not a real blood sample (Pribyl and Skladal, 2006).

2.2 Electrochemical properties and detection of haemoglobin

The main problems in electrochemical studies of Hb are slow electron transfer, poor signal and electrode passivation. This is due to the large 3-dimensional structure of Hb and hidden heme group inside the molecule, which results in inaccessibility of heme group (Hart *et al*., 2003). Other difficulties concern the amount of electroactive protein,

denaturation (Yan *et al*., 2005) and unfavourable orientation of haemoglobin at the electrode surface (Yin *et al*., 2006).

2.2.1 Mediated sensor

Numerous studies have been conducted using a mediator to facilitate electron transfer in biosensors. Commonly used mediators are ferrocene and its derivatives, ferricyanide, N,N,N'N'-tetramethyl-p-phenylenediamine (TMPD) and osmium complexes (Turner *et al.*, 1987; Reiter *et al.*, 2001). Their application can also reduce possible interferences from substances present in blood samples by reducing the potential required to monitor the reaction. For instance, ascorbic acid has a redox potential more positive than 0 V vs Ag/AgCl on a pyrolytic graphite (PG) electrode and at a lower potential it is electroinactive (Liu et al., 2004). All the most successful home blood glucose monitoring companies now use mediated electrochemistry (Turner *et al.*, 1999).

The electrochemical response in the presence of Hb on an electrode with a mediator, such as ferricyanide results from the oxidation of the heme group and reduction of the mediator. Oxidation of the reduced mediator produces current flow which is proportional to the amount of haemoglobin (Son *et al.*, 2006).

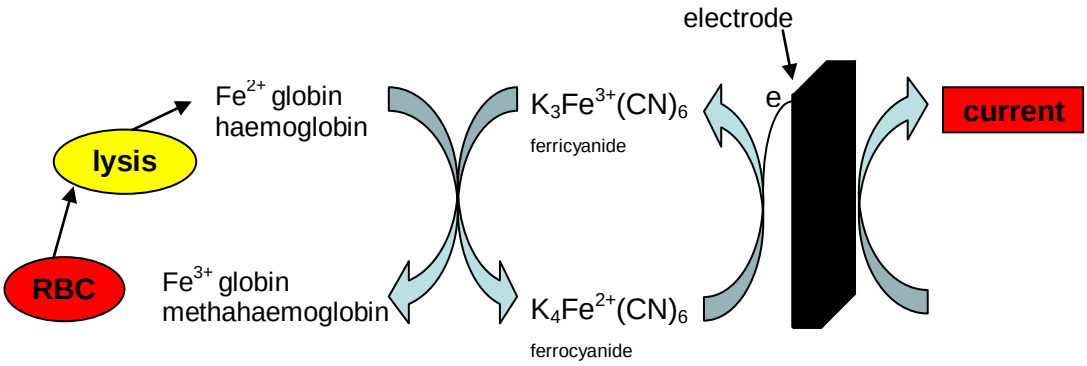

Figure 7. The mechanism of electrochemical detection of haemoglobin using a mediator. RBC-red blood cell (Son *et al*., 2006).

Jiang *et al.* (2005) also prepared a haemoglobin sensor using potassium ferricyanide (100 mM) in the solution. Studies were performed on two-electrode system, based on thin-film technology. The plastic substrate (0.5 mm x 6 mm x 1 mm) was coated with

NiCr/Au film (10 nm / 100 nm thickness) and afterwards electrodes were coated with carboxymethyl cellulose to form a hydrophilic membrane. Sensor response was studied with an amperometric method at the working potential of +200 mV. Authors suggested the catalytic mechanism as follows.

$$HbFe^{2+} + Fe(CN)_6^{3-} \longrightarrow Fe(CN)_6^{4-} + HbFe^{3+}$$

$$Fe(CN)_6^{4-} \rightleftharpoons Fe(CN)_6^{3-} + e^-$$

The first chemical reaction suggests that the process is irreversible. The calibration curve for the sensor was in the linear range of 10 – 3000 μM, which correlate well with clinical demand of 1600 – 2400 μM. The experiment was performed over 280 seconds and Hb was added to the mediator solution after 50 seconds. The correlation coefficient was 0.996 and standard deviation 2.3% for six measurements (2000 μM Hb). The response time of the sensor was 90 seconds and the detection limit for the sensor was 5 μM (Jiang *et al.,* 2005). They also showed detection of Hb using methylene blue as a mediator. A three-electrode system, which was used in the studies, consisted of a working electrode, Ag/AgCl as a reference electrode and platinum as a counter electrode. A cyclic voltammogram was performed at a scan rate 100 mV/s from 0 to -400 mV. The anodic peak potential was recorded at the potential -200 mV. The linear range was lower than in the previous experiment (from 0 to 200 μM).

A carbon electrode modified by ferrocene carboxylic acid (FcCOOH) was developed by Chen *et al.* (2003) who used a chronoamperometric method for the measurement of Hb in blood samples. The sensor was operated at +400 mV versus Ag/AgCl (reference/counter electrode). The authors reported a fast response with Hb solution and plateau after 10 seconds. The measurement time was less than 1 minute. Linearity was obtained with concentration of Hb up to 93 μM. Therefore, real blood sample with much higher level of haemoglobin required to be diluted. This step can also minimise possible interferences. It was reported that the amount of FcCOOH had an influence on sensitivity. A 1:1 ratio of carbon: FcOOH was chosen for electrode fabrication. The variation coefficient was 8.6% with n=10. The sensor could not be used many times because of mediator leaching out in a wash step (Chen *et al*., 2003).

Determination of haemoglobin ($HbFe^{2+}$) via its electrooxidation have been carried out on modified poly(methylene blue) electrodes. The electrochemical cell consisted of a glassy carbon electrode, SCE reference electrode and a platinum counter electrode. Tests were done using chronoamperometric methods with a potential of +550 mV vs SCE. Positive results were obtained with human whole blood samples (dilution 1:5), but not with commercial Hb at this potential; perhaps because commercial Hb is almost 100% MetHb. A standard deviation of 5% for n=3 was obtained (Brett *et al*. 1999).

2.2.2 Mediator-free sensor

Direct electron transfer between haemoglobin and an electrode is another approach. Construction of sensors without a mediator is based on application of nanoparticles, polymers and other materials. HbA0 / HbA1c detection is possible due to their properties and material characteristics. They play a role not only in electrochemistry, but also in providing analyte recognition matrices, which will be discussed in the next sections.

Metal nanoparticles have good conductivity and catalytic properties. They can decrease overpotentials of electrochemical reactions and promote the reversibility of some redox reactions. Nanoparticles play a role in immobilisation without a loss of bioactivity (Luo *et al.,* 2005). They can modify biological material or form nanotubes or nanowires on the electrode surface (Murphy, 2006). Nanoparticles have recently been more often used to enhance electron transfer between redox centres in haemoglobin and electrode surfaces. Their application is presented below.

Liu *et al*. (2006) studied direct electrochemistry of haemoglobin with cyclic voltammetry on zirconium dioxide (ZrO_2) nanoparticles in the presence of didodecyldimethylammonium bromide (DDAB). The electrochemical cell contained a platinum auxilary electrode and an Ag/AgCl reference electrode. Immobilisation of haemoglobin on particles showed a faster electron transfer $149s^{-1}$ in comparison with Hb immobilised in DDAB solution ($2.3+-0.4\ s^{-1}$) (Lu *et al.,* 1997), ZrO_2/DMSO ($7.9+-0.93s^{-1}$) or in clay colloid nanoparticles ($79s^{-1}$) (Lei *et al.,* 2002). Application of nanoparticles was used for determination of HbA1c. For this purpose researchers used

ZrO_2/DDAB nanoparticles, a pyrolytic graphite modified electrode (PG) and ferroceneboronic acid (FcBA). Ferroceneboronic acid was used for the recognition and detection of HbA1c. Recognition of HbA1c was possible due to specific affinity of boronic group to glucose. Ferroceneboronic acid is also electroactive compound and therefore it was possible to detect HbA1c with electrochemical methods (square wave voltammetry). Haemoglobin was immobilised onto the particles and cast on the electrode. Then the electrode was incubated with FcBA solution and unbound FcBA was removed in a washing step. The electrochemical signal resulted from FcBA bound to HbA1c. This could be explored to distinguish between Hb and HbA1c. It was calculated that only 4.6% of immobilised haemoglobin was active electrochemically. A high deviation in comparison to the standard method of HPLC (from -10.7 to 31%) was observed by researchers (Liu *et al.*, 2006). Further improvement will be necessary for a precise measurement of HbA1c.

Direct electron transfer was obtained by Han *et al*. (2002) using lipid (DDAB)-protected gold nanoparticles. They used a glassy carbon working electrode and a Ag/AgCl reference electrode. The formal potential was -169 mV vs Ag/AgCl. They showed that Hb can be adsorbed to nanoparticles keeping its natural activity. Most probably lipid provided the microenvironment for both electron transfer and immobilisation. Gold played a role as a link between haemoglobin and electrode.

Another approach is to cover nanoparticles with antibodies specific to haemoglobin and its detection by stripping voltammetry. A preconcentration of an analyte on the electrode may improve a detection limit. Electrochemical metaloimmunoassay was used successfully for myoglobin determination showing promising results as compared to ELISA assay (Szymanski *et al*. 2009).

Electron transfer and detection of analyte can be facilitated also by the application of carbon nanotubes. This probably results from their electrochemical and structural properties; the 3-dimensial structure and thus a larger surface area and a high conductivity (Yan *et al.*, 2005).

Direct electron transfer can be also improved by the application strategies based on other surface modifications. For instance, polymers such as polyaniline and polypyrrole can act as an electrical wire or mediator and thus facilitate electron conduction. This property may be used to detect binding events by monitoring decrease or increase in electron transport. They can be also utilized as a matrix for the preparation of affinity materials (Kriz *et al.*, 1995). Rick and Chou (2006) showed high conductivity of two polymer layers, namely poly-aminophenylboronic acid (APBA), formed on a polypyrrole. Current transmitting capacity was higher for this material than for a film made of p-APBA grafted directly on platinum.

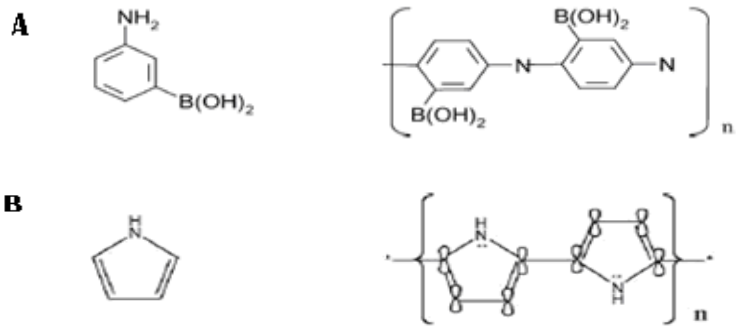

Figure 8. Structures of the monomers used and their polymeric forms. A) 3-amionphenylboronic acid and B) pyrrole (Rick and Chou, 2006).

Hart *et al.* (2003) combined an impregnated carbon-ink printed electrode (carbon/cyclohexanone) and a screen-printed carbon electrode for Hb measurement (lyophilised bovine haemoglobin). CV was run from -300 mV to +800 mV versus Ag/AgCl as a reference electrode. The authors also used an electron transfer promoter, cetyltrimethylammonium bromide (CTAB), with the analyte solution. The result was a quasi-reversible response in a presence of Hb. The voltammetric response for the cathodic and anodic signals was linear up to 8 and 4 mg/mL, respectively (lower than the values obtained by Chen *et al*. (2003) and Jiang *et al.* (2005)). There was no anodic or cathodic peak at a plain unmodified surface-printed carbon electrode with or without CTAB (Hart *et al.*, 2003).

2.2.3 Catalytic activity

Haemoglobin has catalytic activity because of its similarity with peroxidase (Shang *et al.*, 2003). Numerous studies have been performed to construct HRP-based sensor for hydrogen peroxide detection. Figure 9 shows schematic presentation of sensor performance.

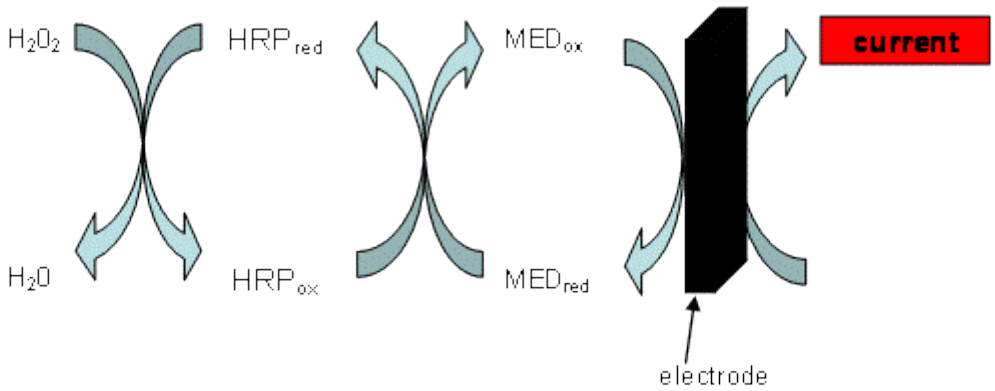

Figure 9. Schematic representation of electrochemical detection of hydrogen peroxide. MED_{ox} and MED_{red} are oxidized and reduced forms of mediator. HRP_{ox} and HRP_{red} are the redox forms of HRP.

Reactions catalyzed by HRP are as follows:

$$\text{HRP Fe}^{3+} + H_20_2 \longrightarrow \text{HRP}^{+\cdot}\ \text{Fe}^{4+}{=}0 + H_20$$

$$\text{HRP}^{+\cdot}\ \text{Fe}^{4+}{=}0 + \text{MED}_{red} \longrightarrow \text{HRP Fe}^{4+}{=}0 + \text{MED}_{ox}$$

$$\text{HRP Fe}^{4+}{=}0 + \text{MED}_{red} + 2H^{+} \longrightarrow \text{HRP Fe}^{3+} + \text{MED}_{ox} + H_20$$

The first reaction leads to a formation of an unstable intermediate which contains Fe^{4+} and porphyrin-radical cation. This compound reacts with a reduced mediator and gives HRP with ferryl cation (Fe^{4+}). The latter is reduced by a mediator to a native state of HRP containing Fe^{3+} (Schubert *et al.*, 1991; Lei *et al.*, 1996; Liu *et al.*, 1996). Detection of redox reactions with a mediator allows minimization of the interferences. The current signal is proportional to the amount of oxidized mediator resulting from an enzymatic reaction. Hydrogen peroxide concentration can be also evaluated by the well-known direct detection of hydrogen peroxide onto the electrode by its oxidation or reduction:

$$H_2O_2 \longrightarrow O_2 + 2H^+ + 2e^-$$
$$2H_2O_2 + 2H^+ + 2e \longrightarrow 2H_2O$$

However, this approach can suffer from interferences from other electroactive species at high potentials, which are required to apply for direct electrochemical detection of H_20_2 (Desai *et al.,* 1993; Stollner *et al.,* 2002; Wang, 2006).

The mechanism of Hb mediated catalytic activity towards hydrogen peroxide could be the same as for horseradish peroxidase. The reduced form of haemoglobin ($HbFe^{2+}$) can also react with hydrogen peroxide. However, reaction between $HbFe^{4+}$ and reduced mediator regenerates $HbFe^{3+}$ and not $HbFe^{2+}$ (Everse and Hsia, 1996).

Masuoka *et al.* (2003) showed an enhanced and faster catalytic activity of haemoglobin and also methaemoglobin A in the presence of reduced pyridine nucleotides. Inhibition of catalytic reaction was caused by oxygen, carbon monoxide, sodium azide and potassium cyanide. They suggested that NAD(P)H could protect haemoglobin from oxidative degradation. The pH 4.5-5.0 was estimated as optimal, however reaction in a neutral condition was also confirmed. In the absence of reducing NAD(P)H, ferryl radical could oxidise another Hb molecule or other biomolecule. Such mechanism was described by Gulivi *et al.* (1990) and Winterbourn (1985). However, catalytic activity of haemoglobin was 43 times lower than catalase. This should be considered in the haemoglobin sensor. Masuoka *et al*. (2003) explained catalytic activity of Hb in vitro by following reactions:

$$2H\text{-}HbFe^{2+}O_2 + H_2O_2 + 2H^+ \longrightarrow 2H\text{-}HbFe^{3+} + H_2O + O_2$$
$$H\text{-}HbFe^{3+} + H_2O_2 \longrightarrow HbFe^{4+}{=}O + H_2O + H^+$$
$$HbFe^{4+}{=}O + NAD(P)H + 2H^+ \longrightarrow H\text{-}HbFe^{3+} + NAD(P)^+ + H_2O$$

Furthermore, interesting studies were performed by Storle and Eyer (1991). In reaction between oxyhaemoglobin ($HbFe^{2+}O_2$) (3 mM) and N,N,N$^{'}$N$^{'}$-Tetramethyl-p-phenylenediamine (TMPD) (0.1 mM) they observed formation of ferrihaemoglobin ($HbFe^{3+}$). Catalytic mechanism was also confirmed with human red blood cells (3 mM

Hb) which were incubated with TMPD (0.1 mM). The reaction rate was lower in the presence of catalase. Therefore, they claimed that this process was enhanced by hydrogen peroxide which was formed during the process. The oxidation of TMPD (0.1 mM) with hydrogen peroxide (0.1 mM) was not observed. These researchers proposed a mechanism as follows:

$$HbO_2 + TMPD \rightleftharpoons HbFe^{3+}[-OO]^{2-} + TMPD^{+\cdot}$$

$$HbFe^{2+} + TMPD^{+\cdot} \rightleftharpoons HbFe^{3+} + TMPD$$

$$HbFe^{3+}[-OO]^{2-} + 2H^{+} \rightleftharpoons HbFe^{3+} + H_2O_2$$

$$HbFe^{2+} + H_2O_2 \dashrightarrow HbFe^{3+} + \text{products}$$

They suggested that the superoxide radical anion plays a minor role in ferrihaemoglobin formation while hydrogen peroxide is a major player. This fact can reflect gradual formation of hydrogen peroxide which itself produces ferrihaemoglobin. These studies indicate possibility for application of TMPD in electrochemical detection of Hb (Storle and Eyer, 1991).

Numerous studies have been dedicated to a catalytic activity of Hb including its application in biosensors. However, it has not been applied to detect haemoglobin using electrochemical techniques so far. This property has been applied to construct H_20_2 sensor. The signal was produced by a reduction of H_20_2 and direct electrochemistry of Hb (Feng *et al*., 2005; Liu *et al*., 2005; Masuoka *et al*., 2003; Li *et al*., 2006). Catalytic activity and direct electron transfer have been also reported to detect substances such as O_2, trichloroacetic acid and bromate (Li et al., 2006) and NO (Fan *et al.*, 2004). These reactions may also be used to detect Hb, but their efficiency might not be sufficient for biosensor application. Materials that may be exploited to assist in catalytic activity of biomolecules include amphiphilic polymeric polyacrylamide, lipid bilayers and other films. They create more suitable environment for protein function and also may have a positive impact on the protein orientation and location close to the electrode surface (Davis and Higson, 2005). The following examples show the detection of catalytic activity by reduction of $HbFe^{3+}$ on the electrode using various immobilisation techniques.

Shang *et al.* (2003) reported that Hb showed direct electron transfer and an increased peroxidase (reduction) activity after being captured in a DNA matrix. They used pyrolytic graphite (PG) as a working electrode, saturated calomel electrode (SCE) as a reference electrode and platinum as a counter electrode. The potential range of the CV was between +300 and -800 mV and the scan rate was 100-1000 mV/s. The last parameter had no influence on the peak shift. Key findings were the importance of pH on the CV (more acidic or basic pH gave higher peak). However, such conditions were not suitable for the stability of Hb-DNA (peak current was decreased about 25% after 20 cycles of a scan at pH 3.0). pH 4.5 was estimated as optimal. The linear range was $1.9x10^{-6}$-$6.8x10^{-4}$ mol/L with a detection limit $1x10^{-6}$ mol/L (Shang *et al.*, 2003).

Enzymatic activity was also obtained after immobilisation of Hb on chitosan films. Electrostatic interaction enhanced the process and Hb was protected in this environment showing good biological activity. Therefore, it can be presumed that an immobilisation can have a protective effect on the protein structure and preserve it from unfolding (Zhang *et al.*, 2006).

Application of phospholipid bilayers appears to be an interesting approach as they are structural elements of biological membranes. Different types of phospholipid bilayers have been constructed, for example Langmuir-Blodgett films (Du *et al.*, 2005; Davis and Higson, 2005). Lipid modified electrodes or surfactant for example polyions complex $2C12N^{+}PSS^{-}$ with similar properties to a lipid membrane (7% Hb was electroactive) have also proved useful for improving protein activity. It was shown that film made from dimyristoyl phosphatidylcholine (DMPC) and Hb immobilised onto pyrolytic graphite forms an ordered multilayer structure. This construction facilitated faster electron transfer. It was confirmed that Hb retained a near native conformation and showed reduction of trichloroacetic acid (Yang and Hu, 1999).

Yin *et al.* (2006) also showed that haemoglobin can be immobilised in a well-ordered form in a linoleic acid monolayer using the Langmuir-Blodgett technique (Hb-LA LB). It was found that LA facilitated electron exchange (ΔEp 80 mV) between Hb and gold

electrode and well-defined redox peaks were observed: Ep_a -180 mV, Ep_c -260 mV, E^o -220 mV (vs Ag/AgCl). Such modified electrodes had an activity towards reduction of H_2O_2 with a linear range of 2.0-100.0 μM and a detection limit of 1.2 μM (Yin *et al.*, 2006). Similar performance could be achieved by encapsulation of haemoglobin in mesoporous silicas. This is a new possibility for the adsorption of large molecules since the size of the pores can be tailored to the size of host molecule. Mesoporous silicas have large surface area and ordered pore structures. The behaviour of Hb on silica is facilitated by coulombic attraction between negative charge on the surface and positive net charge of the protein (at pH 5.0). Direct electron exchange and enhanced electron transfer were achieved. However, significantly better results were obtained with gold particles on silicas. Well-defined and quasi-reversible cathodic and anodic peaks were visible on a cyclic voltammogram, with potentials of -60 mV and +70 mV vs saturated calomel electrode (SCE), respectively. Moreover, the latter experiment showed three times higher catalytic H_2O_2 reduction with a range $5.0x10^{-6}$-$1.0x10^{-2}$ mol/L and a detection limit of $1.0x10^{-6}$ mol/L (Xian *et al.,* 2006).

Many reports discuss the catalytic role of haemoglobin on modified carbon nanotubes CNTs electrode (Zhao *et al.,* 2005; Wang *et al.,* 2005; Yan *et al.,* 2005).

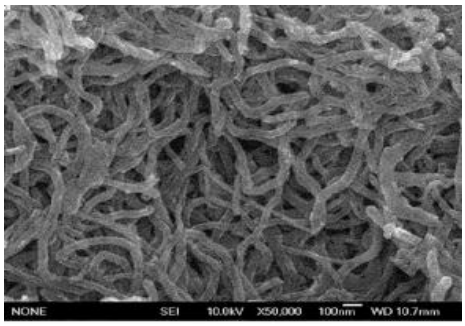

Figure 10. Carbon nanotube, magnification 50000x (Li et al., 2006)

Zhao *et al.* (2005) showed that Hb incorporated in carbon nanotubes had a reversible redox process at formal potential E^o -278 mV, peak separation: 119 mV, Epa -219 mV, Epc -338 mV, scan rate 20 mVs^{-1}. The electron transfer rate constant (k) was estimated as $0.062s^{-1}$. Furthermore, Hb kept its native structure and showed catalytic activity toward H_2O_2 at CNT surface (at a potential of 0.8V). However, the detection limit needed to be improved in this case since it was relatively high: $9x10^{-6}$ mol/L. The linear response was in the range $2.1x10^{-4}$-$9x10^{-4}$ mol/L (Zhao *et al.*, 2005).

Fabrication of H_2O_2 biosensor was also performed by immobilisation of Hb on multi-wall carbon nanotubes (MWNT) and gold colloidal nanoparticles (GNPs). It was based on electrostatic interaction between positively charged Hb and negatively charged particles. The layer Hb/GNPs was assembled on the layer of Hb/MWNT, previously arranged on a glassy carbon (GC) electrode. CVs of Hb/MWNT/GC and Hb/Au/Hb/MWNT/GC gave redox peak at -260 and -370 mV at a scan rate of 50 mV/s. The latter also showed a higher oxidation peak. Increasing amount of H_2O_2 caused a reduction peak increase and an oxidation peak decrease. Peak currents increased linearly with scan rate from 10 to 300 mV/s, which indicated a surface-controlled process. The pH 6.0 and potential -300 mV were chosen for optimal action of the sensor. Linear response was observed for concentrations $2.1x10^{-7}$ – $3.0x10^{-3}$ M H_2O_2 and response time 5 seconds. Possible interferences of uric acid, glucose and ascorbic acid were not observed. Prepared films showed good catalytic activity (Yan *et al.*, 2005).

Catalytic response was observed with H_2O_2 and $NaNO_2$ on polyamidoamine (PAMAM) dendrimers films. PAMAM films seem to be suitable because their structures mimic a three–dimensional structure of bio-macromolecules and have a good biocompatibility. Catalytic response toward $NaNO_2$ was detected with polyanionic poly(vinyl sulfonate)-Hb (PVS-Hb) layer-by-layer modified PG electrodes. Percentage of electroactive Hb was 46% and k_s around 50 s^{-1} (Wang and Hu, 2001; Shen and Hu, 2005).

Another example of an electrochemical biosensor is based on fructosyl – amine oxidase. HbA1c was detected by specific antibodies and then H_2O_2 was produced as a result of fructosyl – valine oxidization, evaluated amperometrically. The sensor (Pt, Ag/AgCl) was protected against non-specific adsorption of protein by cellulose membrane and thus avoided its passivation (Stollner *et al.*, 2002).

Chapter 3: MIP technology

Molecular imprinting is a generic technology for the introduction of recognition properties into synthetic polymers (Piletsky and Turner, 2006). The process includes formation of a complex between the template molecule and a functional monomer. This structure in molecularly imprinted polymers (MIPs) is "stiffened" by co-polymerisation with cross-linker. Eventually, the printed molecule is extracted in a washing step, while binding sites specific to the template are left in the polymeric network (Piletsky and Turner, 2002).

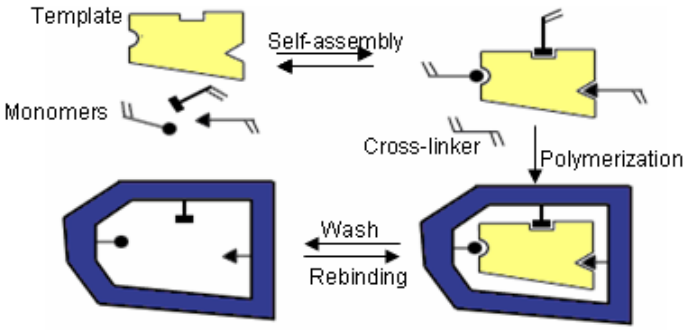

Figure 11. Schematic process of molecular imprinting technology

Functional and cross-linking monomers having vinyl and acrylic group are commonly used in the technology. However other organic polymer and silica are also exploited (Haupt and Mosbach, 2000).

Novel technology for HbA1c determination may include a recognition layer of molecularly imprinted polymer integrated with an electrochemical transducer. Biomimetic sensors, which employ artificially created materials, would be very desirable in the sensor area. Also the application of chemical compounds with high affinity to the analyte can be useful. For instance, Bossi *et al*. (2004) achieved some positive results in separation of haemoglobin by capillary electrophoresis, but further improvement, for example, the extending detection range remains to be done. APBA was polymerised in aqueous environment onto silica capillaries. Such materials could be useful in devices such as microchips (Bossi *et al.*, 2004).

3.1.1 Different approaches in MIP preparation.

MIP can be made as a self-assembly or pre-organised system (Figure 12.). Each of these strategies has its advantages.

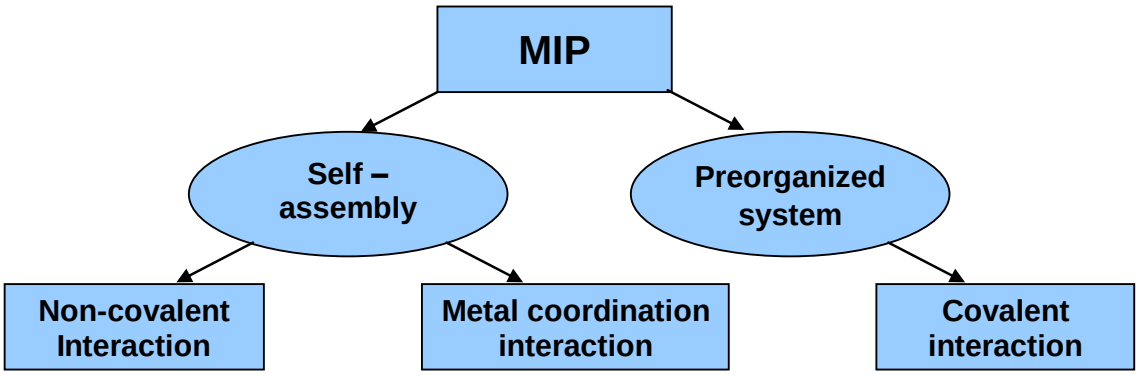

Figure 12. Different approaches in MIP preparation.

Monomers covalently bound with a template create polymers with higher stability and homogenous population of binding sites. On the other hand, non-covalent interactions are more similar to these occurring in natural systems in which most interactions are non-covalent and could be used for imprinting of proteins with a variety of segments and folding motives. Therefore MIP for protein recognition could be more efficient employing weak multipoint interactions instead of a few strong bonds. The non-covalent interactions seem to be more compatible with biological materials (Haupt and Mosbach, 2000; Bossi *et al.*, 2001). These two strategies were combined in Hb imprinting to introduce useful features from both of them (Shiomi *et al*. 2005). Template was covalently attached to the functional monomer, but an analyte was rebound by a noncovalent association. This approach can be used to hydrolyze ester, amide, imine bond and utilise rebinding through electrostatic interaction between for example respective amine and carboxylic acid. However, problems may appear during template removal. Binding capacity and selectivity may be lowered due to high stability of the monomer-template complex. Incorrect positioning of functional group for optimal non-covalent recognition may also have a significant impact (Klein *et al*., 1999; Shiomi *et al*. 2005).

Procedures for MIP preparation encompass a bulk polymerisation (Piletsky and Turner, 2002), surface grafting (Bossi *et al*., 2001) or electropolymerisation (Rick and Chou, 2006). Surface grafting and electropolymerisation are believed to be more suitable for protein imprinting. They can overcome problems, such as destruction of affinity sites, restricted mobility and poor reversibility while ensuring efficiency in the binding process. Surface support can play a stabilisation role and help to achieve correct orientation of functional sites (Bossi *et al*., 2001; Wei *et al*., 2006). The affinity of conventional MIP preparation was 100 times smaller than the affinity of grafted MIP on the polystyrene surface (Bossi *et al*., 2001). These procedures lead to formation of two or three dimensional structures with specific cavities for small and more complex molecules.

3.1.2 Two and three-dimensional MIPs

Grafted layers can be formed by aminophenyl boronic acid (APBA) on the electrode surface in the presence of haemoglobin. The holes remaining after the template extraction have been reported to be complementary in size to the template molecule (Bossi *et al*., 2001).

Phenyl boronic acid (PBA) has been used in many studies because of the boron group, which forms a diol recognition element acting as an antibody – mimicking compound. A covalent bond is formed between aminophenyl boronic acid and cis-diol group of glucose attached to haemoglobin under alkaline conditions (Frantzen *et al*. 1995).

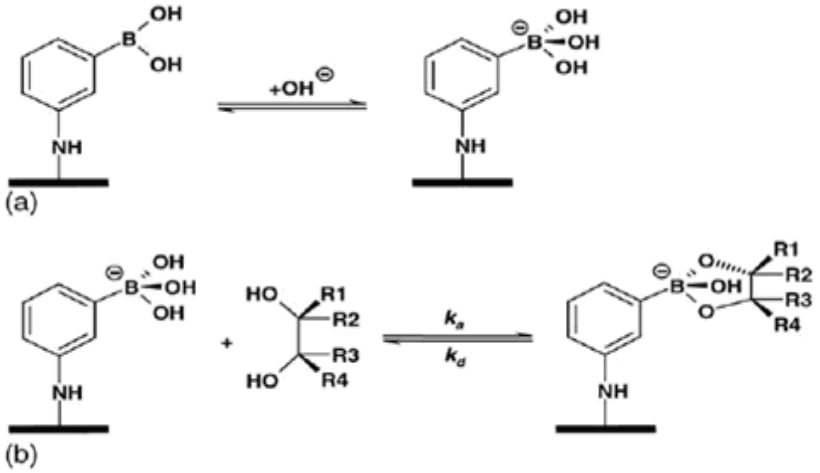

Figure 13. Boronate-diol ester formulation (Pribyl and Skladal, 2006).

An experiment using aniline and APBA for a protein imprinting showed possibility of electrostatic interaction between boronic acid and secondary amino and hydroxyl groups in the template. However aniline has positive charges which might react non-specifically with negative residues of the protein. As a result, discrimination between specific and non-specific binding can be difficult to achieve. In this respect poly-APBA which holds an equal number of positive and negative charges is better suited for protein imprinting (Piletsky, 2001).

MIP formation for detection of glycated haemoglobin (HbA1c) was successfully performed by Bossi *et al*. (2001). The formed MIPs were able to distinguish between HbA0 and HbA1c (Table 2).

Table 2. Analysis of haemoglobin binding to imprinted polymers prepared from APBA (Bossi et al., 2001).

MIP \ Analyte	**HbA1c**	**HbA0**
	K_{dS} (nM)	
HbA1c	65 +/- 2	162 +/- 9
HbA0	188 +/- 10	58 +/- 2

A surface grafting approach was utilized in this research. Polymer was formed around the template on a solid surface of microplates (Bossi *et al*., 2001). As a result, the polymer did not have a high level of cross-linking which probably helped to recognise flexible protein structure (Piletsky *et al*. 2006). Haemoglobin properties, such as shape

and difference in isoelectric points were explored in the detection process (Bossi *et al*., 2001).

APBA was also successfully used to detect another protein. This time two layers of APBA were deposited on the polypyrrole substrate. The outer layer was polymerised in the presence of lysozyme or cytochrome c. An imprinting procedure was successfully employed to detect these proteins. Sensor showed characteristic two-phase response after binding with lysozyme. The first step showed change of concentration, probably due to specific binding sites and the second phase showed smaller change because of a non-specific binding. However, the procedure was not reproduced for other proteins, such as RNAseA and myoglobin (Rick and Chou, 2006).

Table 3. Percentage current decreases with the increasing concentration of protein (Rick and Chou, 2006)

Lysozyme Concentration (ppm)	**Observed electrode % current reduction**	
	Templated	**Control**
1	30.3	4.5
2	48.8	15.9
4.8	62.0	29.8
9.4	68.3	45.8

Electrochemical measurements were performed on screen printed platinum as a working electrode at the potential -100 mV vs Ag/AgCl reference electrode. The passage of current decreased more rapidly when proteins were bound to the imprint than to control electrode. This was probably caused as a result of the specific binding due to shape complementarity of specific binding sites. The thickness of recognition layer seems to be very important. It can influence binding specificity of template entrapment (Rick and Chou, 2006).

In another example Hb was imprinted on a silica surface and showed selective absorption. It was possible to distinguish haemoglobin from myoglobin despite the smaller size of myoglobin (17.5 kDa) and approximately the same pI (Shiomi *et al*. 2005).

Furthermore, the epitope approach, which exploits a short terminal protein as a template shows great promise. The produced recognition site may enable specific determination of unstable proteins, which is problematic using traditional imprinted polymers made using whole protein template (Rachov and Minoura, 2001).

3.1.3 MIC-Molecular imprinted catalyst

It is also possible to create an artificial enzyme by using MIP technology. An attempt to create an artificial fructosylamine dehydrogenase was made by Sode *et al.* (2003). Methyl valine, an analog of fructosyl valine (Fru-val), was used as a template molecule. Allylamine was used as a functional monomer to increase selectivity toward Fru-val. It was possible to distinguish Fru-val from fructosyl-e-lysine. This was comparable with another piece of work (Yamazaki *et al*., 2003) where fru-val was used as a template. Functional monomer, 4-vinylboronate, and cross-linker, EDMA-ethylene glycol dimethacrylate were used. The authors produced a co-polymer, polyvinylimidazole, which functioned as a catalyst of the oxidative hydrolytic reaction of fructosylamine compounds in the presence of electron acceptor and polyvinylphenylboronate. m-PMS (1-methoxyl-5-methylphenazinium methylsulfate) was used as an electron acceptor. To be used in blood this approach however will require pre-treatment with a protease to release fru-val residue from protein molecule (Sode *et al*., 2003).

3.1.4 Parameters influencing MIP performance

There are many parameters affecting MIPs structure and their molecular recognition properties as shown in Table 4. These parameters often interplay together, thus it is necessary to understand them to create the most suitable balance among them. For example, both higher concentration of initiator and increased time of polymerisation are able to decrease polymer swelling (Mijangos *et al*., 2006). It was assumed that MIP should be synthesised using low concentrations of initiator and low temperatures, but time of this process should be increased. Temperature should also be optimised along with pressure and polymer swelling. It is necessary to optimise incubation conditions during analyte detection as well (Piletsky *et al*., 2004; Romanaviciene and Romanavicious, 2004).

Table 4. Influence of parameters on efficiency of molecular imprinting.

Polymerisation parameters	**Influence**	**References**
Type and concentration of monomers, cross-linker, solvent	bonds generation polymer affinity and specificity polymer structure stabilisation	(Piletsky *et al*., 2001; Piletsky *et al*., 2002 ; Romanaviciene, Romanavicious 2004; Karim *et al*., 2005)
Template : functional monomer ratio	Complex formation and re-binding of a template	(Lin et al., 2007)
Initiator	higher concentration –higher rigidity, increased temperature, larger surface area, lower amount – decreased temperature, superior recognition abilities	(Mijangos *et al*., 2006)
Solvent	Organic solvent is not suitable media for protein imprinting. Type of solvent affects volume and diameters of pores and swelling process. Polar solvent improves biocompatibility but is responsible for destabilisation of electrostatic interaction between functional monomer and template	(Haupt and Mosbach, 2000 ; Mijangos *et al*., 2006; Wei *et al*., 2006; Piletsky *et al*., 2002; Yilmaz *et al*. 2000)
Template molecule	Size, complexity, functional group, charge (kind and distribution) affects quality of created sites and their accessibility	(Romanaviciene and Romanavicious 2004), (Piletsky *et al*., 2001)
Ionic strength	Influences polymer-ligand binding	(Karlsson *et al*., 2001)
Time	Longer polymerisation time increases polymer rigidity and specificity; possible slow binding kinetics	(Piletsky *et al*., 2002 ; Mijangos *et al*., 2006)
Temperature	Lower temperature increased strength of polar interactions and produces better quality of the imprinting cavities	(Piletsky *et al*., 2002 ; Mijangos *et al*., 2006)
pH	Extreme pH value might cause protein denaturation; has influence on affinity depending on polymer/template properties	(Romanaviciene and Romanavicious 2004)
Pressure	Plays indirect role in morphology and performance of polymers, influence on the boiling temperature of the solvent	(Piletsky *et al*., 2004)

3.1.5 Design of molecularly imprinted polymers

From the theoretical point of view, MIPs can be generated for any substance even if there is no corresponding biological receptor. So far imprinted polymers were developed successfully for inorganic ions, nucleic acids, pharmaceuticals, sugars, proteins and cells (Piletsky *et al*., 2001). This technology has significant advantages in comparison to other methods where biological material is applied. Its application could improve stability, reduce cost and facilitate preparation (Piletsky *et al*., 2006).

Improvement in MIP preparation can be achieved by application of chemometrics (statistical analysis) (Mijanogos *et al*., 2006), computational design and combinatorial chemistry. Simulation experiments facilitate understanding of molecular mechanisms governing molecular imprinting processes (Wei *et al*., 2006). Molecular modelling can enable selecting the most suitable parameters, such as functional monomers, cross-linkers and solvents and thus provide very stable complex in the pre-polymerisation mixture (Piletsky *et al*., 2002; Karim *et al*., 2005). However, the accuracy of these results has to be confirmed by experimental analysis and the validity of the model system should be established (Wei *et al*., 2006).

A thermodynamic model for the MIP design was suggested by Nicholls. The binding site distribution and the binding equilibrium were studied (Nicholls, 1998). Molecular modelling has been applied for optimisation of polymer composition using protocol developed in Cranfield University (Nicholls *et al*., 2005). The studies showed that affinity of computationally designed MIPs is similar to those of polyclonal antibodies. K_d 0.3 ± 0.08 nM (computational MIP) and 0.5 ± 0.07 nM (polyclonal antibodies) and estimated sensitivities are also within similar range (0.1-100 and 0.05-10 µg/L correspondingly) (Piletsky and Turner, 2002).

Until now molecular imprinting has been usually employed for small molecules. Therefore it is still a challenge to obtain a MIP for protein detection, especially where the protein is characterised by a high molecular weight, such as haemoglobin (Karim *et al*., 2005; Piletsky *et al*., 2006). The problems with protein imprinting are mainly caused by an unstable three – dimensional structure, possible rearrangement processes

and a poor solubility of the template in organic solvents (Bossi *et al*. 2006). Also use of epitope approach for the recognition of two similar proteins, such as HbA1c and HbA0 may be problematic.

Chapter 4: Device configuration

It would be desirable to configure a device that integrates laboratory functions on a single small chip, capable of handling extremely small fluid volumes. Miniaturising a chemical analysis system is very important and microsystem technology to deal with "micro" amounts of fluids has to be developed. This approach exploits areas such as micro-electro-mechanic systems (MEMS), lab-on-a-chip technology and micro Total Analysis Systems (μTAS) (Noda *et al*., 2006). Blood analysis has been performed reasonably often using such devices (Son *et al*., 2006; Noda *et al*., 2006 and Tanaka *et al*., 2006).

4.1 MIP and other recognition materials as a chromatographic medium

One approach may encompass separation of HbA1c on a MIP - chromatographic medium combined with electrochemical detection. The chromatographic matrix could increase the number of specific recognition sites and enable efficient detection.

Presently, an ionic exchange matrix and boronic affinity materials are used for separation of glycated haemoglobin. An attempt to measure HbA1c using a chip based on a miniaturised flow immunoassay systems was performed by Tanaka *et al.* (2006). Lab-on-a-chip technology has been developed in which boronate-affinity chromatography was combined with electrochemical detection. The process is showed in Figure 14. Antibodies were conjugated with ferrocene monocarboxylic acid, which is good electrochemical label. Separation of HbA1c was performed on a column packed with agarose beads consisting of m-APBA. Detection took place in an electrochemical cell equipped with a glassy carbon electrode (WE), platinum (CE) and Ag/AgCl (RE) (Tanaka *et al*., 2006). Tanaka *et al*. (2009) also developed a similar electrochemical system with a cation exchange resin for HbA1c separation. HbA1c level was determined in a range from 4 to 12.6 %, spectrophotometrically and electrochemically. Presented results, however, concern mainly spectrophotometric detection of HbA1c.

Moreover the RSD% of electrochemical detection of HbA1c up to 19.1% may suggest that the system did not work efficiently (Tanaka *et al*. 2009).

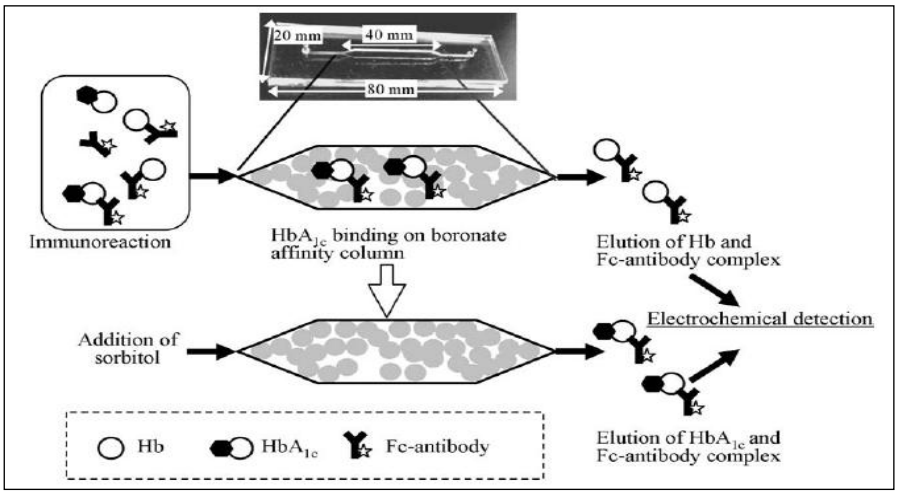

Figure 14. Schematic diagram of an on-chip electrochemical flow immunoassay system (Tanaka *et al*., 2006).

Another approach was introduced by a Son and co-authors (Son *et al*., 2006). They proposed a disposable chip for measurement of HbA1c (%), which involved use of APBA as a separation material but without biological sensing elements. In the description given, the blood suspension was transferred by a micropump which produced oxygen by decomposing hydrogen peroxide with a microheater. In the second step, blood was separated into plasma and red blood cells. The latter was ruptured in the lysis chamber giving a mixture of haemoglobin which flowed into two channels.

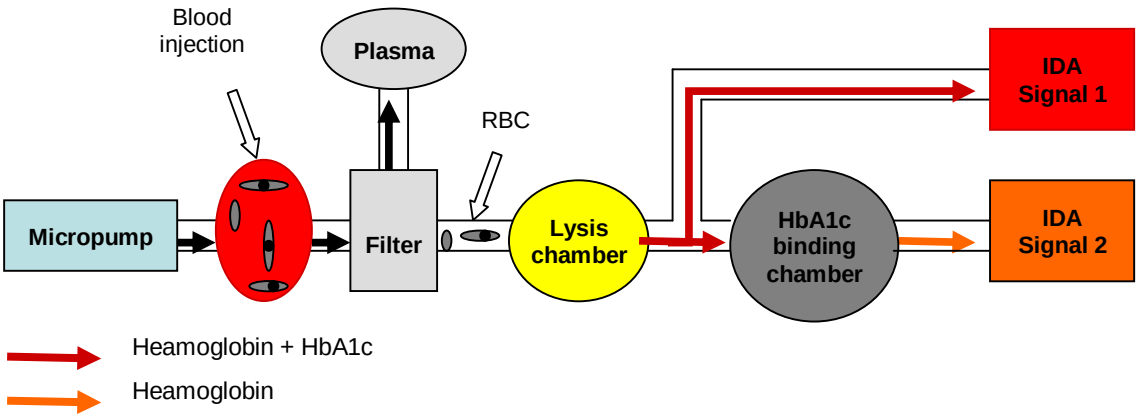

Figure 15. Schematic of the % HbA1c measurement process (modified from Son *et al.*, 2006)

One of the channels led straight to the electrode and gave the signal 1 reflecting the whole content of haemoglobin. The second had a binding chamber, which consisted of m-APBA agarose beads. Therefore the HbA1c was captured and the signal 2 reflected a content of non-glycated haemoglobin. The electrodes were IDA-shaped (interdigitated array electrodes) and had a high-aspect-ratio (elongation) to magnify contact area and improve sensitivity. Electrodes consisted of nickel and gold (Son *et al*., 2005; Son *et al*., 2006). There were however essential details missing in this paper and it would be difficult to construct a working system based on the information provided. The paper also lacks clear evidence that the system worked as an effective assay.

4.2 MIP integration with transducer – biomimetic sensor

Molecularly imprinted polymer technology combined with an electrochemical reader and microfluidics would be very desirable in medical diagnostics. Biomimetic sensor may be a basis for construction of a miniaturized device for HbA1c determination. The device configuration may be similar to the one presented above, but the separation process would be replaced with a sensing of an analyte using MIP-electrodes.

A very important issue in the development of a biomimetic sensor is the integration of a sensitive transducer with the recognition system (MIP). There should be a close contact between them. A device should be able to detect the binding process and transform it into a readable signal. Introduction of automated production process would be an additional advantage. Polymer can be synthesised directly on the transducer or as a thin membrane and then transferred onto the transducer's surface (Haupt and Mosbach, 2000; Piletsky and Turner, 2002).

It was reported that MIP particles could coat the working carbon electrode as a thin layer (Kroger *et al*., 1999). A stable thin membrane could also be photo-deposited on the sensor surface or used directly in conductometric sensors (Piletsky and Turner, 2002). In the case of an electrochemical transducer, molecular imprinting by electropolymerisation appears convenient. Its application allows for a precise placing of MIP layer and coating of the electrode with homogenous film (Kriz *et al*., 1995; Rick

and Chou, 2006). The polymer thickness and deposition density are controlled by the polymerisation conditions, such as applied voltage and deposition time. By such means, polyaniline and polypyrrole layers were prepared (Vinokurov, 1992). Panasyuk *et al.* (1999) successfully prepared electropolymerised o-phenol for phenylalanine detection. Malitesta and co-authors reported an application of the same procedure for o-phenylenediamine, where glucose was imprinted on a QCM transducer (Malitesta *et al.*, 1999). Another example is an amperometric protein sensor made by electrodeposition of a monomer, APBA, on a gold or platinum screen-printed support or electrodeposition of polypyrrole and p-APBA (Rick and Chou, 2006).

Considering whether the sensor should be disposable or not, its stability and regeneration issue have to be taken into account. Problems may result from a partial destruction of the binding sites in the polymer matrix (Bossi *et al.*, 2001; Ramanaviciene and Ramanavicious, 2004). Furthermore, the signal may decrease because of mediator leaching (Scheller *et al.*, 2005), and some residual species may be left after the previous measurement (Brett *et al.*, 1999). These and other considerations restrict the re-use of sensors and favour disposable device construction. It is also less complicated and a relatively cheap procedure to use MIP technology in combination with electrochemistry to create a disposable device (Hart *et al.*, 2003).

Chapter 5: Conclusions

There are no reports of HbA1c sensor incorporating MIP technology and electrochemical detection so far. The development of such a device would be highly innovative. This review has illustrated some different possibilities for total Hb and HbA1c determination including electrochemistry and MIP technology. Despite numerous studies about direct electron transfer and catalytic activity of Hb, the practical application of this approach is still limited. However, some ideas from this area that may help to improve Hb immobilisation or protect the molecule, could be explored in mediated sensors. It should be noted that the catalytic activity of Hb may be reduced by the organic solvent that is used for MIP preparation. Phenyl boronic acid derivatives (PBA) have been many times recognised as having a good affinity towards total glycated haemoglobin (GHb). It seems to be a suitable molecule for further research in the sensor area. MIP technology or a non-imprinting approach together with various transducers may be used to construct a sensor for HbA1c determination.

Some promising results presented in this review show potential for using molecular imprinting combined with mediators in electrochemical detection of glycated haemoglobin (HbA1c). It is clear however that the imprinting of protein is difficult. New protocols both for electrochemical detection, MIP preparation and their integration into a device may be required.

SECTION II: EXPERIMENTAL

Chapter 6: Electrochemical behaviour of mediators on commercial electrodes

6.1 Materials and methods

6.1.1 Reagents

Potassium ferricyanide (CAS No: 13746-66-2), potassium ferrocyanide (CAS No: 14459-95-1), MB (Methylene Blue, CAS No: 7220-79-3), ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, CAS No: 30931-67-0), FcCOOH (ferrocenecarboxylic acid, CAS No: 1271-42-7), HQ (hydroquinone with CAS No: 123-31-9), DCPIP (dichlorophenol indophenol, CAS No: 620-45-1), sodium monobasic phosphate (CAS No: 7558-80-7), sodium dibasic phosphate (CAS No: 7558-79-4), potassium chloride (CAS No: 7447-40-7), sodium chloride (CAS No: 7647-14-5), sodium hydroxide (CAS No: 1310-73-2), were purchased from Sigma-Aldrich (Dorset, UK). Alumina 0.3μm particle and alumina polishing pads (tan, velvet) were purchased from BASi Electrochemistry Instruments (Warwickshire, UK). Chemicals were used as received.

6.1.2 Apparatus

All electrochemical experiments were performed with a Uniscan potentiostat - galvanostat instrument PG580 with UiEChem software (Buxton, UK). The Uniscan instrument was used with a beaker or an electrochemical cell which consisted of Screen Printed Electrodes (SPE) or a three electrode cell, respectively.

The three electrode cell consisted of a gold (A=2.01 mm^2) as a working electrode and platinum (A=2.01 mm^2) as a counter electrode. They were purchased from BASi Electrochemistry Instruments (Warwickshire, UK). Calomel electrode was used as a reference electrode and was purchased from Sigma-Aldrich (Dorset, UK). The gold and

platinum electrodes were cleaned after each measurement. Electrodes were cleaned firstly with alumina 0.3 µm particle on a polishing pad and thoroughly rinsed with distilled water (dH_20). Following this the electrode was polished on a polishing pad soaked with dH_20 and thoroughly rinsed with dH_20.

Screen Printed Electrodes (SPE-D) and Screen Printed Electrodes - Carbon Nanotubes (SPE CNT-D) were purchased from DropSens Ltd. (Oviedo, Spain). Electrodes were fabricated on ceramic substrate: L33 x W10 x H0.5 mm, electric contacts and a reference electrode were made from silver, a working electrode (A=12.56 mm^2) and a counter electrode was made with carbon. Other type of SPEs (SPE-S) was provided by Microarray Ltd. They consisted of two carbon working electrodes (A=22.53 mm^2 each), counter electrode was made with a carbon and reference electrode was made of the Ag/AgCl paste. Conductive paths were masked with dielectric coat. Additional type of SPEs (SPE-G) was also kindly supplied by GEM Ltd. (Pontypool, UK). They were fabricated on a polystyrene substrate with a carbon working electrode (A=3.14 mm^2), a carbon counter electrode and Ag/AgCl as a reference electrode. Conductive paths were covered with a dielectric layer. Pelikan Technologies Inc. provided SPE (SPE-P) which consisted of a conductive layer, an insulating layer, Ag/AgCl as a reference electrode, Ag/AgCl as a counter electrode, a carbon working electrode (A=0.25 mm^2) and a spacer layer. SPEs were also printed in Cranfield (SPE-C) on a polyester sheets, and they consisted of carbon as a working electrode (A=19.63 mm^2), carbon as a counter electrode and Ag/AgCl as a reference electrode. Conductive paths were covered with an insulating ink. SPEs were used without any pre-conditioning.

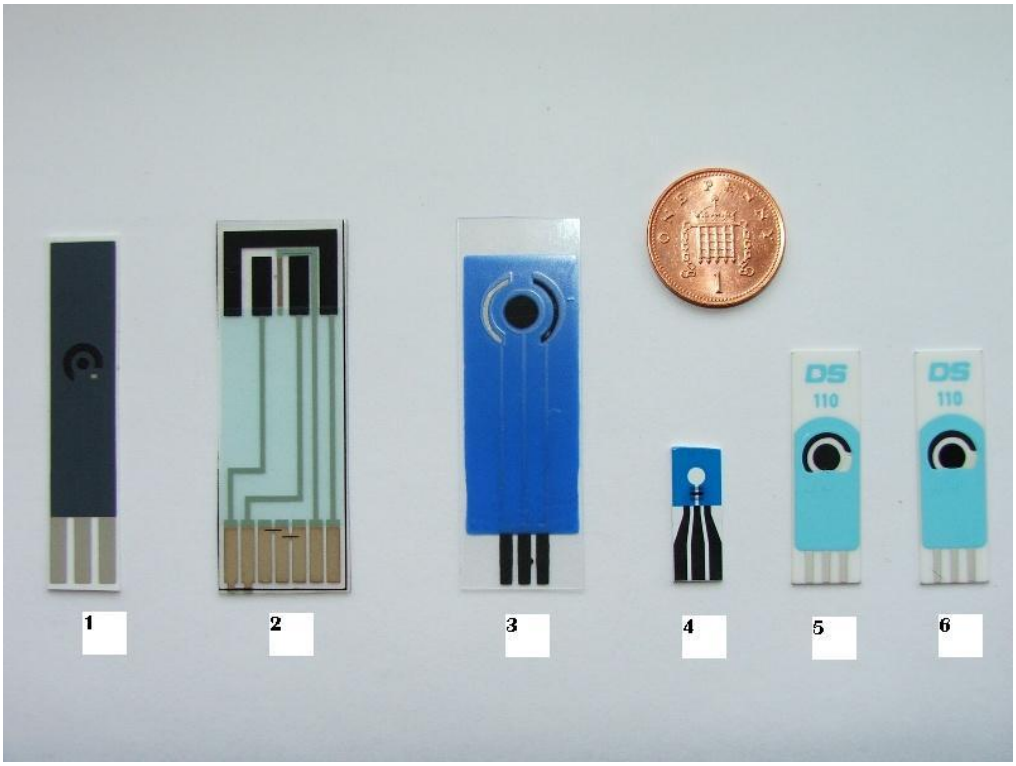

Figure 16. SPE electrodes, 1) SPE-G, 2) SPE-S, 3) SPE-C, 4) SPE-P, 5) SPE-D, 6) SPE CNT-D

pH meter, Hanna Instruments 8519 was purchased from Hanna Instruments Inc. (Woonsocket, USA) and was used for preparation of buffer solutions.

6.1.3 Electrochemical analysis

Cyclic voltammetry and linear voltammetry were used for the qualitative characterisation of electrodes. Linear voltammetry was run from positive toward negative potentials. The cyclic voltammetry was started from negative potentials, except studies with MB and DCPIP. All solutions used for electrochemical analysis were prepared daily. Mediator was prepared in a buffer deoxygenated by purging with nitrogen and was kept in 4^{o}C during measurements. Measured samples contained 0 or 0.5 mM mediator. Phosphate buffer (40 mM) consisted of sodium monobasic phosphate, sodium dibasic phosphate and a supportive electrolyte potassium chloride (100 mM) at pH 7.0.

Measurements were performed at room temperature (22^{o}C). Experiments were performed using SPE without modification. Electrodes were immersed in a vertical

position in a 10 mL beaker. Experiments were also performed in a 10 mL electrochemical cell, which consisted of a gold working electrode, a platinum counter electrode and a calomel reference electrode. The electrode configuration described above was employed in all experiments. CV was performed under unstirred conditions. All measurements were run in triplicate. Data were transferred to Microsoft Office Excel and average values calculated.

6.2 Results and Discussion

6.2.1 Electrochemical behaviour of chosen mediators using an electrochemical cell

Preliminary studies were performed in a classical electrochemical cell with bulk electrodes. They might have had better repeatability and characteristic than SPEs which is crucial in the biosensor field. Redox behaviour of mediators is shown on cyclic voltammograms (Figures 17-22). Three cycles were performed for each mediator at a scan rate 50 mV/s. Table 5 shows a characterisation of mediators using the second cycle and also a first cycle for DCPIP.

Table 5. Mediator characteristic performed in the electrochemical cell (second cycle).

	E_{pa}	E_{pc}	ΔEp	i_{pa}	i_{pc}
Mediator	**mV**			**µA**	
FcCOOH	315	245	70	1.62	-1.4
HQ	305	-105	410	3.06	-2.45
Ferrocyanide	225	150	75	1.67	-1.58
MB	-215	-255	40	1.06	-1.17
ABTS	505	405	100	1.17	-0.814
DCPIP first cycle	65	-270	335	1.15	-1.24
DCPIP second cycle	510	-270	780	1.39	-1.24

CVs of DCPIP exhibited significantly different positions of reduction and oxidation peaks to the other redox compounds. Increased peak separation was observed with each subsequent cycle (Figure 20). Other mediators did not show significantly different peaks positions in subsequent cycles. Mediators showed a higher oxidation peak in a first cycle, except methylene blue which showed a higher oxidation and reduction peak in the subsequent cycles (Figure 18). Hydroquinone showed a useful reduction potential. The reduction peak was slightly shifted toward positive potential with each cycle, E_{pc} for the first, second and third cycle was -110 mV, -100 mV and -90 mV, respectively

(Figure 21). This mediator showed the biggest peak separation, 410 mV and also the highest current response with values of anodic and cathodic current estimated to be 3.06 and -2.45 µA, respectively (Table 4). Separation of redox peaks, larger than 59 mV for mediators, such as potassium ferrocyanide, ferrocenecarboxylic acid, and 29.5 mV for mediators, such as hydroquinone, methylene blue, ABTS and DCPIP indicate a quasi-reversible process. However, relatively good separation of peaks was achieved for potassium ferrocyanide, ferrocenecarboxylic acid, methylene blue and ABTS.

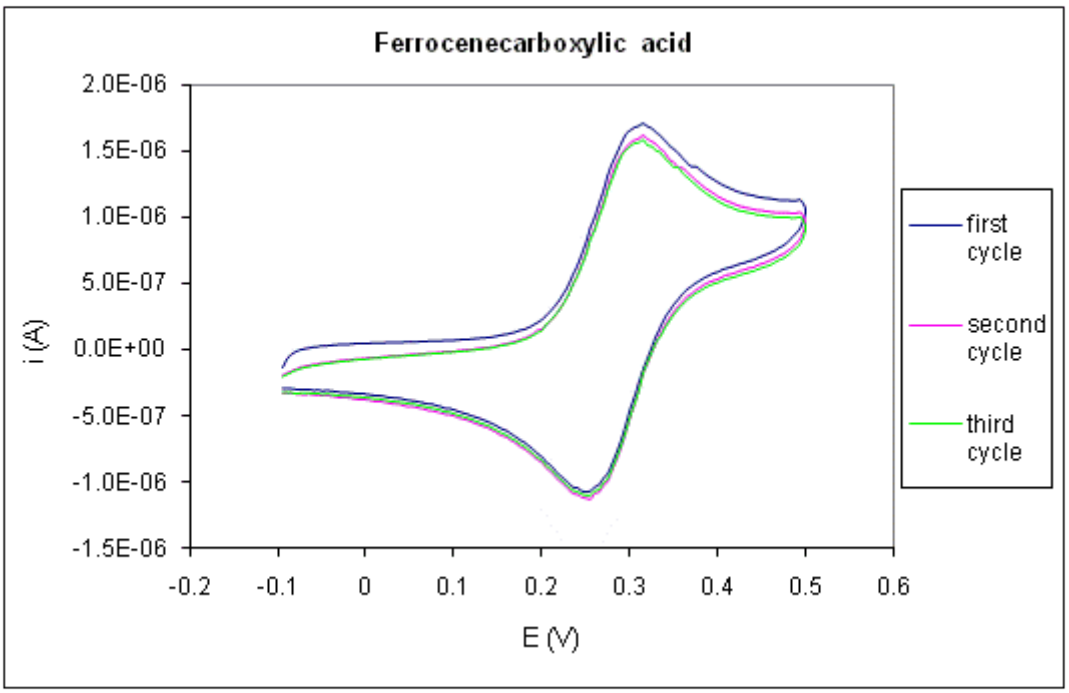

Figure 17. Cyclic voltammetry of FcCOOH (0.5 mM) in PB buffer at pH 7.0. The electrochemical cell: Au-WE, Pt-CE, calomel-RE. Scan rate 50 mV/s.

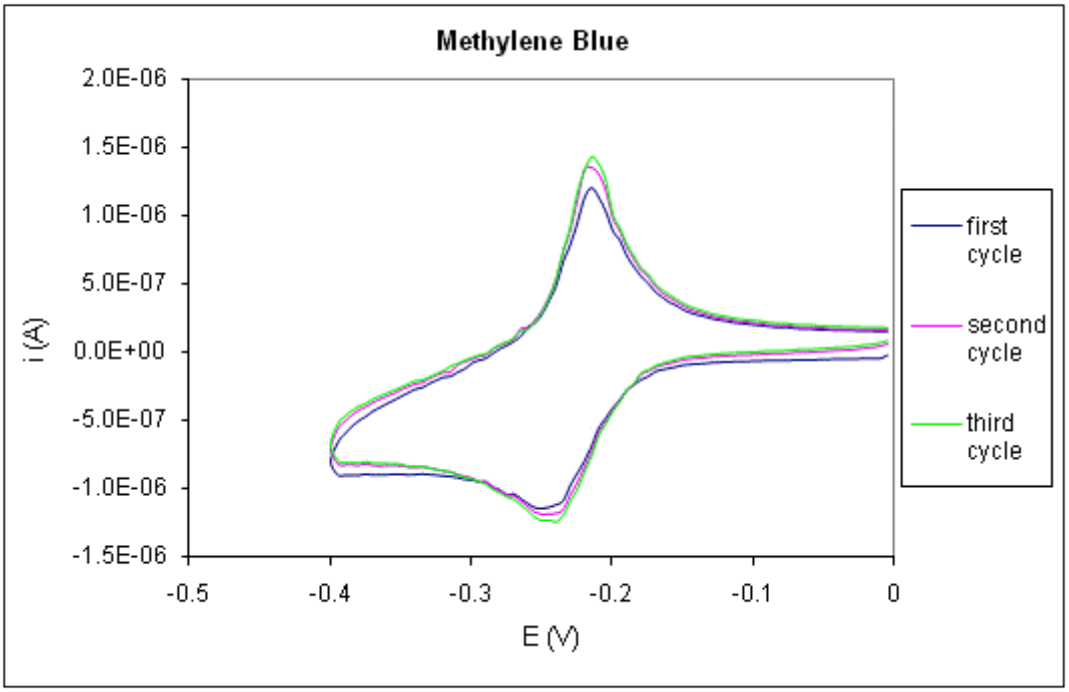

Figure 18. Cyclic voltammetry of MB (0.5 mM) in PB buffer at pH 7.0. The electrochemical cell: Au-WE, Pt-CE, calomel-RE. Scan rate 50 mV/s.

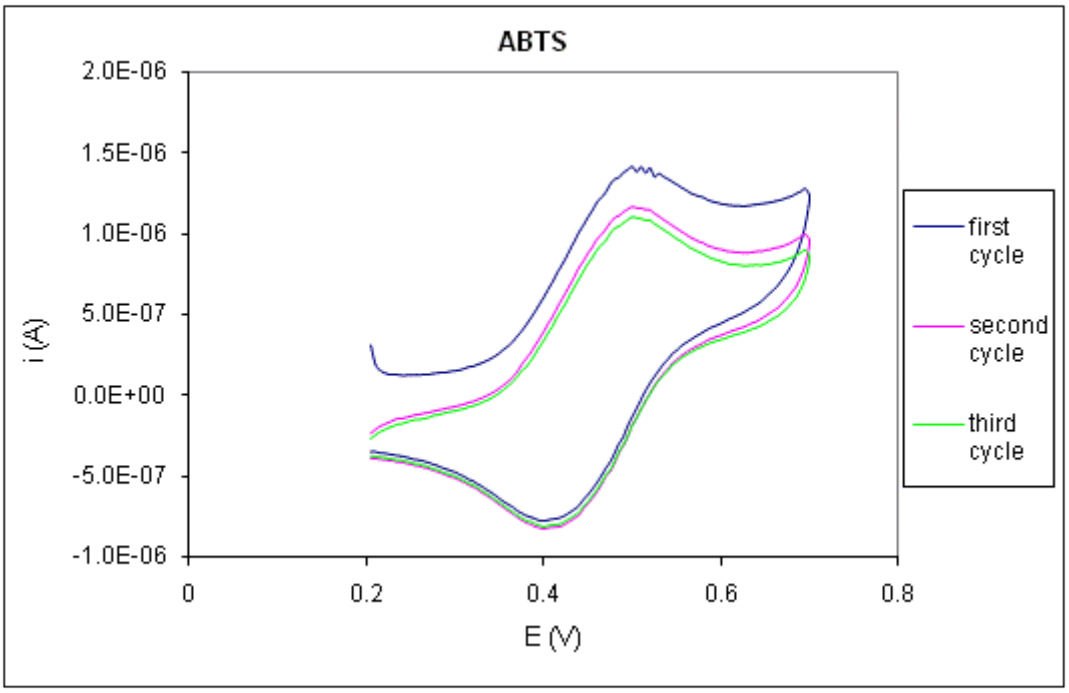

Figure 19. Cyclic voltammetry of ABTS (0.5 mM) in PB buffer at pH 7.0. The electrochemical cell: Au-WE, Pt-CE, calomel-RE. Scan rate 50 mV/s.

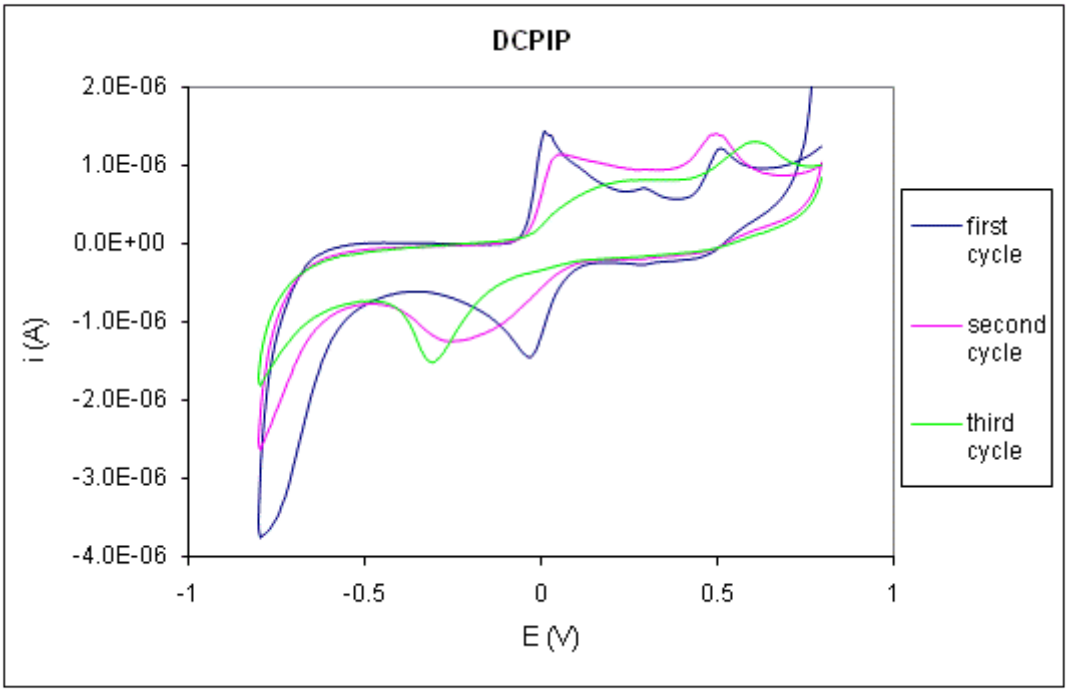

Figure 20. Cyclic voltammetry of DCPIP (0.5 mM) in PB buffer at pH 7.0. The electrochemical cell: Au-WE, Pt-CE, calomel-RE. Scan rate 50 mV/s.

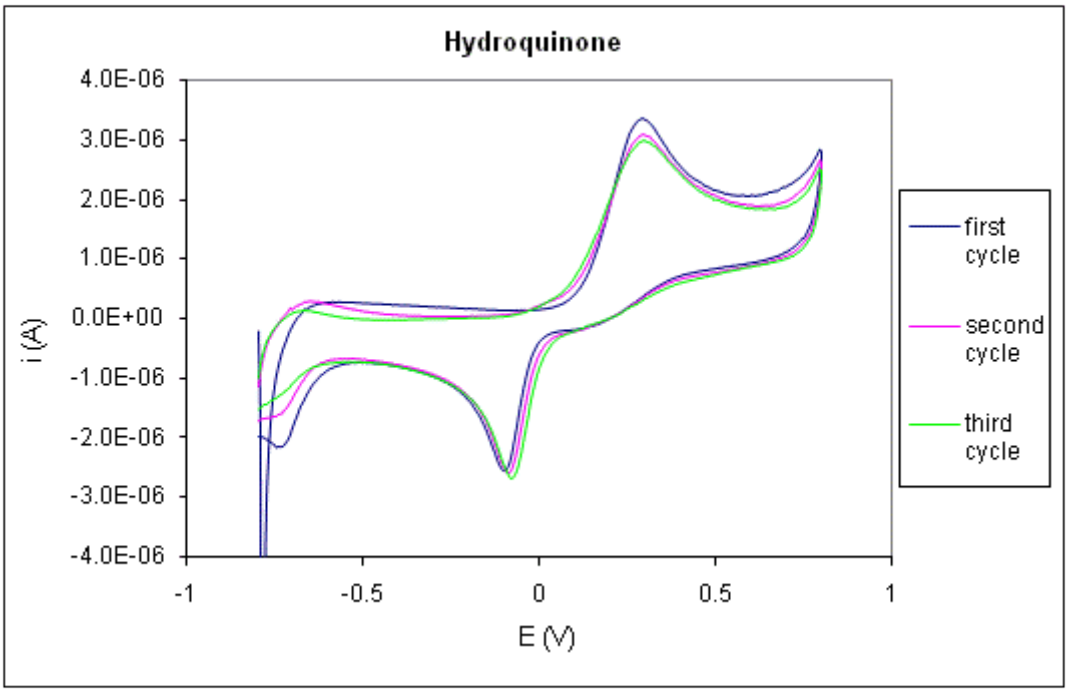

Figure 21. Cyclic voltammetry of HQ (0.5 mM) in PB buffer at pH 7.0. The electrochemical cell: Au-WE, Pt-CE, calomel-RE. Scan rate 50 mV/s.

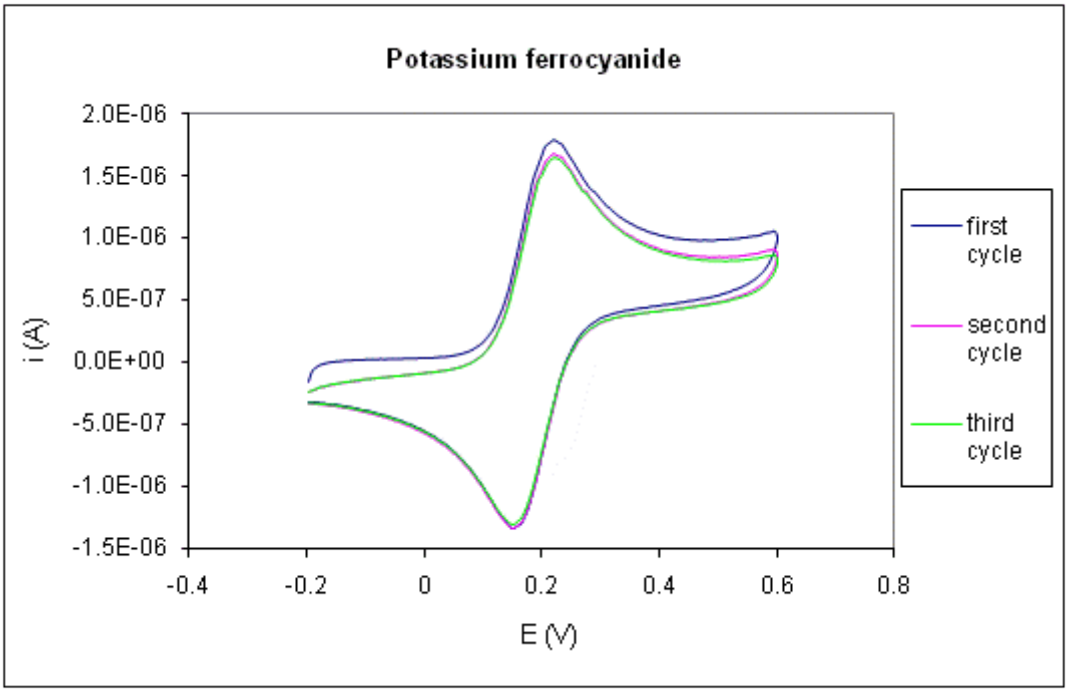

Figure 22. Cyclic voltammetry of potassium ferrocyanide (0.5 mM) in PB buffer at pH 7.0. The electrochemical cell: Au-WE, Pt-CE, calomel-RE. Scan rate 50 mV/s.

6.2.2 Comparison of Screen Printed Electrodes

Screen printed carbon electrodes were tested as electrodes of choice since they are relatively inexpensive, characterised by a wide potential window and a low background current (Wang, 2006). Additionally it is more convenient to conduct research on disposable electrodes and avoid a time-consuming cleaning procedure. Studies can be also performed in a small volume of reaction mixture which is essential in case of work with expensive reagents.

Linear voltammetry and cyclic voltammetry were used to characterise screen printed electrodes (SPEs). The SPE-P showed the broadest potential window. Current resulting from hydrogen and oxygen evolution should not be recorded. The SPE-D, SPE-CNT-D and SPE-G showed narrower cathodic potential. Cathodic current started to increase at a potential above -600 mV for SPE-D, -400 mV for SPE-CNT-D and -200 mV for SPE-G electrodes. Electrochemical cell showed noise at a potential about +500 mV and significant increase in cathodic current at a potential around -800 mV (Figure 23). The properties of working electrode surface might have had an influence on presented

results. Cyclic voltammetry was used to characterise commercial SPEs using potassium ferricyanide and HQ at a scan rate 50 mV/s (Figures 24-35). Tables 6-7 show SPEs characteristics measured for the second cycle.

Table 6. Characterisation of SPEs with potassium ferricyanide.

	Potassium ferricyanide						
SPE	**E_{pa}**	**E_{pc}**	**ΔE_p**	**i_{pa}**	**i_{pc}**	**j_a**	**j_c**
	mV			µA		µA/cm^2	
Pelikan	405	-235	**640**	0.0602	-0.108	**24.08**	**-43.20**
DropSens	190	55	**135**	5.85	-5.77	**46.57**	**-45.94**
DropSens CNT	160	70	**90**	4.91	-5.24	**39.09**	**-41.72**
GEM	175	95	**80**	1.75	-1.69	**55.73**	**-53.82**
SPE-S	515	-260	**775**	9.02	-3.72	**40.04**	**-16.51**
SPE-C	455	-140	**595**	8.22	-5.56	**41.89**	**-28.33**

Table 7. Characterisation of SPEs with hydroquinone.

	Hydroquinone						
SPE	**E_{pa}**	**E_{pc}**	**ΔE_p**	**i_{pa}**	**i_{pc}**	**j_a**	**j_c**
	mV			µA		µA/cm^2	
Pelikan	445	-240	**685**	0.264	-0.109	**105.60**	**-43.60**
DropSens	160	-110	**270**	17.3	-18.3	**137.74**	**-145.70**
DropSens CNT	100	-90	**190**	6.04	-6.54	**48.09**	**-52.07**
GEM	165	-120	**285**	2.87	-2.75	**91.40**	**-87.58**
SPE-S	410	-230	**640**	25.5	-21	**113.20**	**-93.22**
SPE-C	520	-370	**890**	16.7	-13.1	**85.10**	**-66.75**

CVs for potassium ferricyanide and HQ performed on SPE-D and SPE-G electrodes showed the most reversible behaviour in comparison to the other SPEs (Figures 28-31). Peak position and separation for potassium ferricyanide was similar to the gold electrode used with the electrochemical cell. The CV of HQ even showed decreased peak to peak separation in comparison to the electrochemical cell. DropSens CNT-D showed the smallest peak separation for hydroquinone (190 mV). However the anodic

and cathodic peak currents were not the same. A significant difference was observed on the SPE CNT-D for potassium ferricyanide with each cycle and the same effect was visible for HQ (first cycle) on the same electrodes (Figures 30, 31). Other screen printed electrodes showed an increased peak separation, thus slower electron transfer. It can be described as a quasi-reversible process (Pletcher, 1991). Probably the results were caused by different carbon ink used for the preparation of the SPEs. Research performed by Wang *et al.* also showed differences in peak separation of potassium ferrocyanide on different types of carbon. Similar tendency was obtained for catechol and acetaminophen (Wang *et al*., 1998).

The highest anodic and cathodic current density for potassium ferricyanide was obtained for SPE-G electrodes, 55.73 and -53.82 $\mu A/cm^2$, respectively. However, SPE-G electrodes showed also very irregular CVs without clear reduction peak (Figure 34). The highest current density for hydroquinone was obtained on SPE-D electrodes, where the anodic current was calculated to be 137.74 $\mu A/cm^2$ and the cathodic current -145.70 $\mu A/cm^2$. The cyclic voltammogram of hydroquinone showed an increase of the anodic and cathodic current on the SPE CNT-D with each subsequent cycle (Figures 30, 31). The CV of potassium ferricyanide only showed an increase in anodic current with the each subsequent cycle (Figure 31). The summarized data are presented in Table 8.

Table 8. Peaks of anodic and cathodic current on SPE CNT-D for HQ and potassium ferricyanide

	HQ		Potassium ferricyanide	
cycle number	i_{pa}	i_{pc}	i_{pa}	i_{pc}
	i(μA)			
1	5.6	-3.57	3.48	-3.38
2	6.04	-6.54	4.91	-5.21
3	7.81	-8.04	5.01	-4.05

Preparation of SPEs, namely composition of material and fabrication condition might have had a significant influence on a rate of electron transfer and behaviour of redox system at a working electrode, such as background and faradaic current, peaks position and their separation. Wang *et al.* (1996) demonstrated that curing temperature has

positive effect on electrode performances due to removal of organic solvents. They also proved that electrochemical pre-treatment, anodization and cathodization could partially improve electrodes characteristic. Functional groups (quinone and carboxyl group), which are introduced at a high positive polarization potential, facilitate electron transfer (Wang *et al.*, 1996).

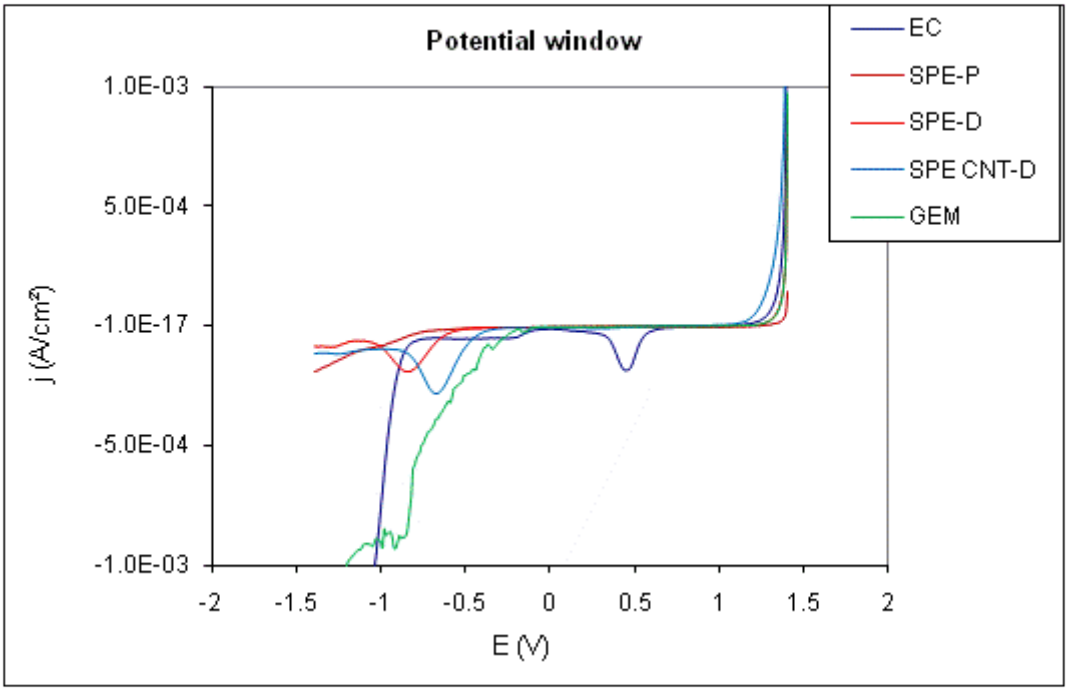

Figure 23 Scan from positive toward negative potential on different electrodes in PB pH 7.0

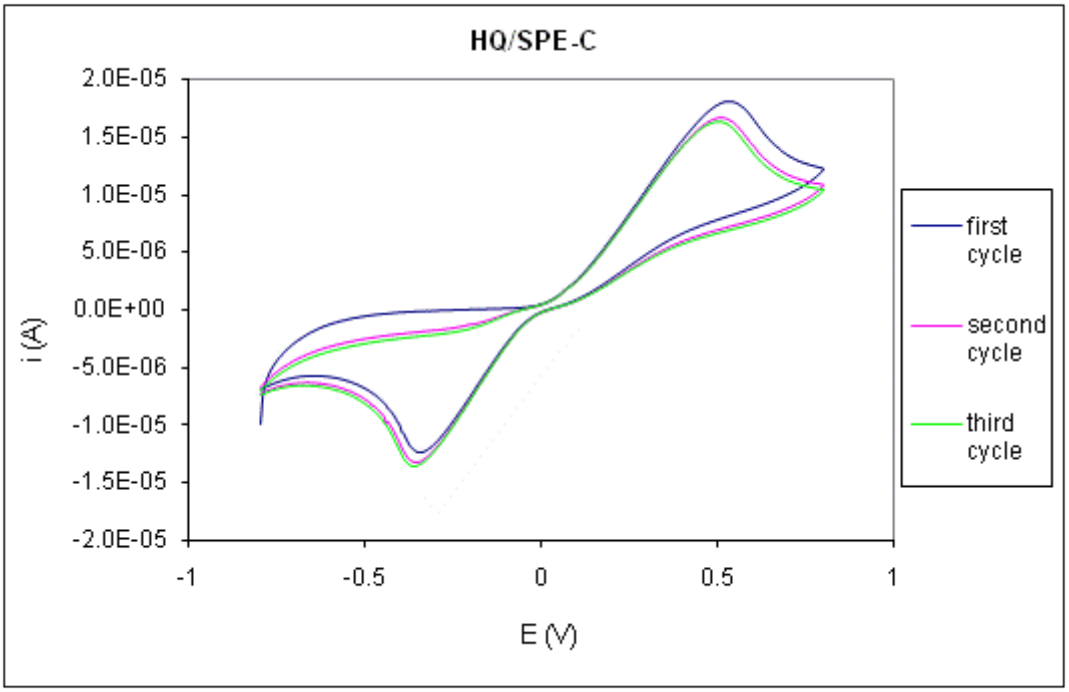

Figure 24. Cyclic voltammetry of HQ (0.5 mM) in PB buffer at pH 7.0 on SPE-C electrodes. Scan rate 50 mV/s.

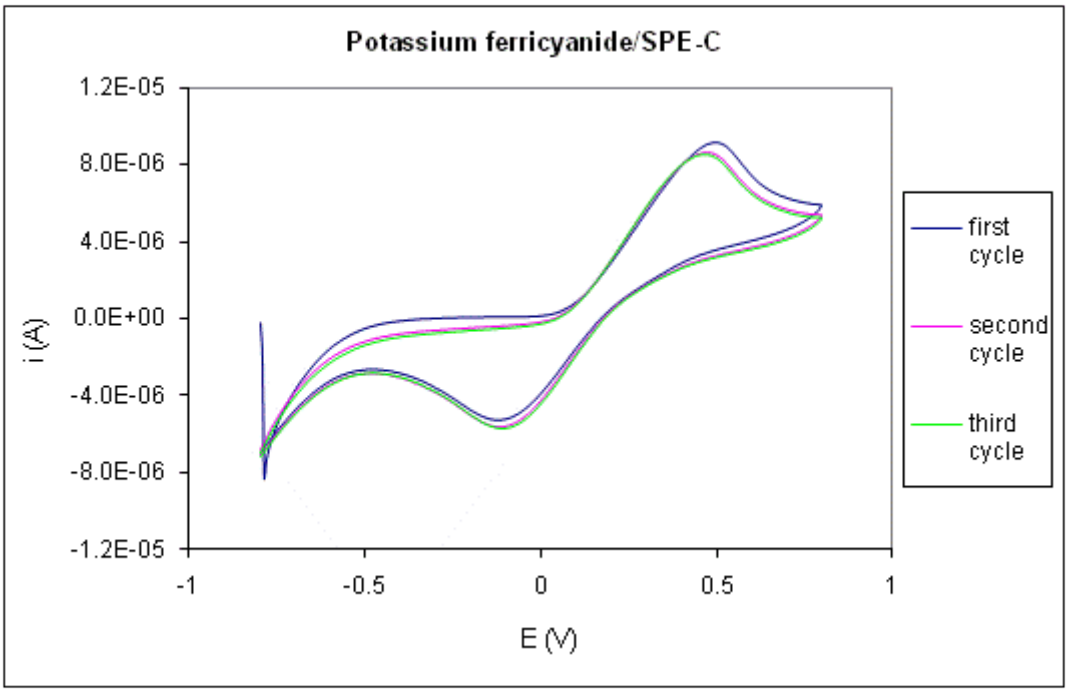

Figure 25. Cyclic voltammetry of potassium ferricyanide (0.5 mM) in PB buffer at pH 7.0 on SPE-C electrodes. Scan rate 50 mV/s.

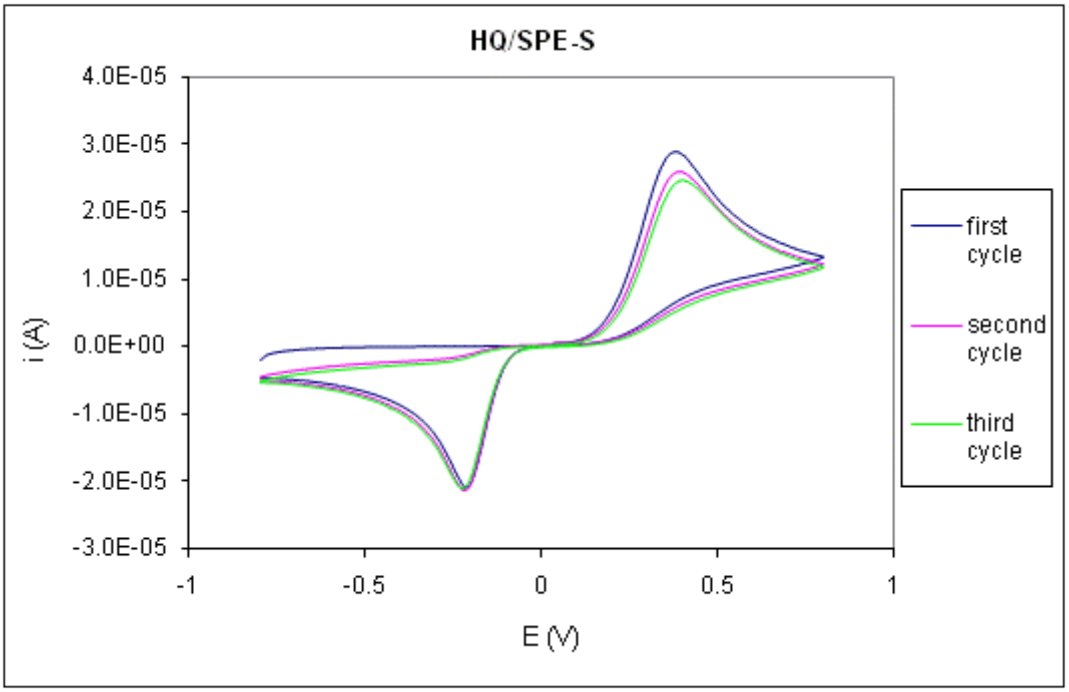

Figure 26. Cyclic voltammetry of HQ (0.5 mM) in PB buffer at pH 7.0 on SPE-S electrodes. Scan rate 50 mV/s.

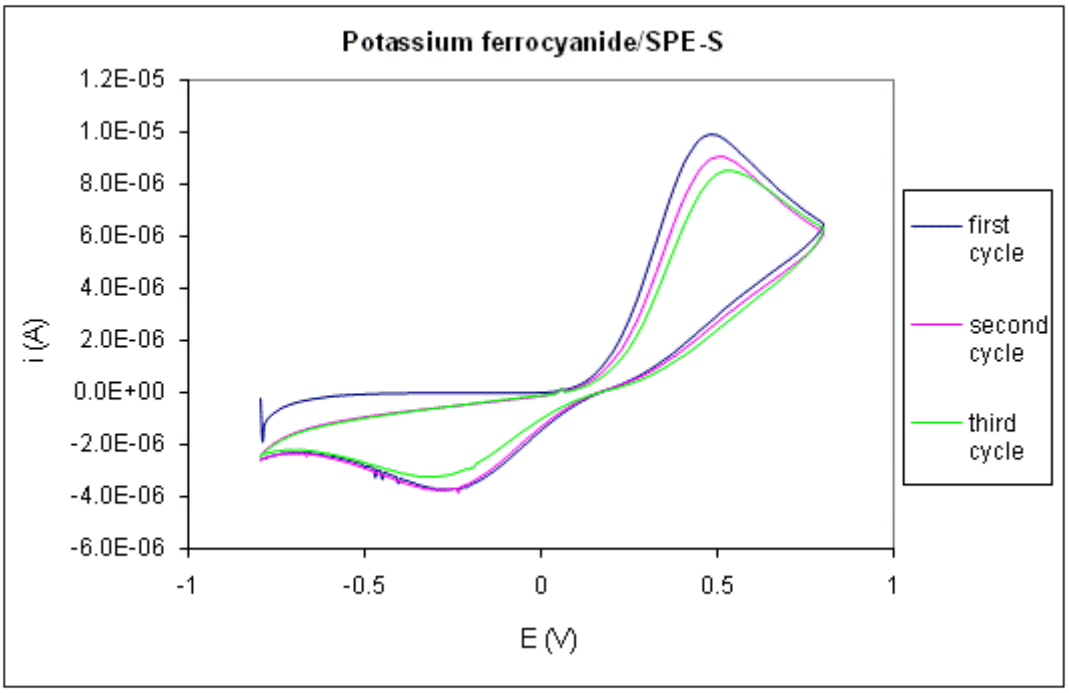

Figure 27. Cyclic voltammetry of potassium ferricyanide (0.5 mM) in PB buffer at pH 7.0 on SPE-S electrodes. Scan rate 50 mV/s.

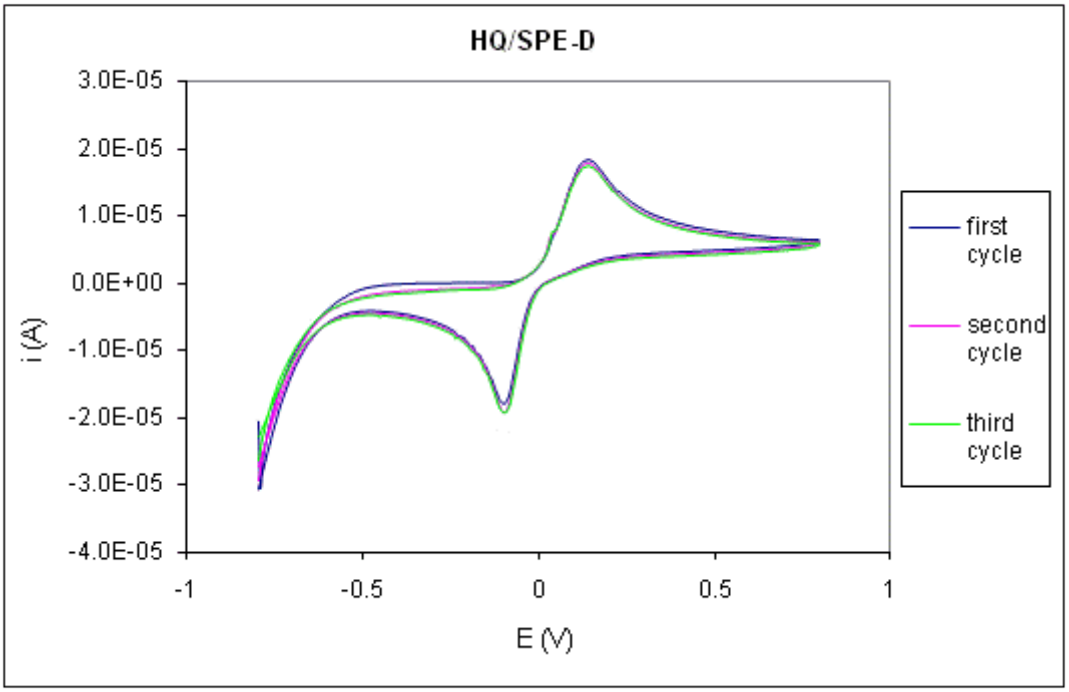

Figure 28. Cyclic voltammetry of HQ (0.5 mM) in PB buffer at pH 7.0 on SPE-D electrodes. Scan rate 50 mV/s.

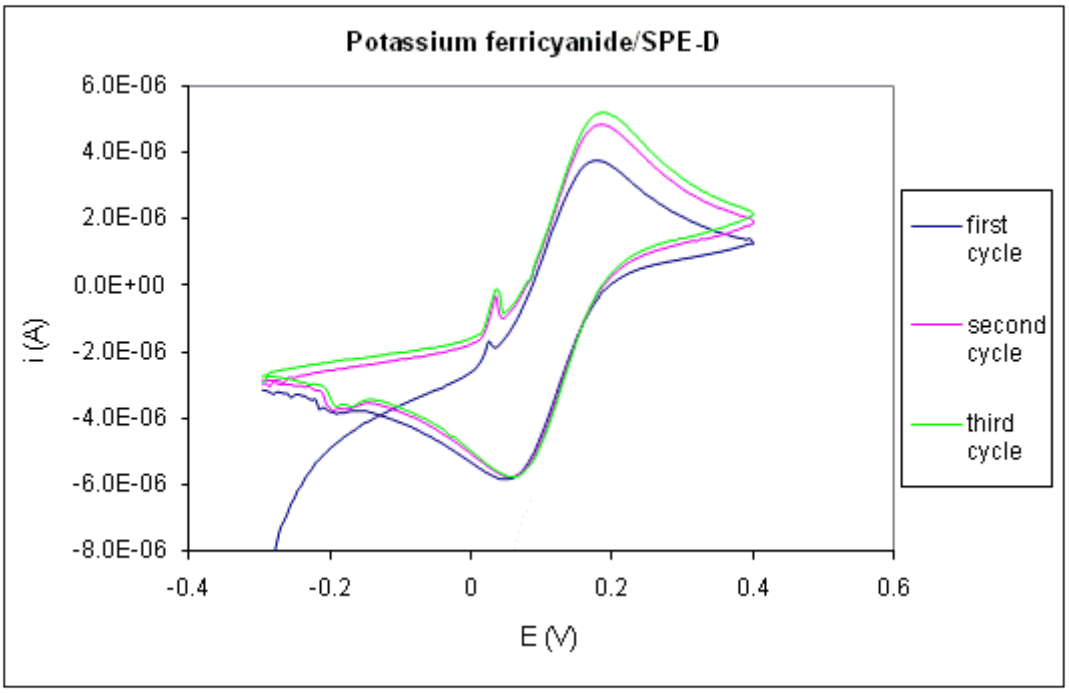

Figure 29. Cyclic voltammetry of potassium ferricyanide (0.5 mM) in PB buffer at pH 7.0 on SPE-D electrodes. Scan rate 50 mV/s.

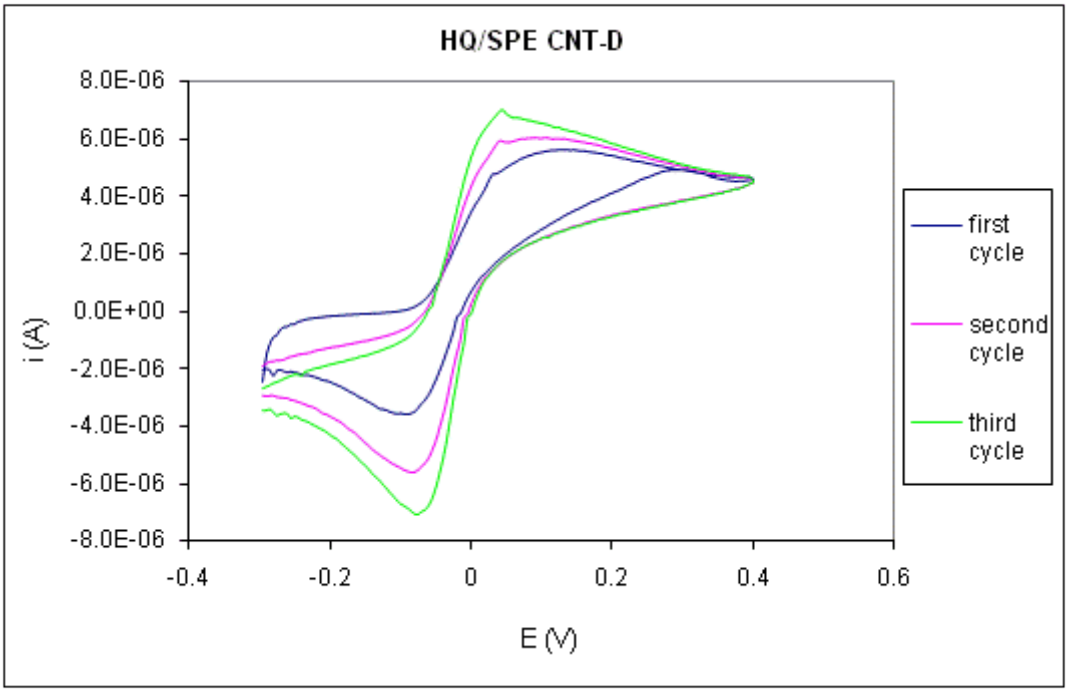

Figure 30. Cyclic voltammetry of HQ (0.5 mM) in PB buffer at pH 7.0 on SPE CNT-D electrodes. Scan rate 50 mV/s.

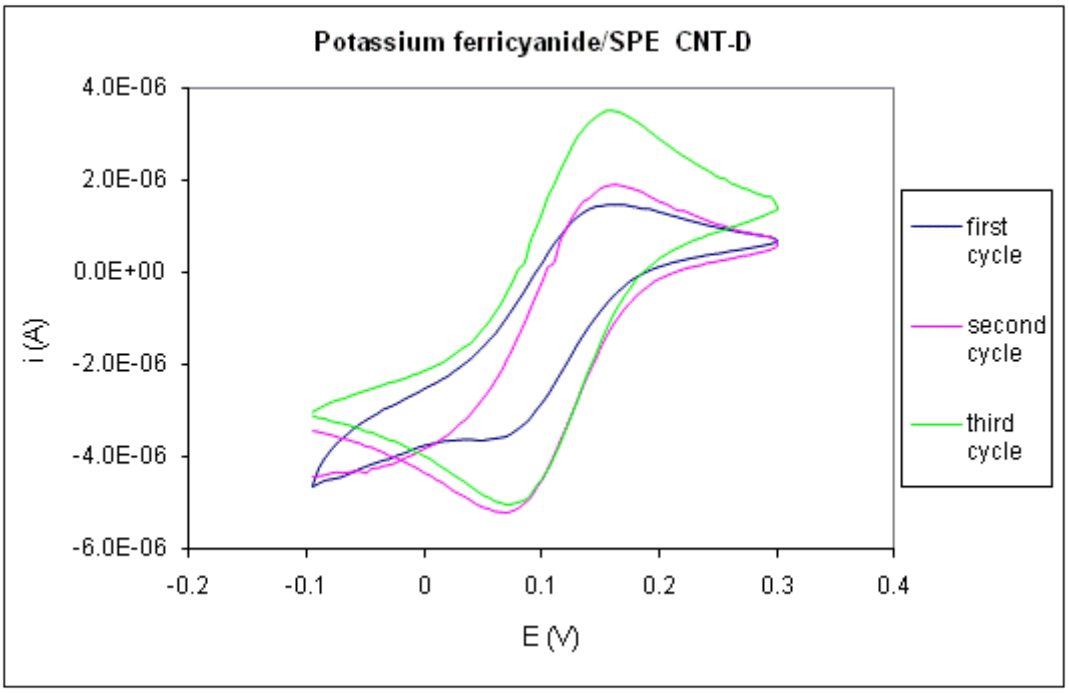

Figure 31. Cyclic voltammetry of potassium ferricyanide (0.5 mM) in PB buffer at pH 7.0 on SPE CNT-D electrodes. Scan rate 50 mV/s.

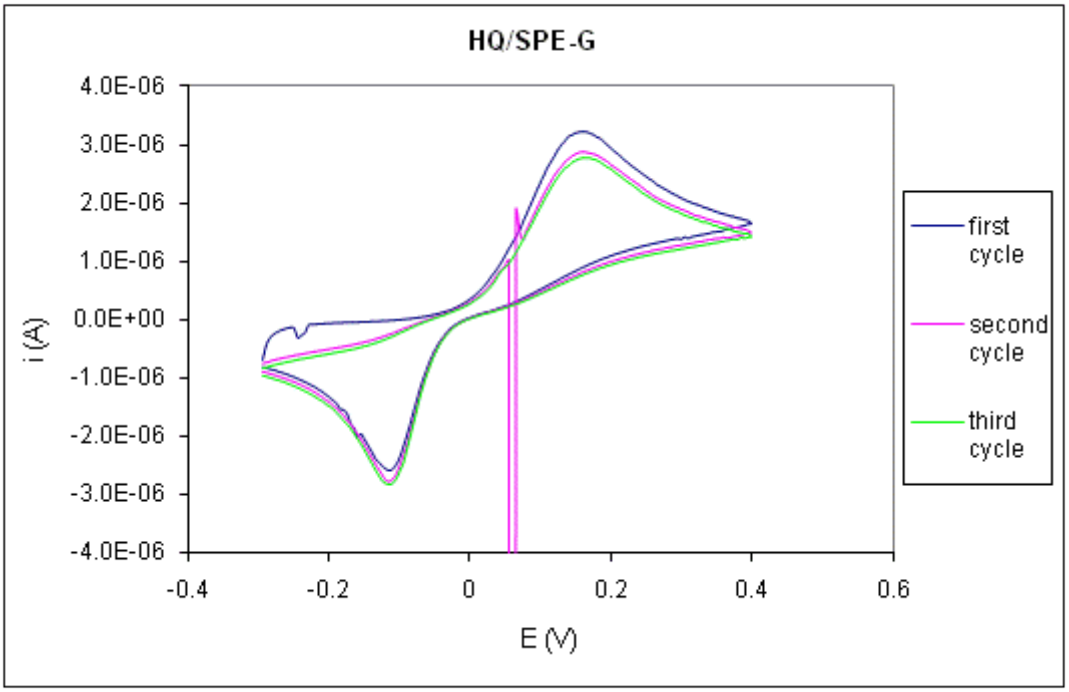

Figure 32. Cyclic voltammetry of HQ (0.5 mM) in PB buffer at pH 7.0 on SPE-G electrodes. Scan rate 50 mV/s.

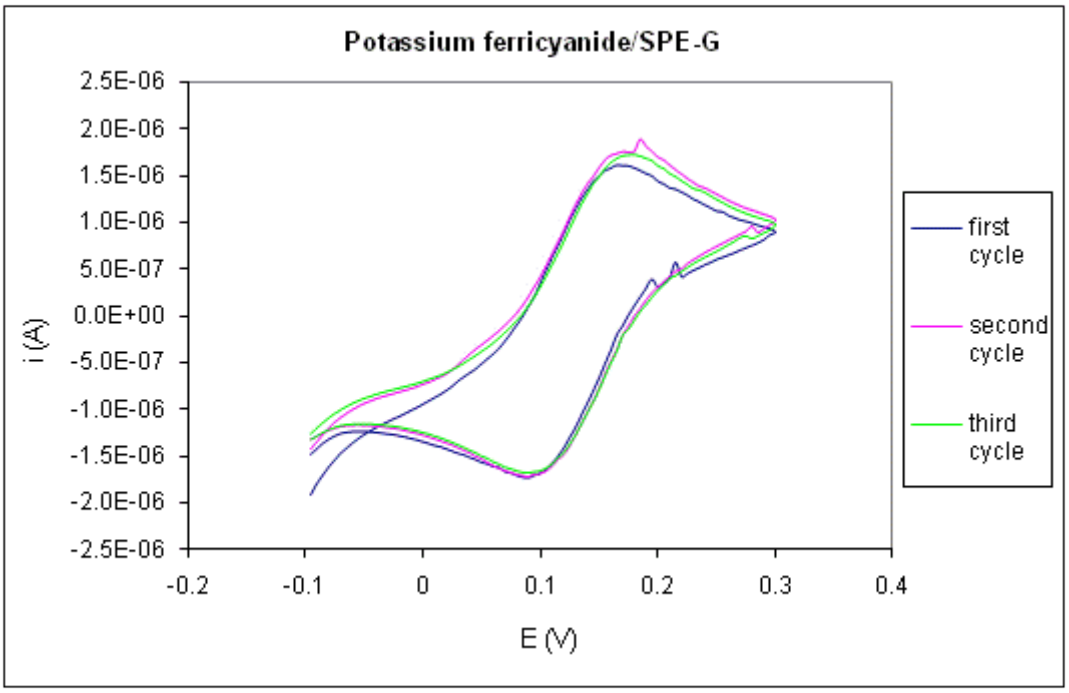

Figure 33. Cyclic voltammetry of potassium ferricyanide (0.5 mM) in PB buffer at pH 7.0 on SPE-G electrodes. Scan rate 50 mV/s.

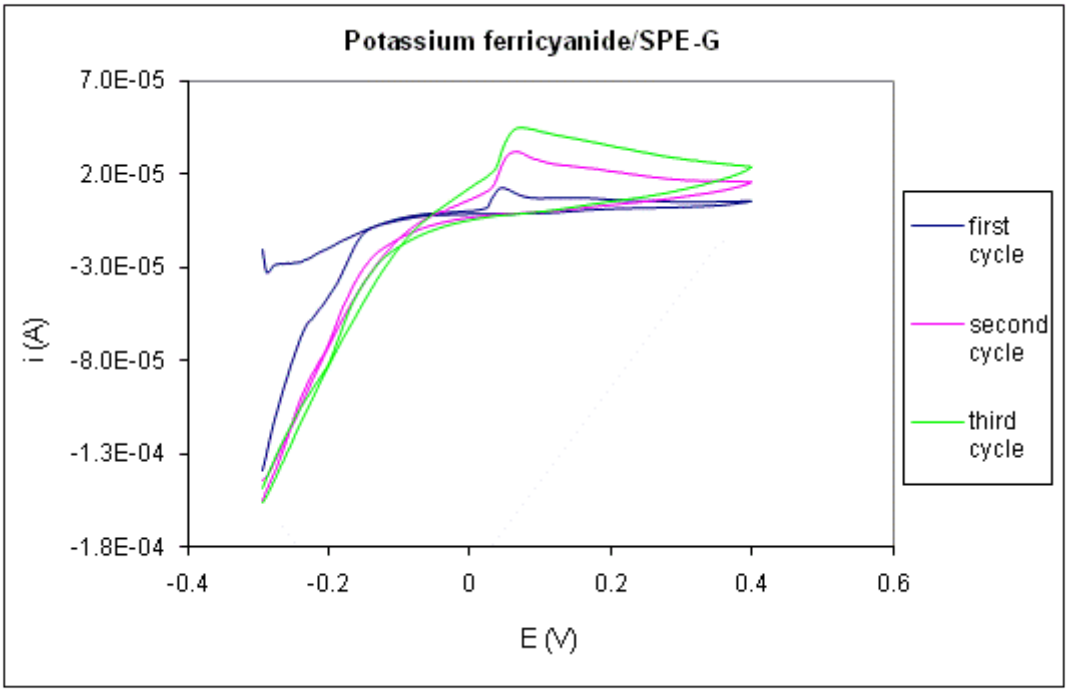

Figure 34. Cyclic voltammetry of potassium ferricyanide (0.5 mM) in PB buffer at pH 7.0 on SPE-G electrodes. Scan rate 50 mV/s.

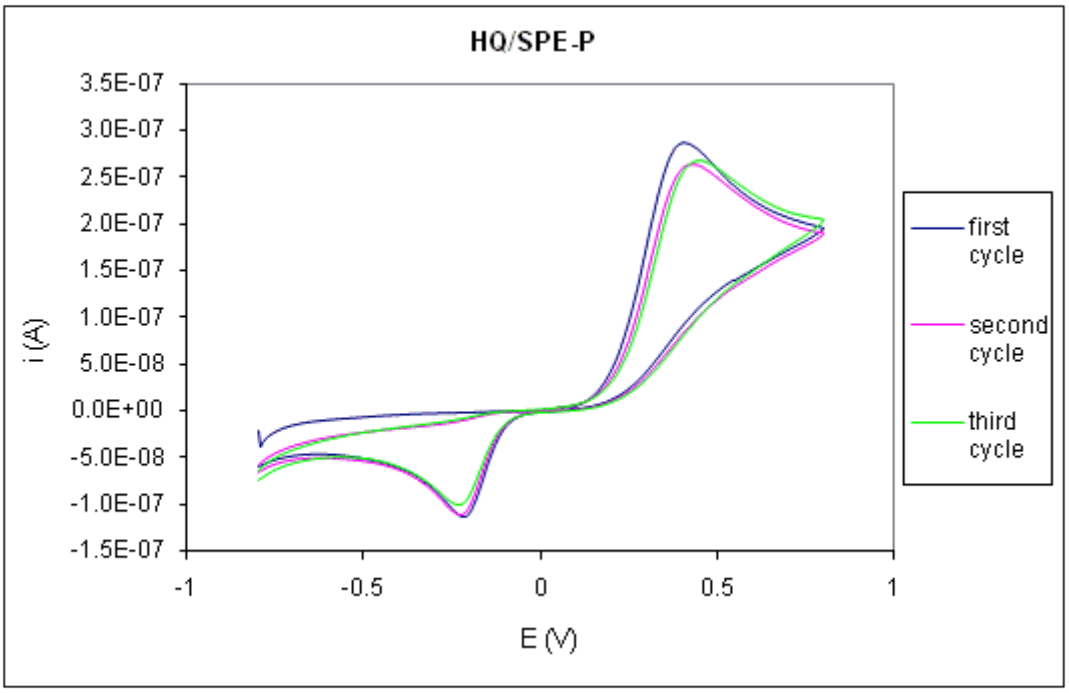

Figure 35. Cyclic voltammetry of HQ (0.5 mM) in PB buffer at pH 7.0 on SPE-P electrodes. Scan rate 50 mV/s.

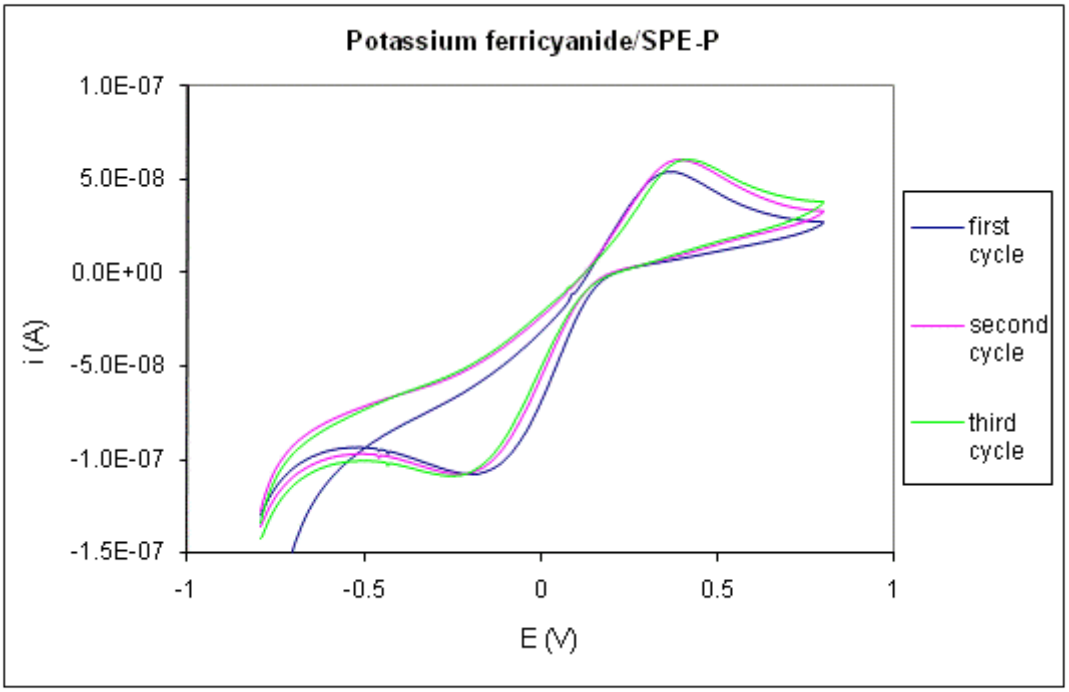

Figure 36. Cyclic voltammetry of potassium ferricyanide (0.5 mM) in PB buffer at pH 7.0 on SPE-P electrodes. Scan rate 50 mV/s.

6.2.3 Influence of scan rate on performance of SPE-D electrodes and electrochemical cell

SPE-D electrodes were compared to the electrochemical cell. Cyclic voltammetry was used to compare the SPE-D with the electrochemical cell using the potassium ferricyanide at different scan rate (Figures 37-40).

Table 9. Electrochemical cell with the potassium ferricyanide at different scan rates.

Scan rate	**Electrochemical cell**							
V	**$V^{1/2}$**	**i_{pa}**	**i_{pc}**	**E_{pa}**	**E_{pc}**	**ΔE_p**	**j_a**	**j_c**
V/s		µA		mV			µA/cm^2	
0.005	0.070711	0.59	-0.295	225	155	**70**	**29.36**	**-14.68**
0.02	0.141421	1.1	-0.784	225	155	**70**	**54.74**	**-39.01**
0.05	0.223607	1.67	-1.32	225	150	**75**	**83.10**	**-65.68**
0.08	0.282843	2.15	-1.77	225	150	**75**	**106.99**	**-88.08**
0.1	0.316228	2.35	-1.98	230	150	**80**	**116.94**	**-98.53**
0.15	0.387298	2.86	-2.44	230	140	**90**	**142.32**	**-121.42**

Table 10. SPE DropSens with the potassium ferricyanide at different scan rates.

Scan rate	**SPE DropSens**							
V	**$V^{1/2}$**	**i_{pa}**	**i_{pc}**	**E_{pa}**	**E_{pc}**	**ΔE_p**	**j_a**	**j_c**
V/s		µA		mV			µA/cm^2	
0.005	0.070711	0.93	-1.55	180	75	**105**	**7.41**	**-12.34**
0.02	0.141421	3.8	-4.23	185	70	**115**	**30.25**	**-33.68**
0.03	0.173205	4.04	-4.79	185	60	**125**	**32.17**	**-38.14**
0.05	0.223607	4.85	-5.77	190	50	**140**	**38.61**	**-45.94**
0.08	0.282843	7.02	-6.91	205	50	**155**	**55.89**	**-55.02**
0.1	0.316228	7.99	-7.47	210	50	**160**	**63.61**	**-59.47**

A linear dependence i_p vs square root of scan rate was obtained with the electrochemical cell and also SPE-D electrodes. Electrochemical cell showed linearity described by the equation y (i_{pa}) =7.19x+0.081 with R^2=0.9995 and y (i_{pc}) =-6.81+0.184 with R^2=0.9995. The SPEs displayed linearity according to the equation y (i_{pa}) =25.02x-0.152 with R^2=0.9567 and y (i_{pc}) =-18.76x-1.567 with R^2=0.9996. Linear dependence of peak current from the square root of the scan rate indicates reversible reaction (Wang, 2006). Peak separation was different at applied scan rates for both examples, but for the electrochemical cell, this was not significant (Tables 9-10). Higher anodic and cathodic current density was obtained with the electrochemical cell.

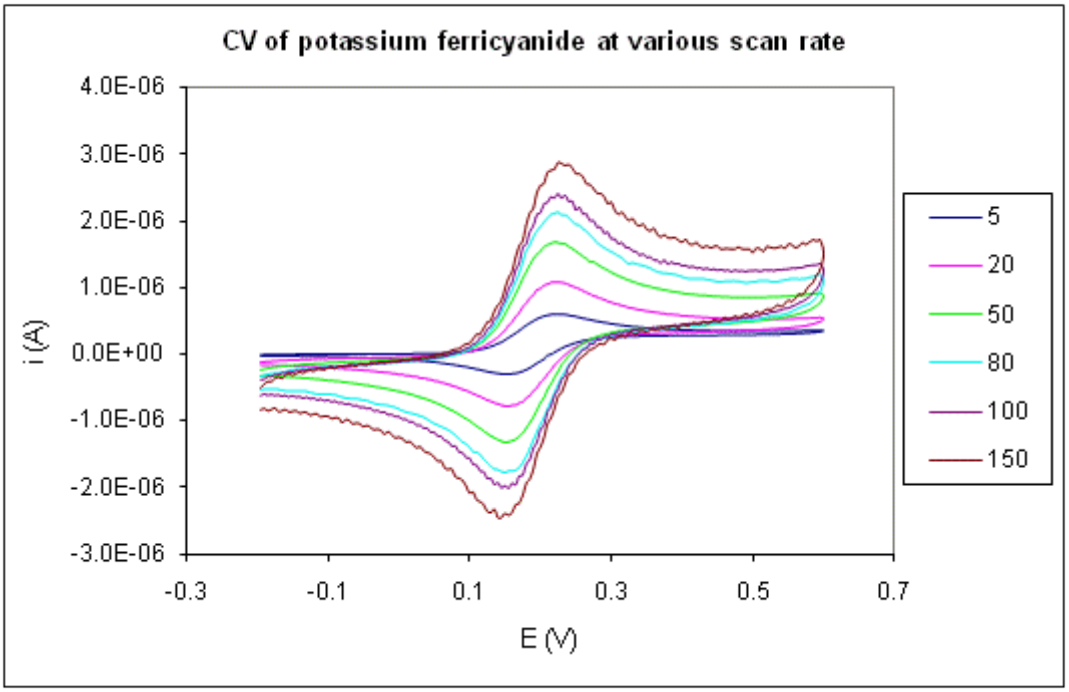

Figure 37. Cyclic voltammetry of potassium ferricyanide (0.5 mM) in PB buffer at pH 7.0. The electrochemical cell: Au-WE, Pt-CE, calomel-RE. Scan rates from 5 to 150 mV/s.

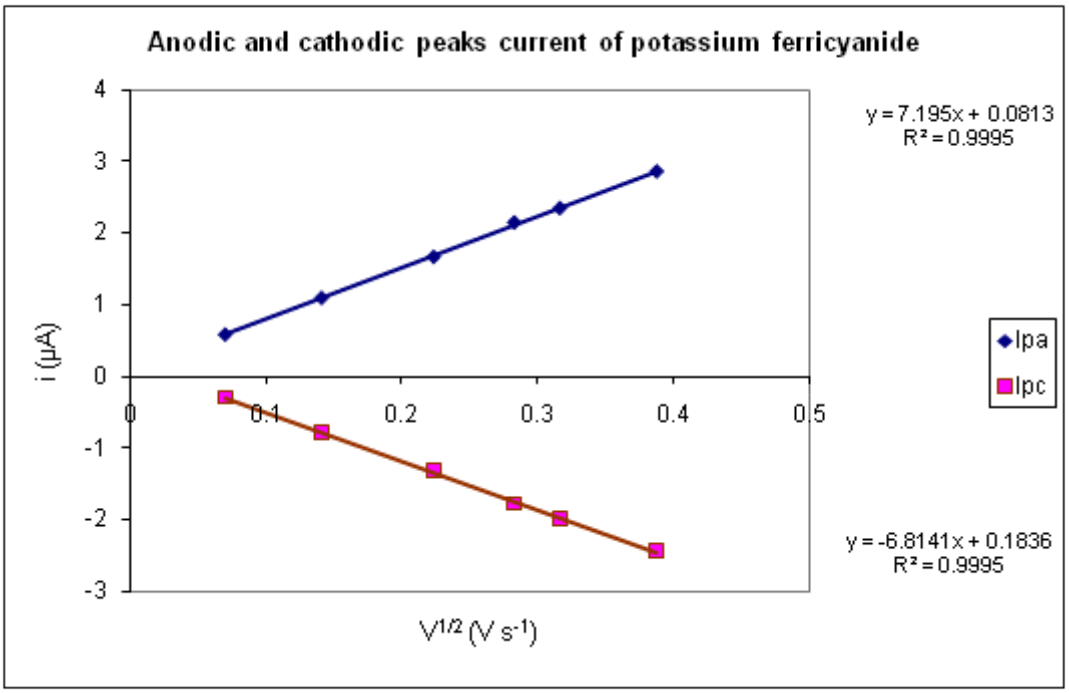

Figure 38. Plots of anodic (i_{pa}) and cathodic (i_{pc}) peaks current of potassium ferricyanide vs. $v^{1/2}$ from CVs of Figure 37.

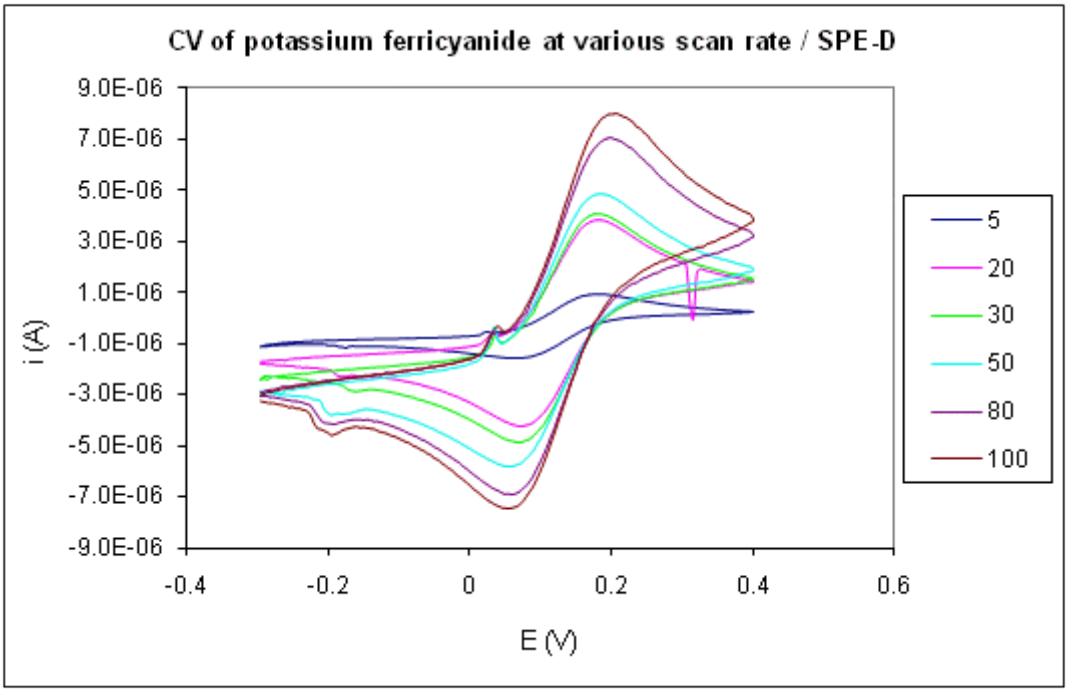

Figure 39. Cyclic voltammetry of potassium ferricyanide (0.5 mM) in PB buffer at pH 7.0 on SPE-D electrodes. Different scan rate from 5 to 150 mV/s.

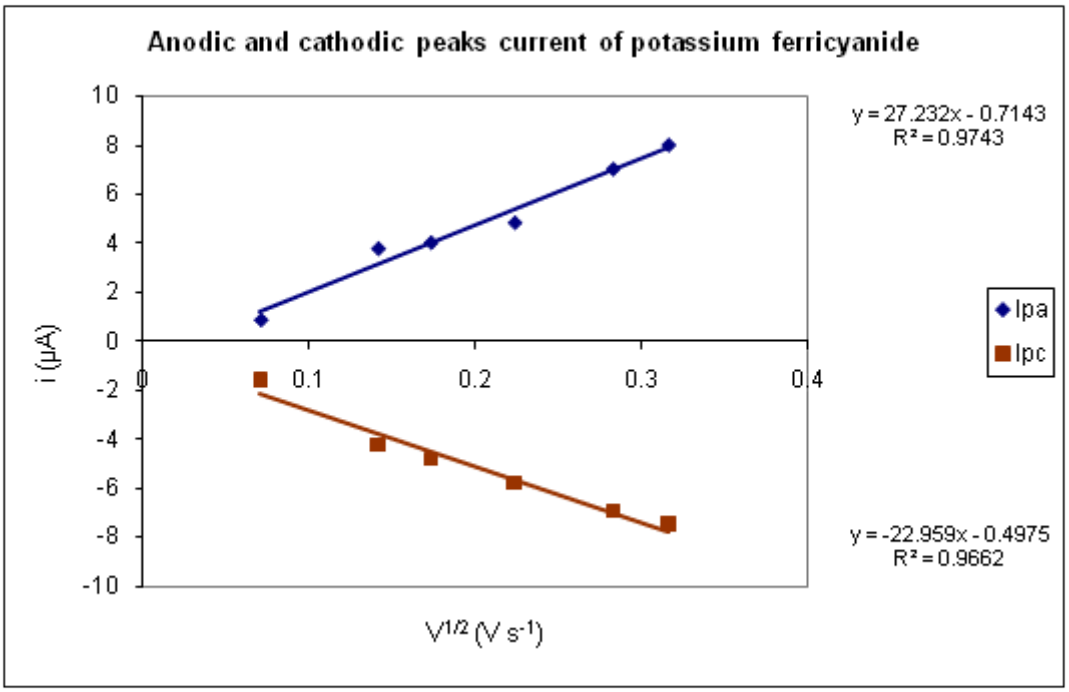

Figure 40. Plots of anodic (i_{pa}) and cathodic (i_{pc}) peaks current of potassium ferricyanide vs. $v^{1/2}$ from CVs of Figure 39.

6.3 Conclusions

The electrochemical performance of several disposable screen printed carbon electrodes and the classical electrochemical cell with bulk electrodes were compared. SPE-D electrodes were chosen for further research since they showed the best characteristics. SPEs would allow conducting research with a small volume of samples, which is required to study an expensive analyte such as glycated haemoglobin (HbA1c).

Chapter 7: Peroxidase system optimisation

Initial experiments were focussed on developing a model system for investigation of electrochemical properties of haemoglobin. The aim of this chapter was to select parameters, such as type of mediator, potential, pH, concentration of substrates, which would give the highest sensor response. HRP was selected to develop a model system due to its similarity to haemoglobin, high activity and low price. Furthermore HRP is one of the most commonly used enzyme in amperometric biosensors for the detection of H_20_2. The model system was developed to eliminate possible, complex and overlapping problems resulting from lower activity of haemoglobin and electrochemical detection of proteins. Cyclic voltammetry and chronoamperometry methods were used for the peroxidase system optimisation.

7.1 Materials and methods

7.1.1 Reagents

HQ (hydroquinone with CAS No: 123-31-9), hydrogen peroxide 30% (CAS No: 7722-84-1), sodium monobasic phosphate (CAS No: 7558-80-7), sodium dibasic phosphate (CAS No: 7558-79-4), potassium chloride (CAS No: 7447-40-7), sodium chloride (CAS No: 7647-14-5), sodium hydroxide (CAS No: 1310-73-2), L-ascorbic acid (CAS No: 50-81-7), urea (CAS No: 57-13-6), acetaminophen (CAS No: 103-90-2) and horseradish peroxidase type II (CAS No: 9003-99-0) were purchased from Sigma-Aldrich (Dorset, UK). Chemicals were used as received.

7.1.2 Apparatus

All electrochemical experiments were performed with the Uniscan potentiostat - galvanostat instrument PG580 with UiEChem software (Buxton, UK).

Screen Printed Electrodes (SPE-D) were purchased from DropSens Ltd. (Oviedo, Spain). Three electrodes cell were purchased from BASi Electrochemistry Instruments

(Warwickshire, UK). Electrodes were described in Chapter 6.1.2. SPEs were used without any treatment.

7.1.3 Electrochemical analysis

All solutions used for electrochemical analysis were prepared daily. Mediators were prepared in a buffer deoxygenated by purging with nitrogen. Hydroquinone was used within 3 hours. Hydrogen peroxide was prepared in dH_20 deoxygenated by purging with nitrogen. Mediator and hydrogen peroxide solutions were kept at 4^oC during measurements. Phosphate buffer (40 mM) consisted of sodium monobasic phosphate and sodium dibasic phosphate and potassium chloride (100 mM). Alkaline pH was adjusted with 1 M sodium hydrooxide. Measurements were performed at room temperature (22 ^oC).

Selection of mediator was made using solution containing 100 µM H_20_2, 1.5 µg/mL HRP and 0.5 mM mediator. HRP and mediator stock solutions were prepared in 40 mM phosphate buffer, pH 7.0, with supporting electrolyte containing 100 mM KCl.

The effect of applied potential was tested in the presence of 0.5 mM HQ, 200 µM H_20_2 and 1.5 or 100 µg/mL HRP. Peroxidase system has been optimised with HRP (100 µg/mL). Enzyme and mediator stock solutions were prepared in 40 mM phosphate buffer, pH 7.0, with supporting electrolyte of 100 mM KCl.

The effect of pH on the electrode response was studied in the presence of 0.5 mM hydroquinone, 200 µM H_20_2 and 100 µg/mL HRP. The measured samples were prepared in 40 mM phosphate buffer at different pH with supporting electrolyte of 100 mM KCl. Mediator and HRP stock solutions were prepared in dH_20 deoxygenated by purging with nitrogen.

The influence of different concentration of hydroquinone and hydrogen peroxide was studied in 40 mM phosphate buffer, pH 7.5, with supporting electrolyte of 100 mM KCl. Different concentrations of hydroquinone and hydrogen peroxide were tested with HRP (100 µg/mL).

The influence of interfering substances was studied in the presence of 0.5 or 50 mM hydroquinone, 200 µM H_20_2 and 100 µg/mL HRP. Additionally measured samples contained 0.11 mM L-ascorbic acid, 0.17 mM acetaminophen or 4.3 mM urea. Samples were prepared in 40 mM phosphate buffer, pH 7.5, with supporting electrolyte of 100 mM KCl.

Cyclic voltammetry was used for the qualitative characterisation of the peroxidise system. Experiments were performed on SPEs immersed in a vertical position in a 10 mL beaker or in the electrochemical cell with bulk electrodes. Measured samples were stirred for 1 minute before performing cyclic voltammetry. CV was performed under unstirred conditions. Experiments were run at a scan rate of 50 mV/s and the second cycle was analyzed. All measurements were run in duplicate. Data were transferred to Microsoft Office Excel and average figures calculated.

Chronoamperometry was used for the peroxidase system optimisation and selection of parameters such as, type of mediator, applied potential, pH and concentration of peroxidise substrate. The effect of interferences was also investigated. SPEs were immersed in a vertical position in a 10 mL beaker, solutions were stirred for 1 minute and current was recorded. Measurements were performed in duplicate. A background current resulting from peroxidase substrates, hydrogen peroxide and mediator (H_20_2/MED) was subtracted from a signal current resulting from the enzymatic reaction of horseradish peroxidase with hydrogen peroxide and mediator (H_20_2/HRP/MED). Data were processed in Microsoft Office Excel and average values calculated.

7.2 Results and Discussion

7.2.1 Choice of mediator

Mediators such as hydroquinone, potassium ferrocyanide, ferrocenecarboxylic acid, ABTS, dichlorophenol indophenol were tested. They were previously used to construct hydrogen peroxide biosensors (Miao *et al*., 2001; Liu *et al*., 1996; Davis *et al*., 1995). ABTS was already recognised to react with haemoglobin and hydrogen peroxide but this reaction was previously detected spectrophotometrically (Everse *et al.,* 1994).

Cyclic voltammetry and chronoamperometry were used for the qualitative and quantitative analysis of enzymatic reaction, respectively. Measurements were performed in the electrochemical cell. Mediators, except dichlorophenol indophenols, in samples with horseradish peroxidise and hydrogen peroxide (MED/HRP/H_20_2) showed higher cathodic current density in comparison to the samples which contained only mediator (second cycle). Also the first cycle of cyclic voltammetry for DCPIP did not show a positive response for an addition of an enzyme and hydrogen peroxide. DCPIP did not show any reduction and oxidation peak with H_20_2 and HRP. A significant change of E_{pc} was not observed for other samples containing only a mediator and samples: MED/HRP/H_20_2 (Figures 41-45) The highest cathodic current density ($-j_{pc}$) was obtained in a sample with HQ/HRP/H_20_2, namely 157.25 µA/cm^2 with the reduction peak recorded at the potential -90 mV and a difference in current between a mediator and a sample with MED/HRP/H_20_2 was also the highest with HQ, namely 32.34 µA (Figure 41).

Chronoamperometry was performed at a reduction potential. The applied potential (E_{pc}) was read from CV (reduction peak potential, second cycle) for each mediator, except DCPIP which was studied at the potential -40 mV (first cycle). The largest current passage was obtained with HQ/HRP/H_20_2 A difference in current passage between a background current and a signal current, recorded at the time 60 seconds was as follows: 9.7 µA/cm^2 for HQ, 4.2 µA/cm^2 for ABTS, 6.5 µA/cm^2 for DCPIP and 0.72

$\mu A/cm^2$ for potassium ferrocyanide (PFo). Trials with DCPIP showed higher current passage recorded for a background as compared to a signal current (Figure 46).

Measurements were also performed on screen printed electrodes (SPE-D). Cyclic voltammetry was mainly used to obtain fast information about the electrochemical behaviour of chosen mediators on the SPE-D, required for further quantitative studies. Figures 47-51 show an electrochemical response of chosen mediators and also with addition of hydrogen peroxide and horseradish peroxidase. Data are summarized in Table 11. Redox peaks and their separation for each mediator were comparable to the electrochemical cell, except HQ and DCPIP which showed decreased peaks separation. Potential of redox peaks for samples MED and MED/HRP/H_20_2 was not significantly changed. The CV of HQ and FcCOOH containing H_20_2 and HRP showed a higher cathodic peak current as compared to samples with mediator, however, the difference was small. It might have been caused by direct, partial reduction of H_20_2 at negative potentials. Cyclic voltammetry was also not sensitive enough to record higher response of sample (MED/HRP/H_20_2) with chosen parameters. The CV of DCPIP/HRP/H_20_2 showed characteristic two oxidation peaks and one reduction peak, however, a significantly lower current was recorded in comparison to a sample containing only DCPIP.

Table 11. Analysis of cyclic voltammograms performed for mediators on SPE-D. Data were taken from Figures 47-50.

	Mediator		
	E_{pa}	**E_{pc}**	**ΔE_p**
	mV		
HQ	145	-100	245
Potassium Ferrocyanide	195	70	125
ABTS	445	350	95
FcCOOH	260	200	60
DCPIP first peak	-30	-150	120

Further experiments concerned qualitative analysis of HRP activity in a presence of mediators and H_20_2 using chronoamperometry method (Figure 52). Measurements were performed at a potential which was selected from cyclic voltammograms. The applied potential was -100 mV negative of the mediator reduction peak (first peak, second cycle). The highest enzyme response, recorded at the time 60 seconds, was obtained with hydroquinone (6.61 $\mu A/cm^2$). The difference in current passage between a signal and background current for another mediators was calculated to be 3.11 $\mu A/cm^2$ (ABTS), 1.83 $\mu A/cm^2$ (FcCOOH), 0.96 $\mu A/cm^2$ (potassium ferrocyanide) and -4.38 $\mu A/cm^2$ (DCPIP). Studies which were performed with DCPIP showed a lower response with HRP and H_20_2 as compared to a background current (DCPIP/H_20_2).

An increased enzymatic response and a higher difference in signal between samples: MED/H_20_2 and MED/HRP/H_20_2 can be expected with a reduced form of mediator. This is important especially in further studies with haemoglobin since its catalytic properties are not as much pronounced as in the case of peroxidase (Everse *et al.*, 1994). Studies performed with hydroquinone showed the highest enzymatic response. Furthermore, its reduction potential should not be sensitive for oxidation of interfering substances, such as ascorbic acid. Therefore HQ was selected for further research.

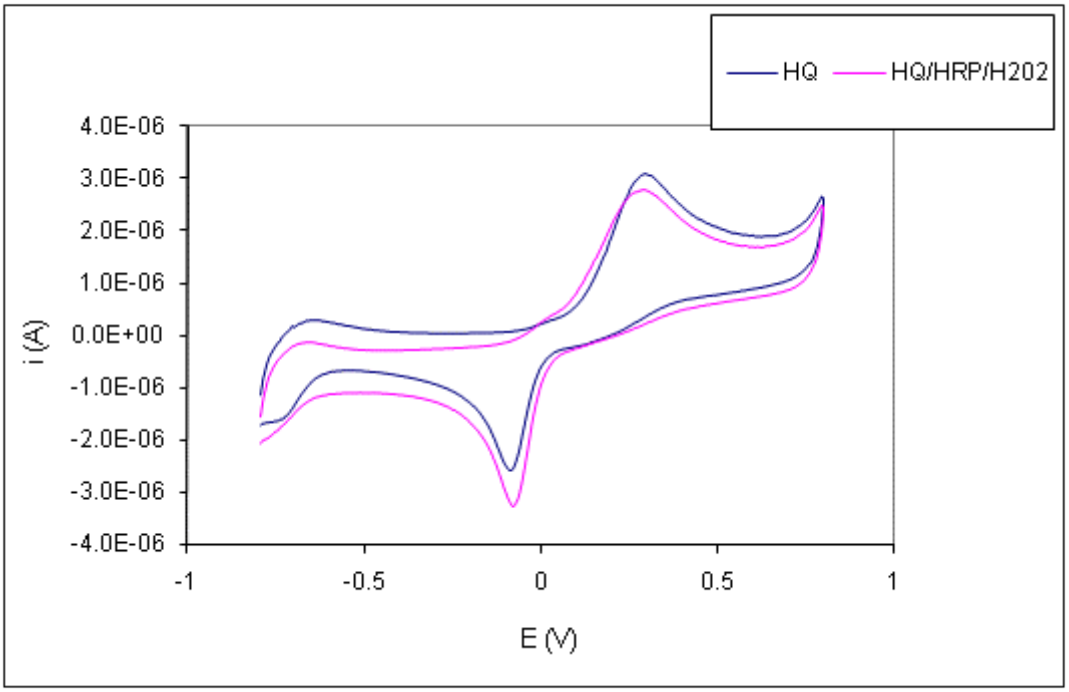

Figure 41. Cyclic voltammetry of HQ (0.5 mM) and in the presence of HRP (1.5 µg/mL) and H_20_2 (100 µM) in PB at pH 7.0. Electrochemical cell: Au-WE, Pt-CE, calomel-RE. Second cycle. Scan rate 50 mV/s.

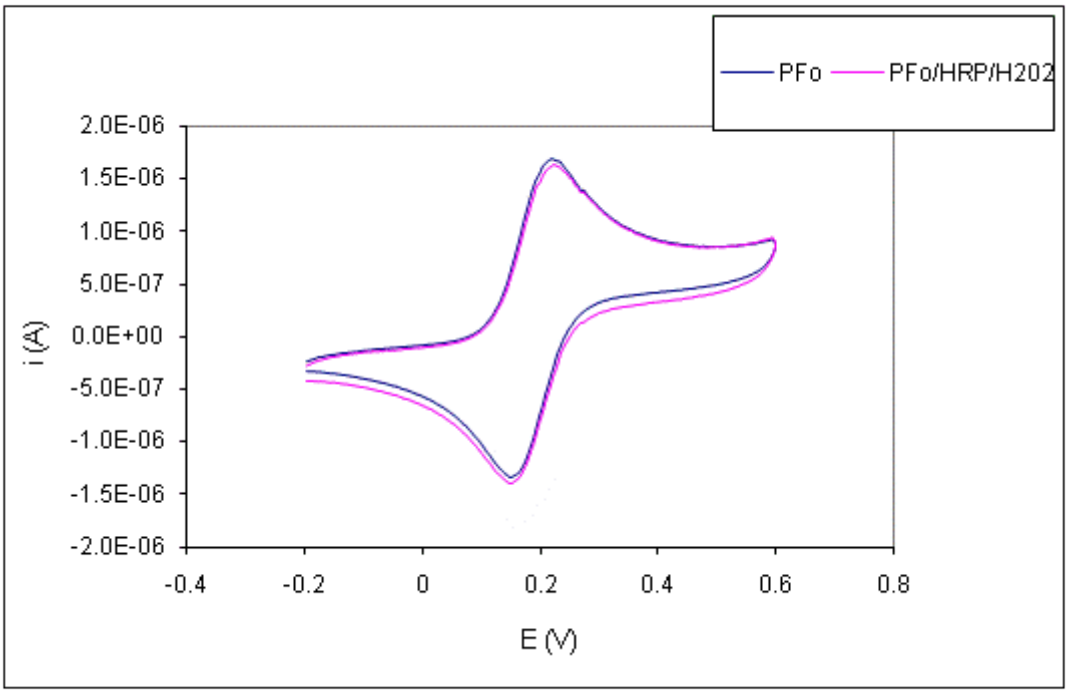

Figure 42. Cyclic voltammetry of potassium ferrocyanide (0.5 mM) in the presence of HRP (1.5 µg/mL) and H_20_2 (100 µM) in PB at pH 7.0. Electrochemical cell: Au-WE, Pt-CE, calomel-RE. Second cycle. Scan rate 50 mV/s.

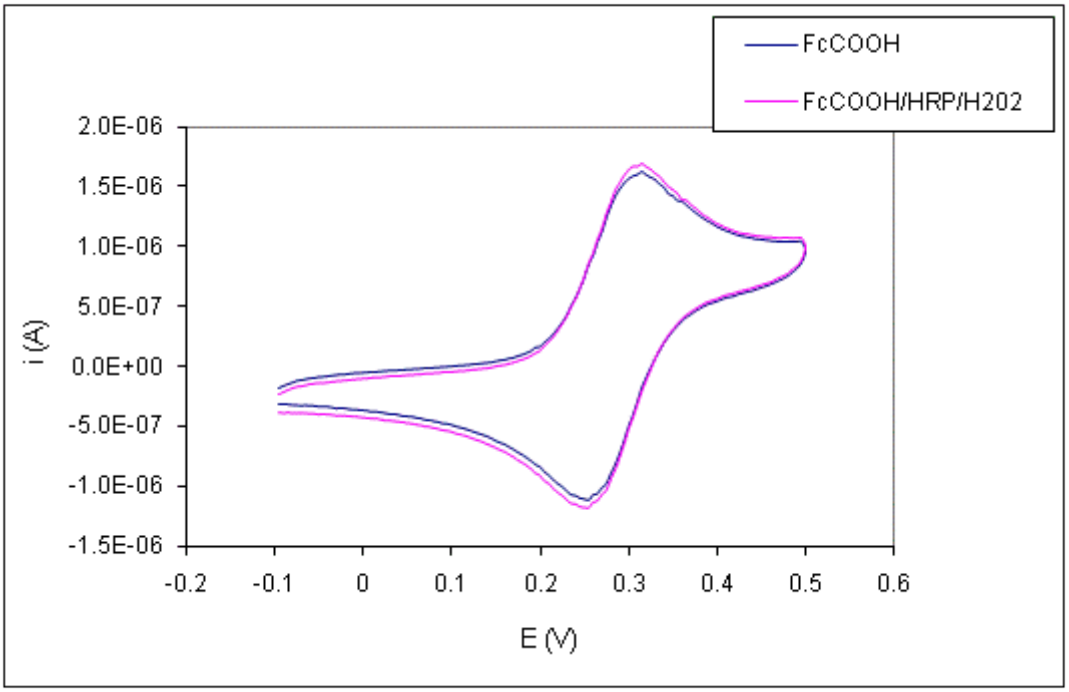

Figure 43. Cyclic voltammetry of ferrocenecarboxylic acid (0.5 mM) in the presence of HRP (1.5 µg/mL) and H_20_2 (100 µM) in PB at pH 7.0. Electrochemical cell: Au-WE, Pt-CE, calomel-RE. Second cycle. Scan rate 50 mV/s.

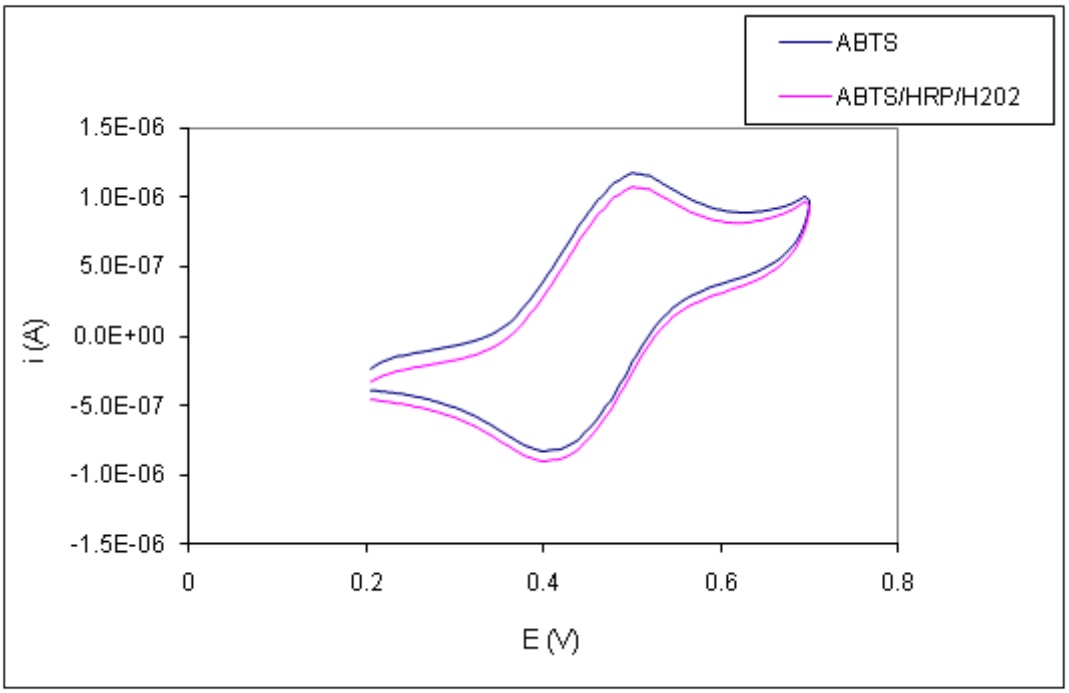

Figure 44. Cyclic voltammetry of ABTS (0.5 mM) in the presence of HRP (1.5 µg/mL) and H_20_2 (100 µM) in PB at pH 7.0. Electrochemical cell: Au-WE, Pt-CE, calomel-RE. Second cycle. Scan rate 50 mV/s.

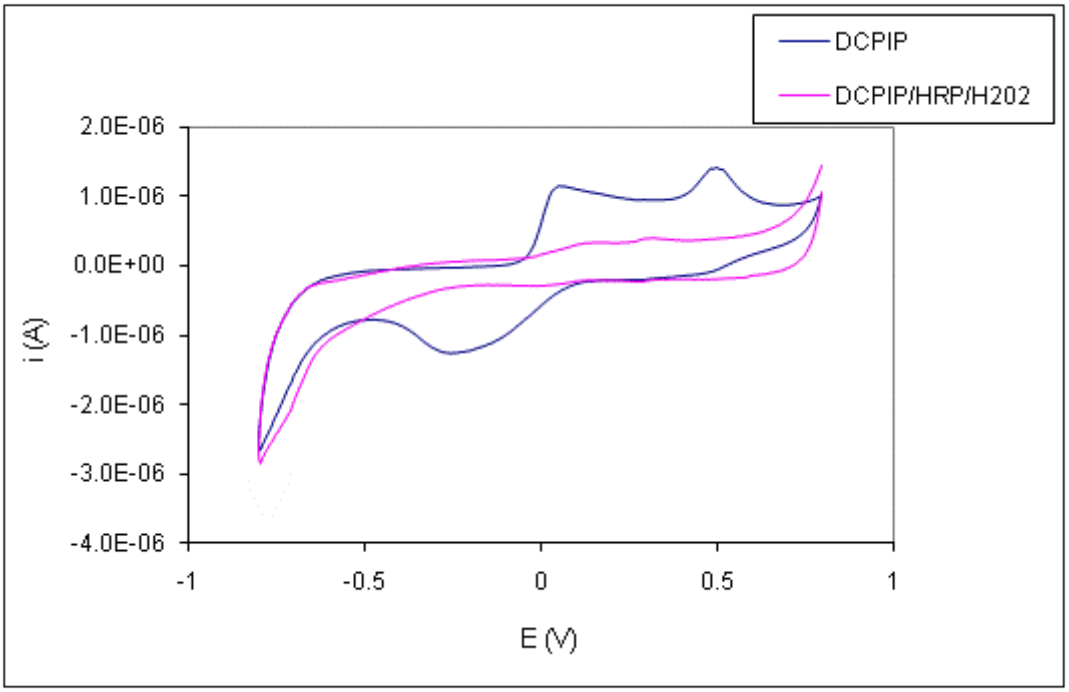

Figure 45. Cyclic voltammetry of DCPIP (0.5 mM) in the presence of HRP (1.5 µg/mL) and H_20_2 (100 µM) in PB at pH 7.0. Electrochemical cell: Au-WE, Pt-CE, calomel-RE. Second cycle. Scan rate 50 mV/s.

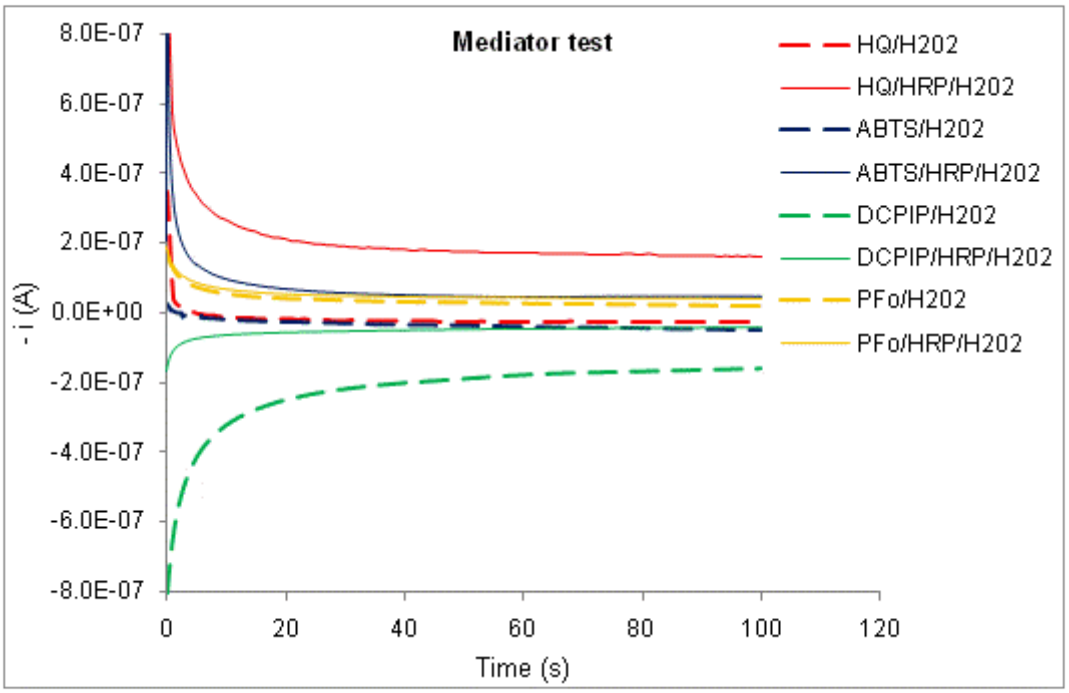

Figure 46. Amperometric response of sensor for addition H_20_2 (100 µM), HRP (1.5 µg/mL) and tested mediator (0.5 mM).

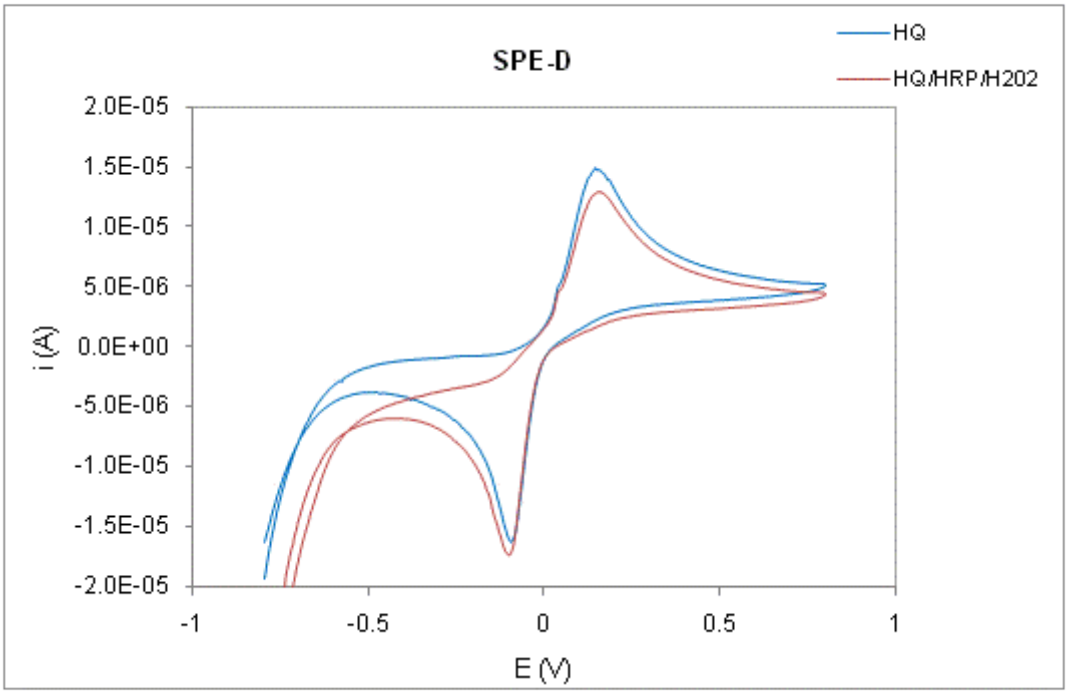

Figure 47. Cyclic voltammetry of HQ (0.5 mM) in the presence of HRP (1.5 µg/mL) and H_20_2 (100 µM) in PB at pH 7.0 on SPE-D. Second cycle. Scan rate 50 mV/s.

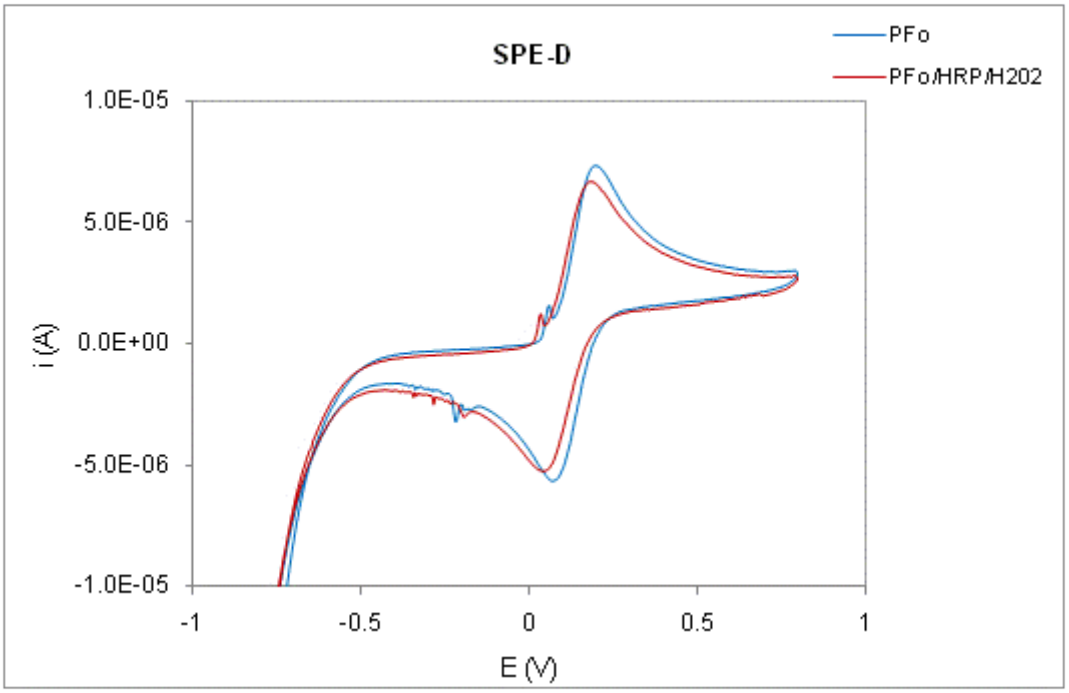

Figure 48. Cyclic voltammetry of potassium ferrocyanide (0.5 mM) in the presence of HRP (1.5 µg/mL) and H_20_2 (100 µM) in PB at pH 7.0 on SPE-C. Second cycle. Scan rate 50 mV/s.

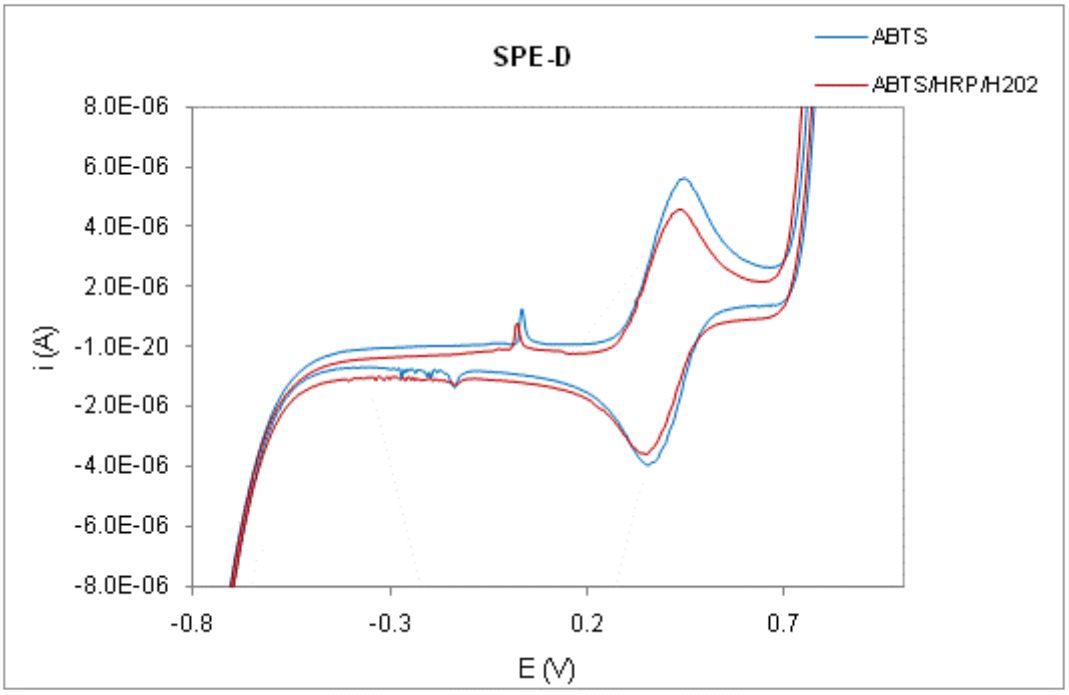

Figure 49. Cyclic voltammetry of ABTS (0.5 mM) in the presence of HRP (1.5 µg/mL) and H_20_2 (100 µM) in PB at pH 7.0 on SPE-D. Second cycle. Scan rate 50 mV/s.

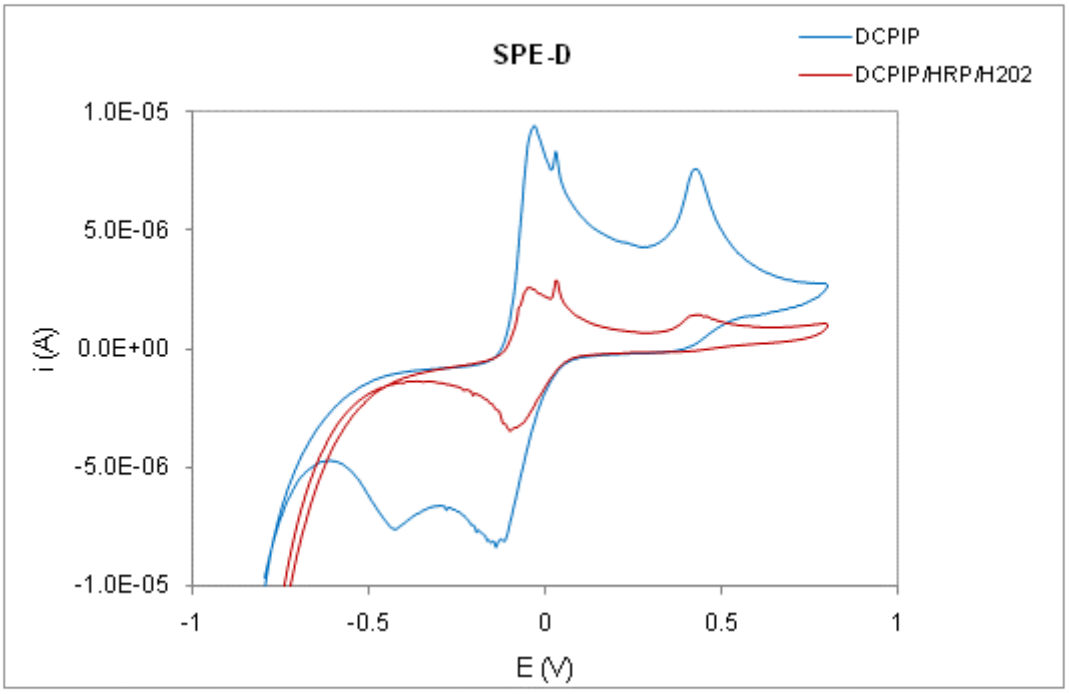

Figure 50. Cyclic voltammetry of DCPIP (0.5 mM) in the presence of HRP (1.5 µg/mL) and H_20_2 (100 µM) in PB at pH 7.0 on SPE-D. Second cycle. Scan rate 50 mV/s.

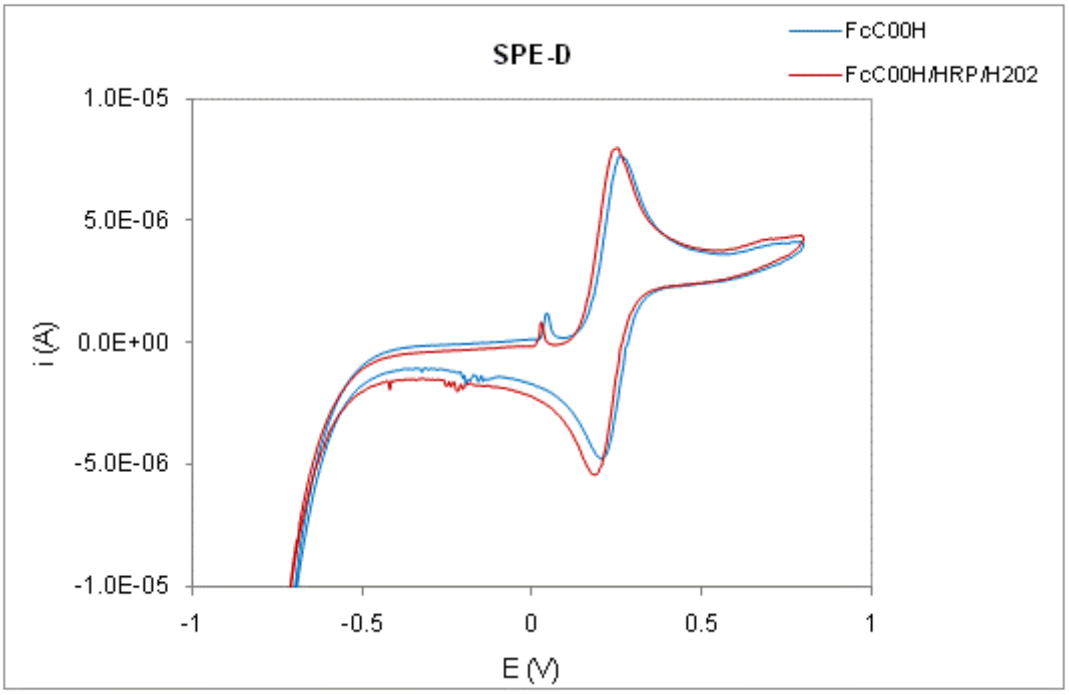

Figure 51. Cyclic voltammetry of FcCOOH (0.5 mM) in the presence of HRP (1.5 µg/mL) and H_20_2 (100 µM) in PB at pH 7.0 on SPE-D. Second cycle. Scan rate 50 mV/s.

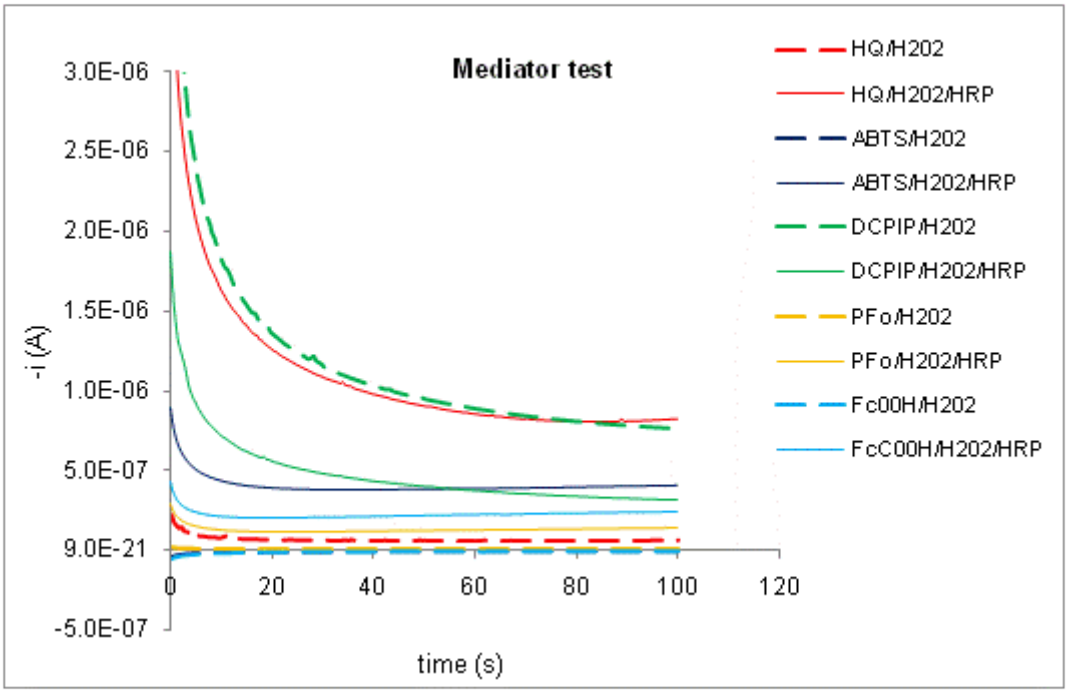

Figure 52. Amperometric response of sensor (SPE-D) for addition H_20_2 (100 µM), HRP (1.5 µg/mL) and tested mediator (0.5 mM).

7.2.2 Peroxidase system optimisation with HQ

Peroxidase system optimisation was continued at SPE-D electrodes. They were recognized to have a good quality and also showed the best response with HQ. The area of working electrode (12.56 mm^2) is sufficient to modify it with a polymer. Additionally, contribution of a background current such as non-faradaic current associated with double layer charging is lower with a smaller working electrode. It is essential to minimise this effect which is especially important for detecting a small amount of analyte. Cyclic voltammetry and chronoamperometry were used to optimise a system H_20_2/HRP/HQ.

Figures 53 and 54 show cyclic voltammograms for HQ and H_20_2/HRP/HQ with different concentrations of HRP. Data from cyclic voltammograms are presented in Table 12. The sample with HRP (100 µg/mL) showed a reduction potential of HQ which was shifted about 70 mV and 55 mV in comparison to the sample without HRP and with 1.5 µg/mL HRP, respectively. Measurements with HRP (100 µg/mL) showed also increased peak to peak separations, about 150 mV in comparison to measurements without the enzyme and decreased cathodic peak current. Most probably it was caused by the adsorption of protein on the electrode and its partial blocking. Results suggest that the adsorption process occur very fast and during about 30 seconds of the first scan. Similar effect caused by the adsorption of other proteins, such as human serum albumin (HSA) and immunoglobulin G (IgG) with the protein concentration of 100 µg/mL was demonstrated by Moulton *et al*. (2003). Cyclic voltammograms of ferricyanide showed wider peaks separation and decreased anodic and cathodic peak current. Peaks separation increased from 150 mV to 390 mV after 30 seconds of the adsorption process of HSA under open circuit potential and after 10 minutes, an oxidation peak of ferricyanide was not detected anymore (Moulton *et al*., 2003).

Table 12. Cathodic and anodic peak potential and current for a different concentration of HRP

HRP	**E_{pc}**	**E_{pa}**	**ΔEp**	**i_{pc}**	**i_{pa}**
µg/mL	mV			µA	
0	-85	115	200	-18.9	16.7
1.5	-95	115	210	-21.4	14.05
100	-140	210	350	-17.5	12.08

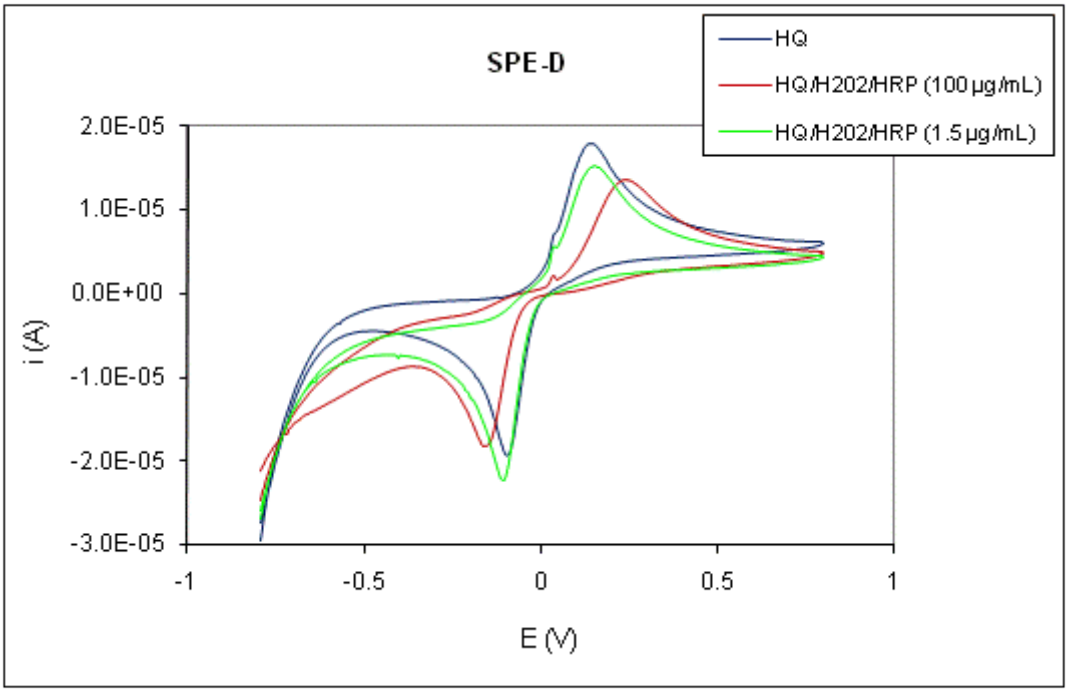

Figure 53. Cyclic voltammetry of HQ (0.5 mM) in the presence of HRP (1.5 or 100 µg/mL) and H_20_2 (200 µM) in PB at pH 7.0 on SPE-D. Second cycle. Scan rate 50 mV/s.

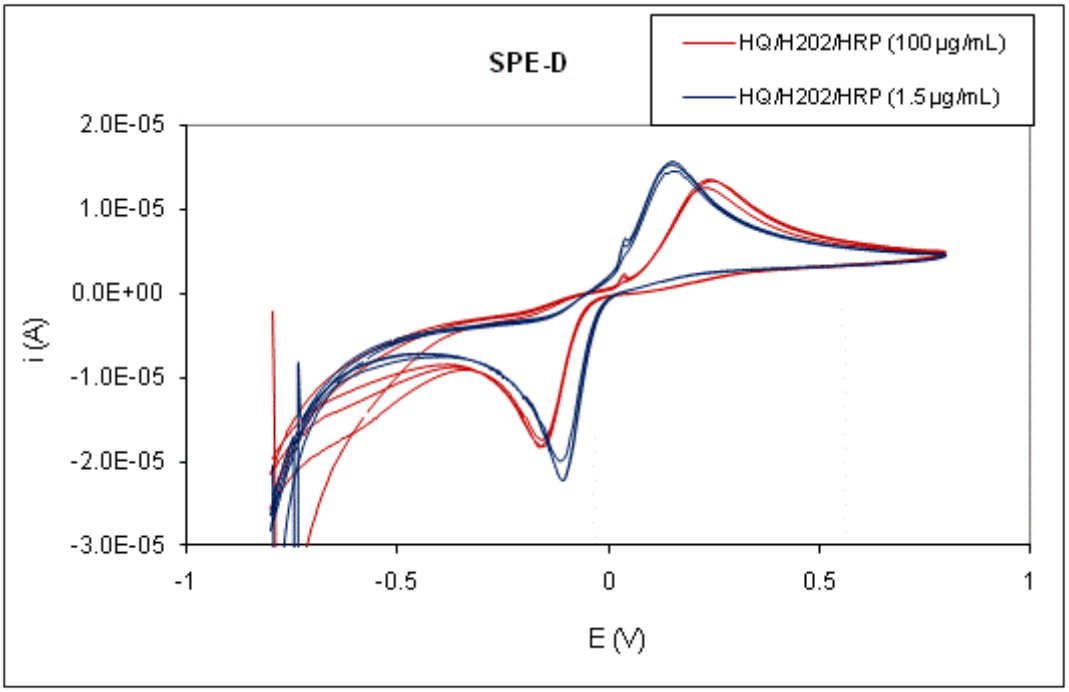

Figure 54. Cyclic voltammetry of HQ (0.5 mM) in the presence of HRP (1.5 or 100 µg/mL) and H_20_2 (200 µM) in PB at pH 7.0 on SPE-D. Cycle 1-3. Scan rate 50 mV/s.

Chronoamperometry was applied to study an impact of different reduction potential (Figure 55). Table 13 shows current recorded (at approximately steady-state current) at the time 50 seconds at a different potential. It was observed that for the higher reduction potential the current recorded for the sample was higher. Significantly higher current was recorded for HQ/H_20_2 and H_20_2/HRP/HQ at a potential of -500 mV. This can be attributed to interferences in the system at a high potential, such as hydrogen evolution and partial direct reduction of H_20_2 on the electrode.

Taking into account obtained results and the fact that an electrode is likely to be fouled by a higher concentration of proteins, a reduction potential of -400 mV was selected for further experiments. The adsorption process of protein on the electrode surface slow an electron transfer process and therefore reactions must be driven by the application of a significant overpotential (Pletcher, 1991).

Table 13. Effect of applied potential on recorded current in samples: HQ/ H_20_2 and H_20_2/HRP/HQ.

	HQ/H_20_2					
Potential	mV	-145	-200	-300	-400	-500
Current	µA	0.02	0.03	0.03	0.14	0.7
	H_20_2/HRP/HQ					
Potential	mV	-145	-200	-300	-400	-500
Current	µA	1.62	1.79	2.00	2.37	4.29

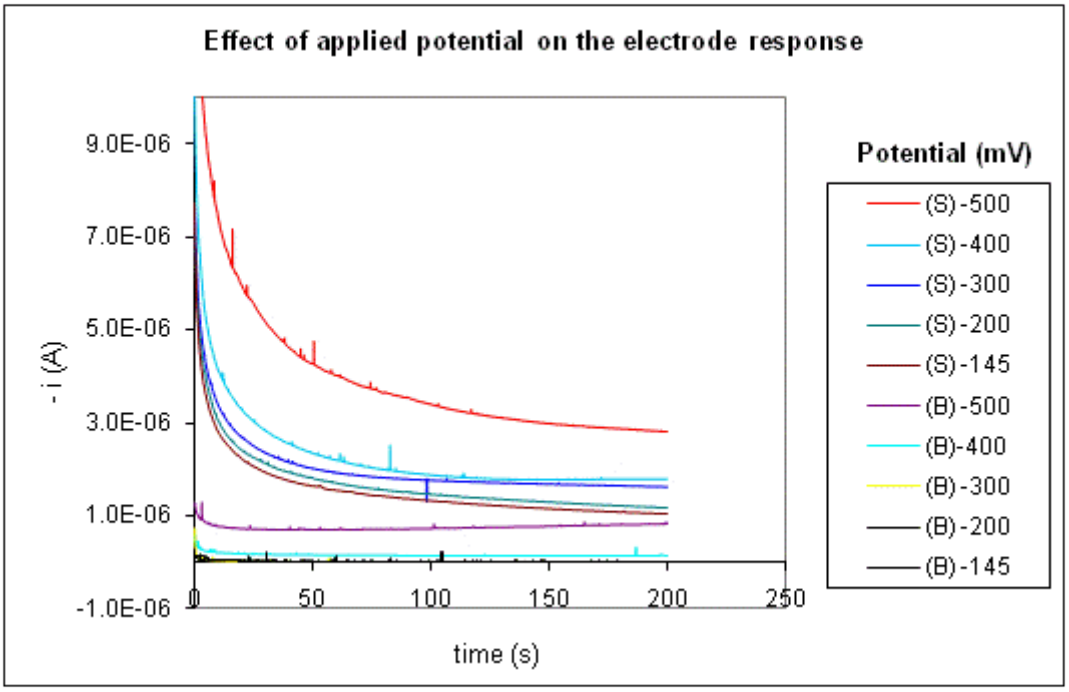

Figure 55. Effect of applied negative potential on electrode response in samples containing HQ (0.5 mM) and H_20_2 (200 µM) in the absence or presence of HRP (100 µg/mL) in PB at pH 7.0. Legend: (B) – background, (S) – signal.

Different pHs were tested to enhance enzymatic performance of HRP (Figure 56). The current recorded at approximately steady-state, at the time 50 seconds, is shown in Table 14.

The highest current was recorded at pH 8.5 and pH 9.0. However interferences were visible in the system, namely current decay and its increase with time (Figure 56). The highest response without interferences was obtained at pH 7.5 and pH 8.0. Therefore pH 7.5 was chosen for a further work. Furthermore, the effect of passivation might have

been decreased at pH above the isoelectric point of HRP which is 7.1 (data provided by the producer), at the negative potential. Electrostatic forces might have had an impact on adsorption and orientation of HRP on the electrode. The influence of electrostatic repulsion and attraction of proteins on a charged electrode surface was reported by Guo *et al*. (1996) and Moulton *et al*. (2003). Stability of protein is also an important factor and its denaturation at high pH may cause an easier adsorption of protein onto the electrode surface.

Table 14. Effect of pH on recorded current at the time 50 seconds.

Sample	**H_20_2/HRP/HQ**						
pH	6	6.5	7	7.5	8	8.5	9
i (µA)	2.56	2.48	2.45	2.81	2.92	5.55	8.40

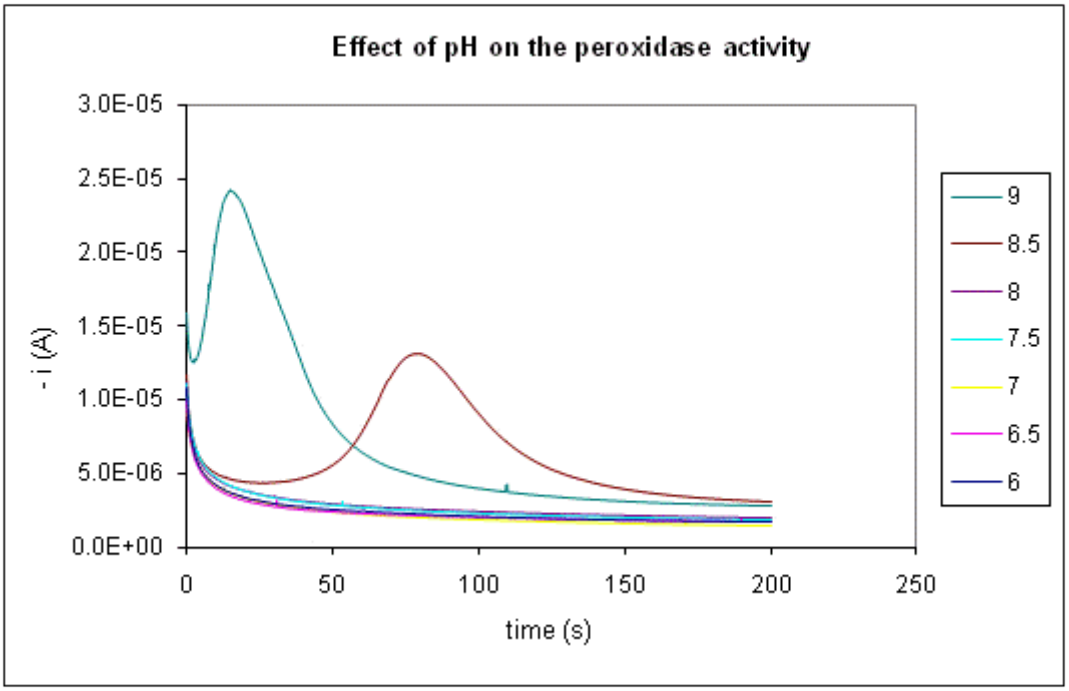

Figure 56. Effect of pH on the peroxidase activity. Samples contained HQ (0.5 mM) in the presence of HRP (100 µg/mL) and H_20_2 (200 µM) in PB buffer at the applied potential -400 mV.

The influence of hydroquinone and hydrogen peroxide concentration was tested. It is important not to lower a rate of the reaction by affecting the enzyme-substrate kinetics. Figure 57 shows the influence of hydrogen peroxide and hydroquinone concentration on response (recorded current) in measured samples. Various concentrations of HQ: 0.5

mM, 5 mM or 20 mM showed a very similar response in a range of H_20_2 concentrations from 0 to 2 mM. A significantly higher current was recorded at 50 seconds for 50 mM HQ. Further increase of HQ concentration to 100 mM did not show an increased current as compared to 50 mM HQ. Optimum concentration of HQ was estimated to be 50 mM and this one was selected for further research. Enzyme saturation was obtained with 4 mM H_20_2 and 50 mM HQ.

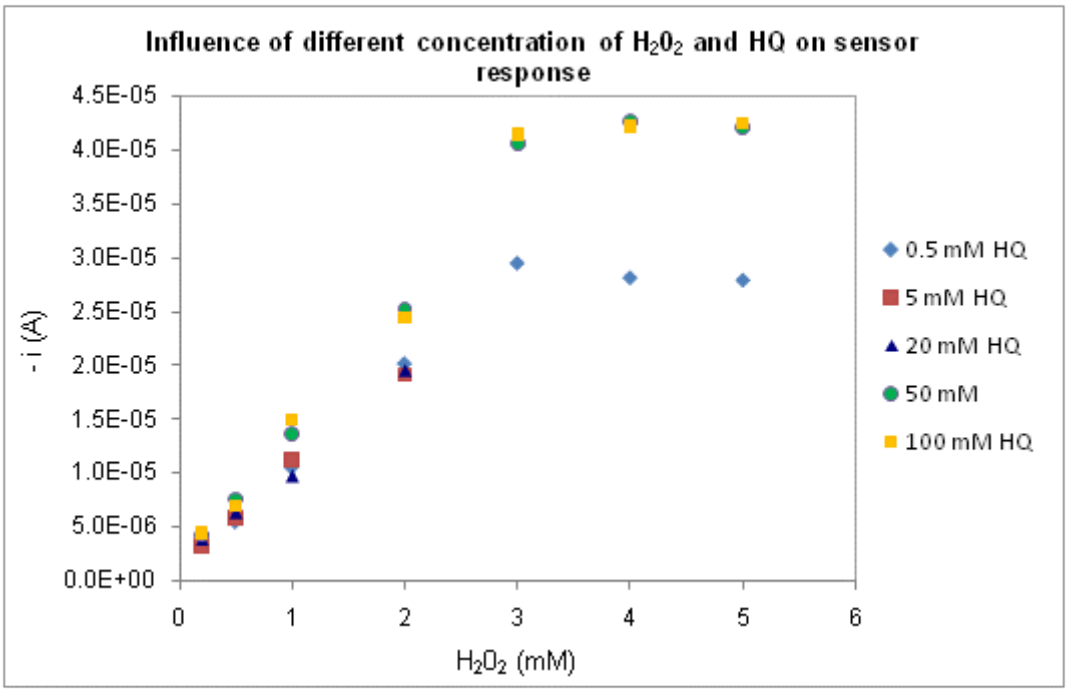

Figure 57. Effect of different concentration of HQ and H_20_2 at PB pH 7.5 in the presence of HRP (100 µg/mL) on its activity at 50 seconds.

The influence of interfering substances was studied. Measured samples contained the highest physiological concentration of interfering substances found in blood, namely 0.11 mM L-ascorbic acid, 0.17 mM acetaminophen, and 4.3 mM urea (Moussy *et al.*, 1993).

Figures 58-59 and Table 15 show the influence of interfering substances on current response at a potential of -400 mV measured at the time 50 seconds. The highest influence was observed with ascorbic acid. Samples of ascorbic acid, acetaminophen or urea in the presence of HRP/HQ showed the highest current in comparison to the samples containing HQ/H_20_2 (except samples ascorbic/HRP/HQ/H_20_2 and HQ/HRP/H_20_2). Probably ascorbic acid reacted with quinone which was produced in the

enzymatic reaction. Quinone was presumably reduced with ascorbic acid, instead of being reduced on the electrode. Research performed by Miao *et al.* showed a reduction of potassium ferricyanide by ascorbic acid (Miao *et al*., 2001). There was no significant influence of interfering substances on a sensor response in the sample with HRP and H_20_2. Results may suggest that these substances do not undergo oxidation or reduction process on the electrode. Figure 60 shows a difference in current between samples: ascorbic/HRP/HQ and ascorbic/HRP/HQ/H_20_2 with different concentration of H_20_2. The highest concentration of hydrogen peroxide produced the highest sensor response. The highest difference in current was obtained for 4.0 mM H_20_2: 36.9 µA (Figure 60).

Table 15. Effect of interfering substances on a sensor response.

	i(µA)
HQ	0.15
HQ/H_20_2	0.18
HRP/H_20_2	0.39
Ascorbic/HRP/HQ	1.07
Urea/HRP/HQ	0.9
Acetaminophen/HRP/HQ	0.83
Ascorbic/HRP/ H_20_2	0.25
Urea/HRP/ H_20_2	0.37
Acetaminophen/HRP/ H_20_2	0.59
Ascorbic/HRP/HQ/ H_20_2	1.86
HQ/HRP/ H_20_2	3.38

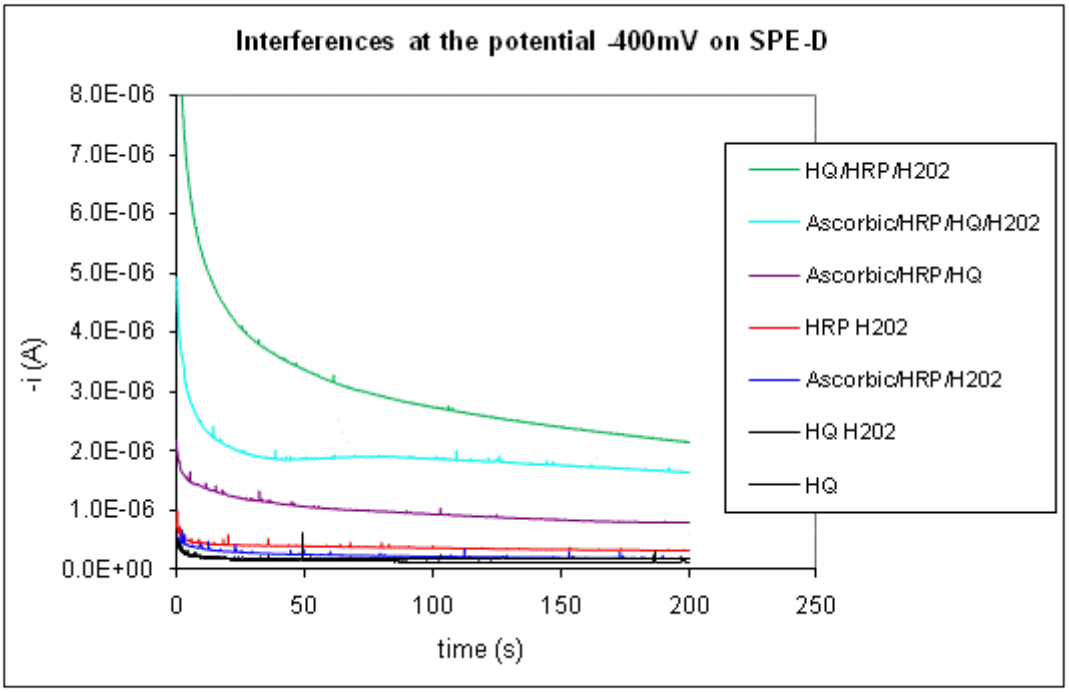

Figure 58. Interferences caused by L-ascorbic acid. Samples contained HQ (0.5 mM), HRP (100 µg/mL) H_20_2 (200 µM), L-ascorbic acid (0.11 mM) in PB pH 7.5 at the applied potential -400 mV.

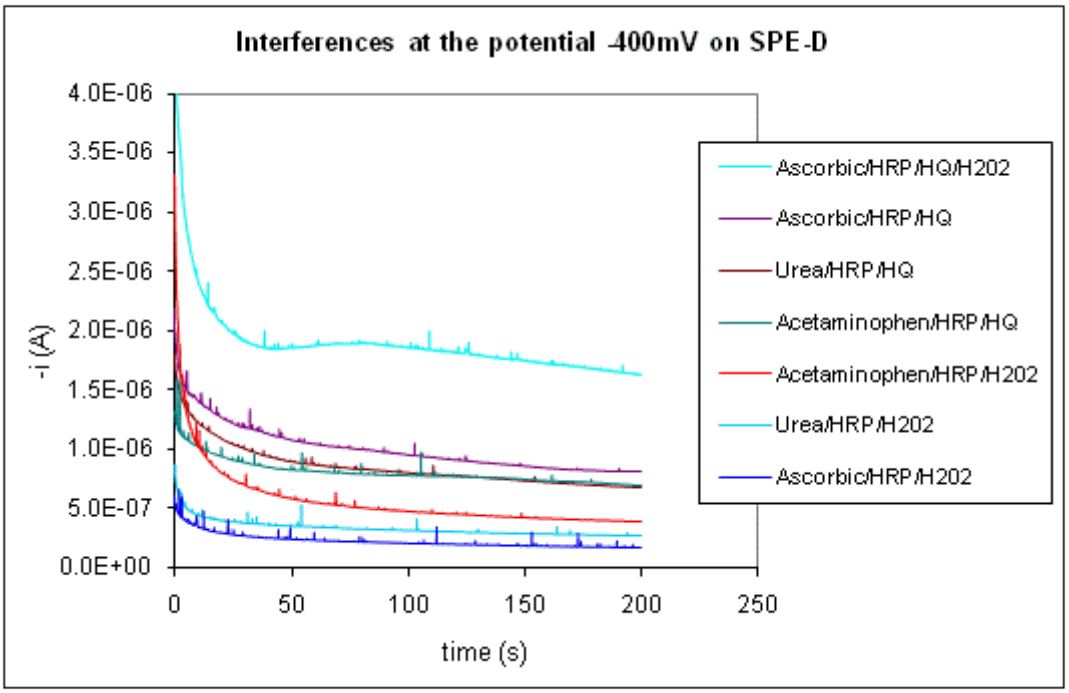

Figure 59. Interferences in the samples containing HQ (0.5 mM), HRP (100 µg/mL), H_20_2 (200 µM), L-ascorbic acid (0.11 mM) or acetaminophen (0.17 mM) or urea (4.3 mM) in PB pH 7.5 at the applied potential -400 mV.

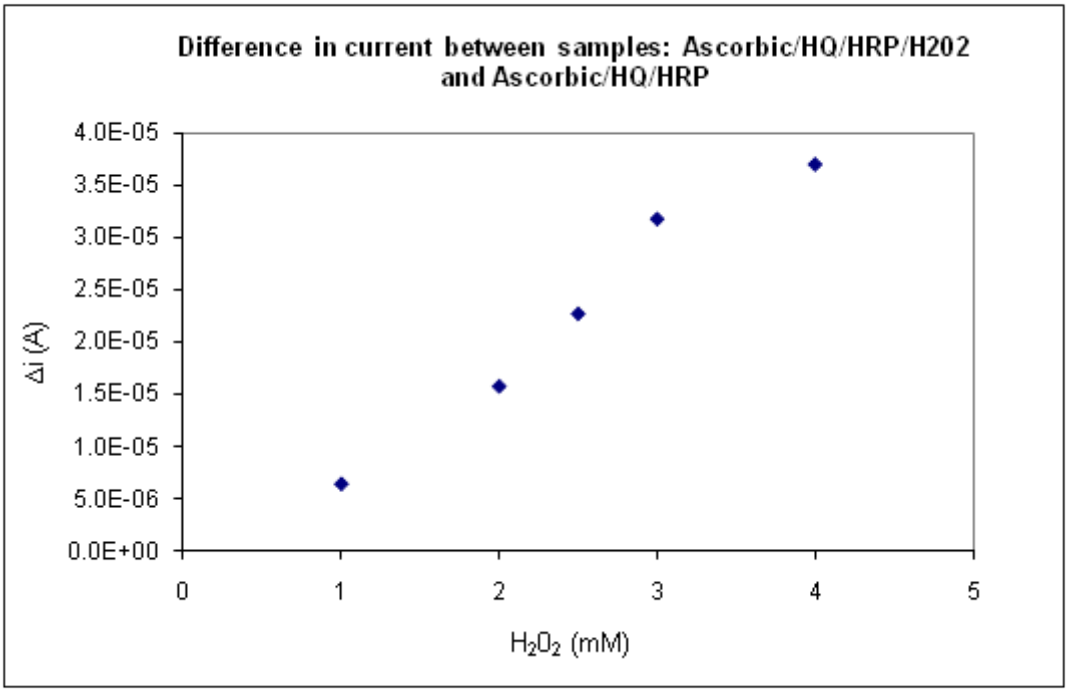

Figure 60. Interferences caused by L-ascorbic acid. Samples contained L-ascorbic acid (0.11 mM), HQ (50 mM), HRP (100 µg/mL) and H_20_2 in PB pH 7.5 at the applied potential -400 mV at 50 seconds.

7.2.3 Conclusions

In this chapter a model system was developed and parameters were optimised for research on electrochemical determination of haemoglobin. Parameters, such as hydroquinone concentration (50 mM), concentration of H_20_2 (4 mM), pH of phosphate buffer (pH 7.5) and the reduction potential (-400 mV) were selected for further experiments. These parameters contributed to a maximal sensor response and a small influence of interfering substances, such as ascorbic acid.

Chapter 8: Electrochemistry of haemoglobin

8.1 Materials and methods

8.1.1 Reagents

Potassium ferricyanide (CAS No: 13746-66-2), MB (Methylene Blue, CAS No: 7220-79-3), HQ (hydroquinone with CAS No: 123-31-9), hydrogen peroxide 30% (CAS No: 7722-84-1), carboxylmethyl cellulose sodium salt (CAS No: 9004-32-4), sodium monobasic phosphate (CAS No: 7558-80-7), sodium dibasic phosphate (CAS No: 7558-79-4), potassium chloride (CAS No: 7447-40-7), sodium chloride (CAS No: 7647-14-5), sodium hydroxide (CAS No: 1310-73-2), phosphate buffer saline tablet, Sephadex G-25 fine (CAS No: 9041-35-4), haemoglobin bovine (CAS No: 9008-02-0), haemoglobin human (CAS No: 9008-02-0), Drabkin's Reagent, 30% Brij 35 solution (9002-92-0) were purchased from Sigma-Aldrich (Dorset, UK). Chemicals were used as received. Human blood was collected into tubes containing EDTA as an anticoagulant. Human blood was kept at 4 ^{o}C and used within two weeks.

8.1.2 Apparatus

All electrochemical experiments were performed with the Uniscan potentiostat - galvanostat instrument PG580 with UiEChem software (Buxton, UK). The Uniscan instrument was used with a beaker or an electrochemical cell which consisted of Screen Printed Electrodes (SPE) or a three electrode cell, respectively.

The three electrode cell consisted of a gold (A=2.01 mm^2) as a working electrode and platinum (A=2.01 mm^2) as a counter electrode and calomel as a reference electrode. They were purchased from BASi Electrochemistry Instruments (Warwickshire, UK). Calomel electrode was purchased from Sigma-Aldrich (Dorset, UK). The gold and platinum electrodes were cleaned after each measurement. Electrodes were cleaned firstly with alumina 0.3 µm particle on a polishing pad and thoroughly rinsed with distilled water (dH_20). Then the electrode was polished on a polishing pad soaked with dH_20 and thoroughly rinsed with dH_20. Screen Printed Electrodes (SPE-D) and Screen

Printed Electrodes - Carbon Nanotubes (SPE CNT-D) were purchased from DropSens Ltd. (Oviedo, Spain). Other type of SPEs (SPE-P) was provided by Pelikan Technologies Inc. Electrodes were described in details in Chapter 6.1.2. SPEs were used without any pre-conditioning or the working electrode surface was modified with 0.5% solution of carboxylmethyl cellulose sodium salt. Also a gold electrode was modified with carboxylmethyl cellulose. CMCNa was used to avoid electrode passivation caused by haemoglobin. CMCNa was prepared in dH_20 and used for modification of working electrode surface. 15 µL of CMCNa was put on SPE-D sensors, 5 µL on SPE-P sensors and the gold electrode. Electrodes were dried 30 minutes in the desiccator at room temperature (24 ^oC).

The following other equipment was used in experiments: Flow-Cell for screen-printed electrodes (wall-jet type) from DropSens (Oviedo, Spain); Minipuls3 Peristaltic Pump Gilson (Middleton, USA); injector Valve V-7 was purchased from GE Healthcare (Amersham, UK); PD-10 column was provided by Sigma-Aldrich (Dorset, UK); Amicon ultra-15 and ultra-4 centrifuge filter (30 kDa cut-off) was provided by Millipore Ltd. (Watford, UK); centrifuge, Jouan B4i was purchased from Thermo Fisher Scientific (Waltham, USA); spectrophotometer, Shimadzu 2100 (Shimadzu, Kyoto, Japan) and pH-meter, Hanna Instruments 8519 was purchased from Hanna Instruments Inc. (Woonsocket, USA).

8.1.3 Haemoglobin isolation from blood

Isolation of Hb was performed at 5 ^oC and purification step was performed at 22 ^oC.
2 mL of human blood was mixed with 6 mL of NaCl (0.9%). Solution was centrifugated for 10 minutes at 1620 g. Supernatant (plasma) was discarded and the washing procedure with NaCl repeated three times. Red blood cells (RBCs) were collected, mixed with 11 mL of distilled water and left for 10 minutes for hemolysis. Solution was centrifugated for 40 minutes at 2880g. Clear, red supernatant containing haemoglobin was collected. Haemoglobin was purified passing over the column which was packed with Sephadex G-25. 2.5 mL of Hb sample was loaded onto the column and eluted with PBS buffer. Hb solution was concentrated on amicon ultra-15 centrifugal filter unit 30 kDa cut-off. Filter unit was rinsed with dH_20 to remove trace amounts of triethylene

glycol and filled with 15 mL of the sample. Centrifugation took 30 minutes at 2880 g. The content of Hb was determined by Drabkin's method.

8.1.4 Spectrophotometric determination of Hb by Drabkin's method

Spectrophotometric determination of Hb was performed according to the Sigma procedure. Drabkin's solution was prepared by reconstitution of one vial of Drabkin's Reagent with 1000 mL of water. Then 0.5 ml of the 30% Brij 35 solution was added. The prepared Drabkin's solution was kept at room temperature 22 ^{o}C and was protected from light. Cyanmethemoglobin standard solution was prepared. It contained 180 mg/mL of human haemoglobin (Hb purchased from Sigma, containing mainly $HbFe^{3+}$) in Drabkin's solution. Cyanmethemoglobin standard solution was diluted as required. 40 µl of the prepared cyanmethemoglobin standard solution was added to 10 mL of the Drabkin's solution. Working standard solutions were prepared by dilution in Drabkin's solution. Absorbance was read versus Drabkin's solution as a reference at 540 nm and calibration curve of absorbance value versus cyanmethemoglobin concentration was plotted. 5 mL of the Drabkin's solution was added to test and blank tubes. Then 20 µL of the whole blood sample was added to test tubes. They were mixed well and left for 20 minutes at room temperature 20 °C. Absorbance was read for test tubes versus the blank as the reference at 540 nm and total haemoglobin concentration (mg/mL) of each test was determined from the calibration curve.

8.1.5 Electrochemical analysis

All solutions used for electrochemical analysis were prepared daily. Mediators, hydrogen peroxide and haemoglobin stock solutions were kept in +4 ^{o}C during measurements. All experiments were performed at room temperature (22 ^{o}C).

Peroxidase activity of $HbFe^{3+}$ was studied with HQ (50 mM) and H_20_2 (4 mM) in deoxygenated or non-deoxygenated phosphate buffer, pH 7.5. Mediator and $HbFe^{3+}$ stock solutions were prepared in a deoxygenated phosphate buffer. Mediator solution was used within 3 hours. Additionally hydroquinone stock solution was deoxygenated by purging with nitrogen over the solution for 5 minutes. Hydrogen peroxide solution

was prepared in deoxygenated dH_20. All solutions were deoxygenated by purging with nitrogen. Stock solutions were kept in +4 ^oC during measurements. Phosphate buffer (40 mM) consisted of sodium monobasic phosphate, sodium dibasic phosphate and potassium chloride (100 mM), pH 7.5.

Potassium ferricyanide, methylene blue and $HbFe^{3+}$ stock solutions were prepared in deoxygenated PBS, pH 7.4. Phosphate Buffer Saline, pH 7.4 was prepared by dissolving one tablet per 200 mL of dH_20.

Two voltammetric techniques were used to investigate electrochemical behaviour and detection of haemoglobin. Cyclic voltammetry was used for the qualitative characterisation of electrodes coated with polymer. Chronoamperometry was used for the detection of Hb ($HbFe^{3+}$ and $HbFe^{2+}$).

Cyclic voltammetry experiments were performed at a scan rate 50 mV/s, starting from negative towards positive potentials, except methylene blue measurements which were started from positive potentials. Measurements were performed on SPEs immersed in a vertical position in a 10 mL beaker or measurements were performed in a 10 mL electrochemical cell, which consisted of a gold working electrode, a platinum counter electrode and a calomel reference electrode. Studies concerning a choice of oxidation potential for potassium ferricyanide and electrodes were performed on SPEs immersed in a vertical position in a 5 mL beaker. Measured samples were stirred for 1 minute before cyclic voltammetry. CV was performed under unstirred conditions. All measurements were run in triplicate. Data were transferred to Microsoft Office Excel and average values calculated.

Chronoamperometric **s**tudies on peroxidase activity of haemoglobin were performed in three different configurations. Initially SPEs electrodes were immersed in a vertical position in a 10 mL beaker with studied samples which consisted of $HbFe^{3+}$, H_20_2 and mediator. Solution was stirred for 10 seconds and current recorded. Enzymatic activity was also investigated by applying a drop on the electrode. 120 µL of studied solution was mixed for 20 seconds and placed on the electrode. Current was recorded at a

potential of -400 mV. Further experiments were focused on detection of haemoglobin in an FIA system at a flow rate 0.5 ml/min. The FIA system was made up of the peristaltic pump, the injector and the flow cell. Samples containing HQ, H_20_2 with or without Hb were injected into the loop (50 µL). Samples were prepared in a phosphate buffer, pH 7.5 and mixed 20 seconds before injection into the running phosphate buffer. Measurements were performed in triplicate. Data were processed in Microsoft Office Excel and average values calculated.

Studies concerning $HbFe^{2+}$ detection with potassium ferricyanide were performed at SPE CNT-D electrodes. Samples which consisted of mediator and different concentration of haemoglobin were incubated for 5 minutes. Afterwards 100 µL of solution was placed on the electrode surface and current was recorded.

8.2 Results and Discussion

8.2.1 Electrochemical detection of haemoglobin using its peroxidase activity

The selection of parameters for Hb detection was based on results obtained with HRP. The chronoamperometric signal was measured at a negative reduction potential of -400 mV on SPE-D electrodes. Haemoglobin ($HbFe^{3+}$) detection was performed with HQ (50 mM) and H_20_2 (4 mM).

Detection of haemoglobin was obtained with three different setups. Amperometric response for different concentrations of haemoglobin is shown in Figures 61-64. A linear calibration curve was obtained for Hb detection in a range from 0.05 to 2 mg/mL (Figures 65-67).

Initial experiments were performed on SPE-D electrodes immersed in a beaker and also by placing a drop of tested solution on an electrode. Measurements were performed under diffusion control. Measured samples gave a calibration curve described by the equation: y = 6.84E-06x + 1.21E-06 and R^2 = 0.9938 for a beaker setup. The amperometric signal was analysed at the time 5 seconds. The characteristic Cottrell behaviour was obtained. However, a deviation from this behaviour is visible after about 100 seconds of experiments (Figure 61) when increased current was detected. It may be attributed to the natural convection that can occur after longer time (over 100 seconds) due to coupled chemical reactions (Wang, 2006). To avoid natural convection current may be recorded in a very short period of time. However problems could result from possible interferences if the data are taken from the first seconds of the experiment where non steady-state current is observed. The recorded current is a result of faradaic current and also a charging current (Pletcher, 1991).

The calibration curve was described by: y = 9.82E-06x + 3.12E-06 and R^2 = 0.9784 for haemoglobin detection on the SPE with 120 µL drop of studied solution and analysed at the time 5 seconds (Figure 65). Calibration curves plotted after a longer time (50 seconds) showed lower sensitivity and were characterised by the equation:

y = 7.17E-06x + 6.04E-07, R2 = 0.9739 (Figure 66). Reduction of quinone on the electrode surface might have been limited by mass transport, especially at the applied high potential in the experiment.

Further studies were performed in the flow injection analysis system (FIA) where the mass transport is not a limiting step. Measurements described by equations: y = 1.97E-05x + 4.21E-06, R2 = 0.982 and y = 1.89E-05x + 4.43E-05, R2 = 0.983 were performed in the flow cell with a deoxygenated or non-deoxygenated buffer as a carrier solution, respectively (Figures 63, 64, 65 and 67). Higher background current was observed for successive injections of samples into a non-deoxygenated stream of carrier solution, however, the sensitivity of the sensor was not significantly decreased (Figure 64). The highest sensitivity was obtained in the FIA system in comparison to previous experiments. Experiments could also have been performed in a stirred solution. However, measurements could be sensitive to the disturbances in the system.

The reduction potential (-400 mV) and also pH 7.5 might have had a positive impact on obtained results. Probably electrode fouling was decreased due to electrostatic forces (repulsion) resulting from negatively charged haemoglobin at pH above its isoelectric point and the negative reduction potential. This effect may be less pronounced at higher protein concentrations. A significant decrease of current passage was detected for Hb (50 mg/mL) which was comparable to the signal recorded for 2 mg/mL of Hb (Figure 62). Detection of haemoglobin over a short time could minimize the fouling effect. An oxidised form of mediator can be also tested since the enzymatic reaction is fast.

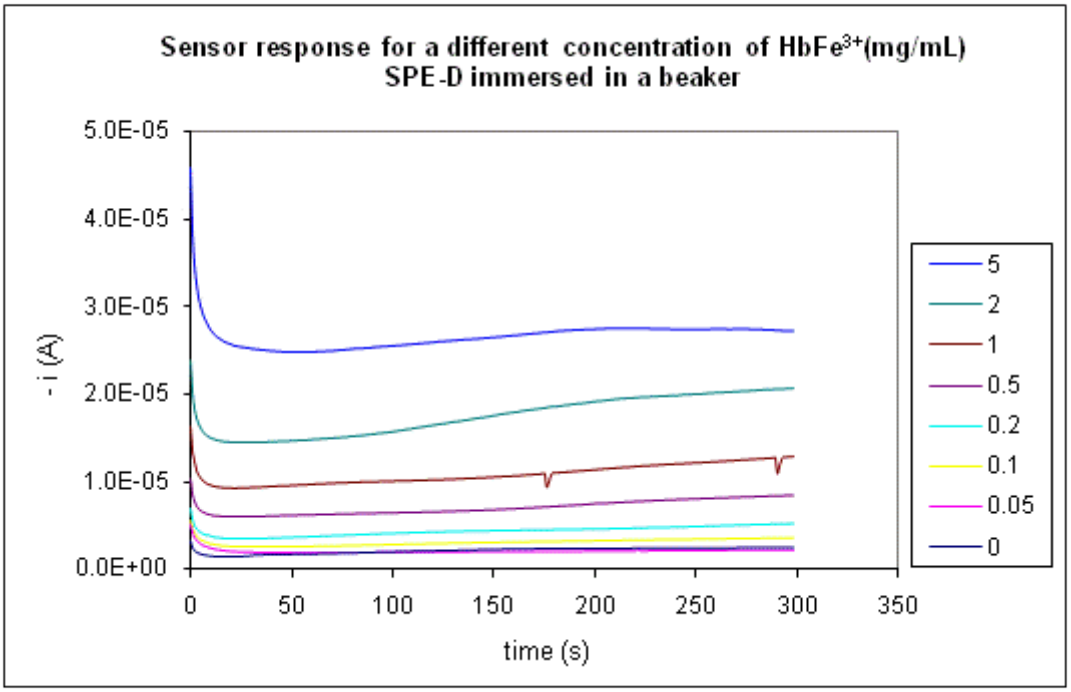

Figure 61. Amperometric response for sample containing different concentration of $HbFe^{3+}$ (mg/mL), HQ (50 mM), H_20_2 (4 mM). SPE-D immersed in a beaker. Signal recorded at a reduction potential -400 mV.

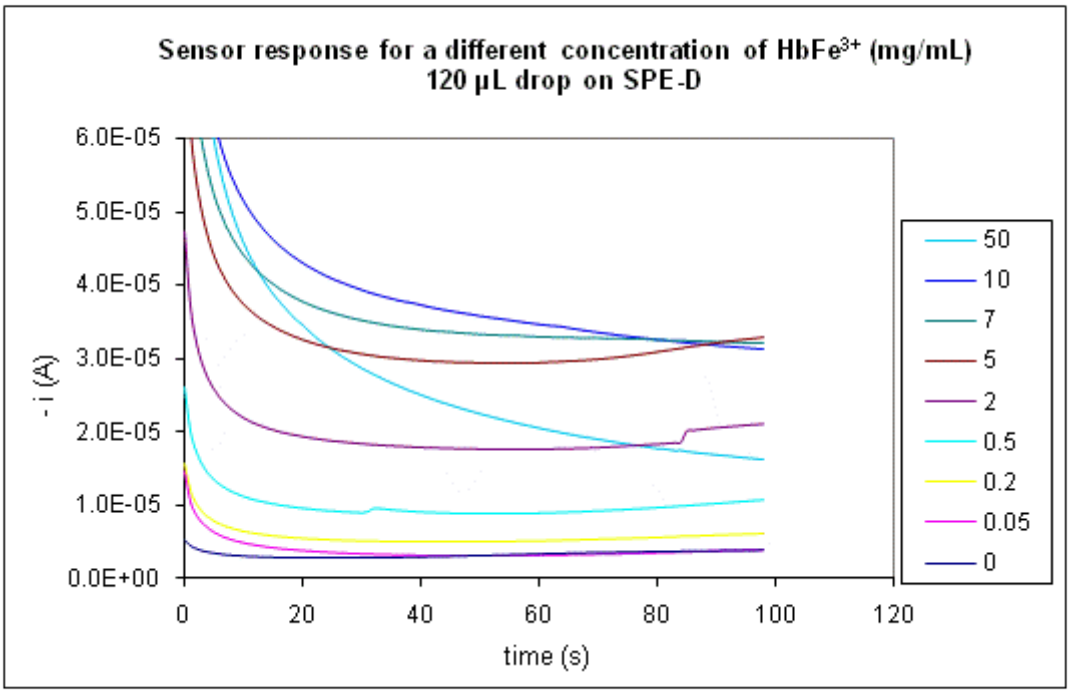

Figure 62. Amperometric response for sample containing different concentration of $HbFe^{3+}$ (mg/mL), HQ (50 mM), H_20_2 (4 mM) at a potential -400 mV. 120 µL of studied solution on SPE-D.

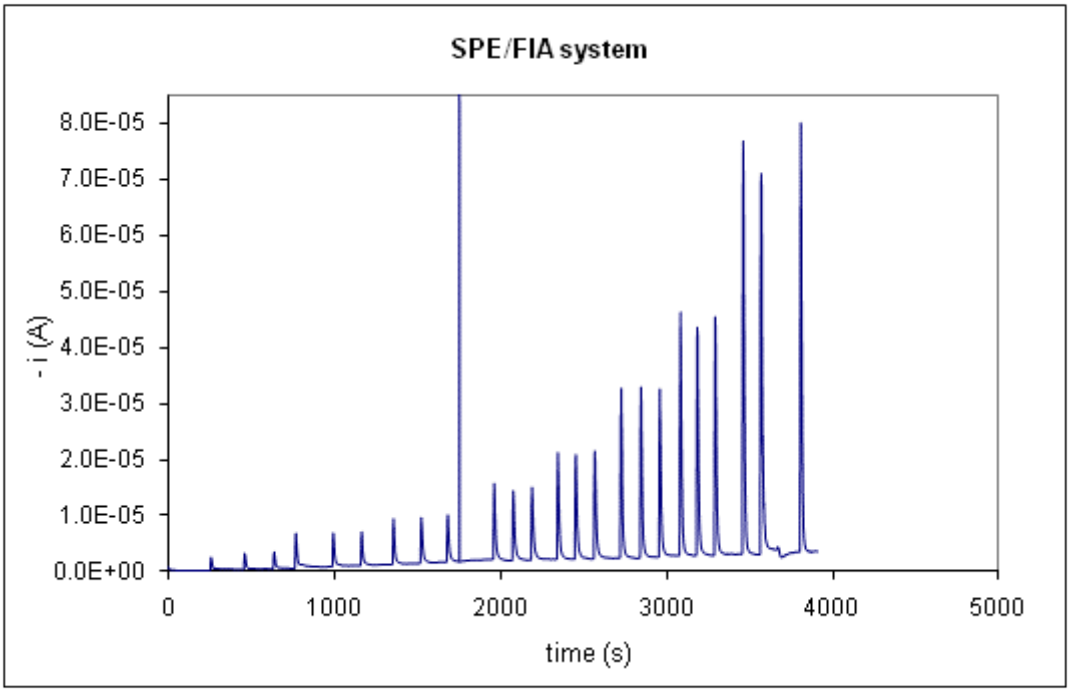

Figure 63. Amerometric response of successive injections of sample (50 µL) containing HQ (50 mM), H_20_2 (4 mM) and $HbFe^{3+}$ (from 0 to 5 mg/mL) on SPE at a potential -400 mV. Carrier solution: deoxygenated PB (pH 7.5). Flow rate 0.5 mL/min.

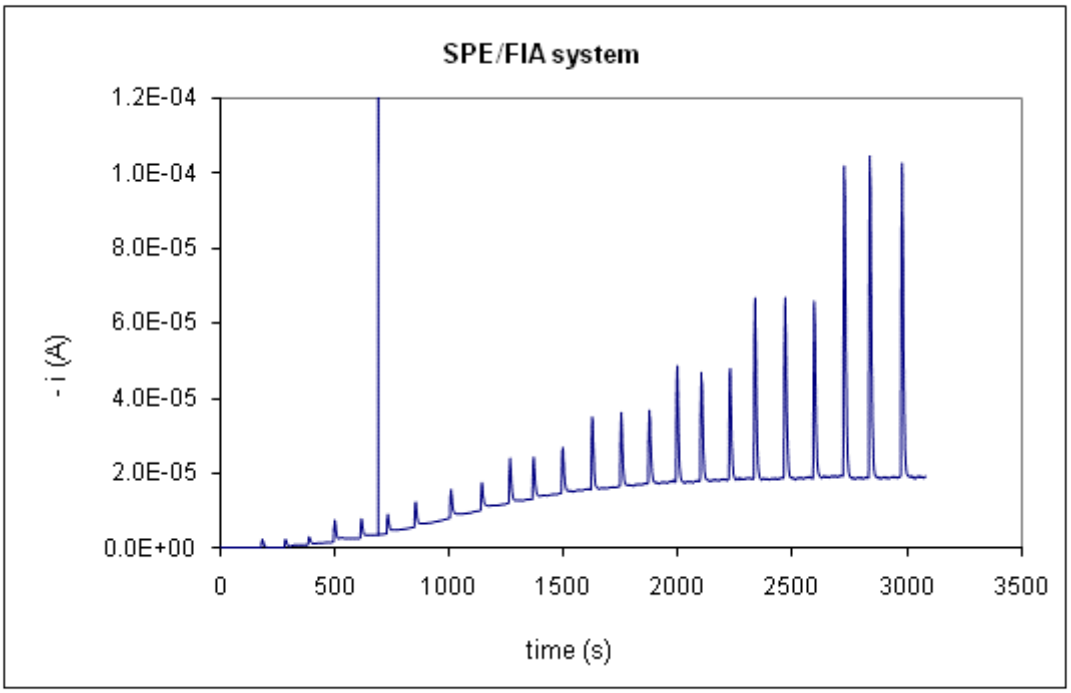

Figure 64. Amerometric response of successive injections of sample (50 µL) containing HQ (50 mM), H_20_2 (4 mM) and $HbFe^{3+}$ (from 0 to 5 mg/mL) on SPE at a potential -400 mV. Carrier solution: non-deoxygenated PB (pH 7.5). Flow rate 0.5 mL/min

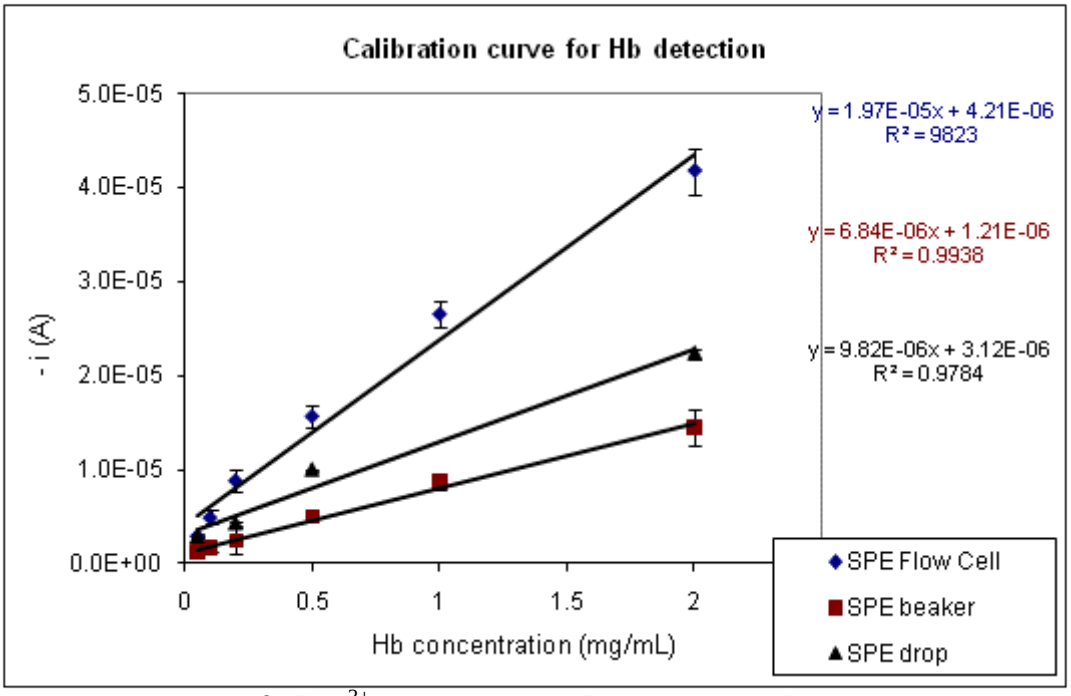

Figure 65. Detection of $HbFe^{3+}$ using its peroxidase activity with HQ (50 mM), H_20_2 (4 mM) in deoxygenated PB at pH 7.5 on SPE-D at a potential -400 mV. Amperometric signal recorded at the time 5 seconds in a drop or beaker setup.

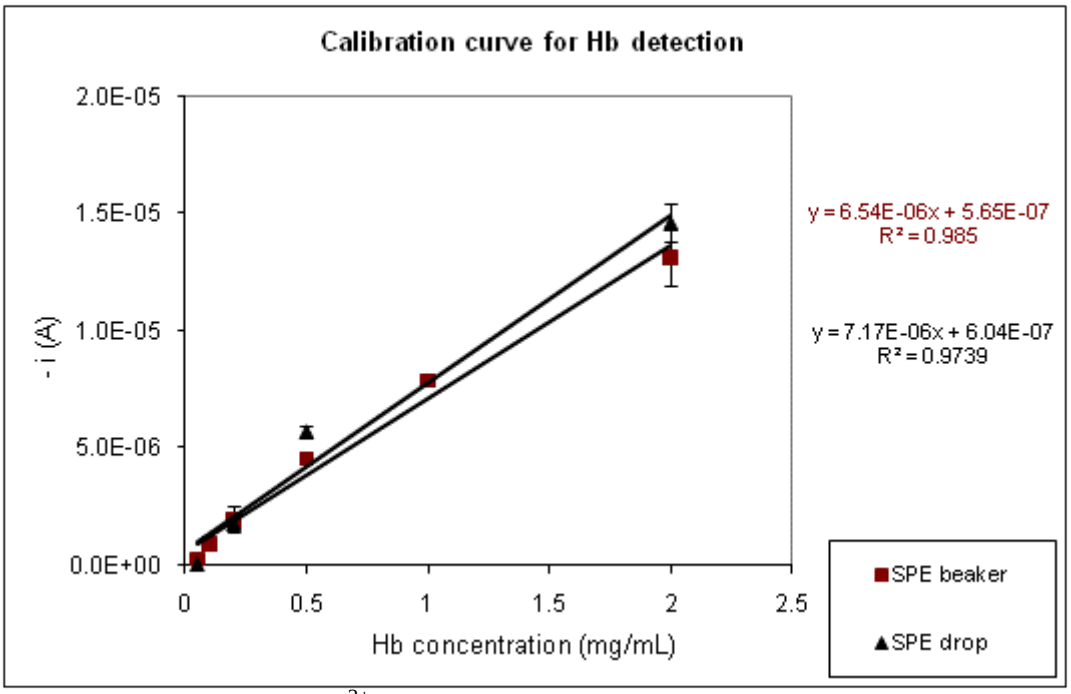

Figure 66. Detection of $HbFe^{3+}$ using its peroxidase activity with HQ (50 mM), H_20_2 (4 mM) in deoxygenated PB at pH 7.5 on SPE-D at a potential -400 mV. Amperometric signal recorded at 50 seconds in a drop or beaker setup.

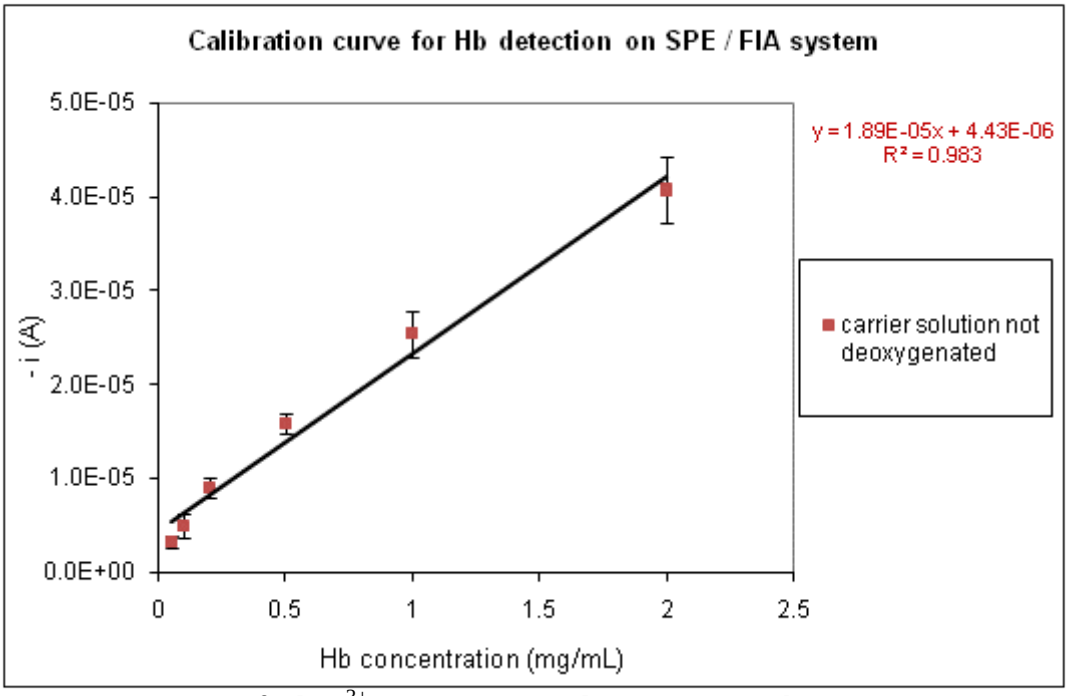

Figure 67. Detection of $HbFe^{3+}$ using its peroxidase activity with HQ (50 mM), H_20_2 (4 mM) in non-deoxygenated PB at pH 7.5 on SPE-D at a potential -400 mV in the FIA system.

8.2.2 Electrochemical detection of haemoglobin with mediator

A simplified system based only on reaction between haemoglobin and mediator was also investigated. Initial experiments included detection of $HbFe^{3+}$. Measurements were performed with the electrochemical cell in PBS buffer pH 7.4. Cyclic voltammetry was used for $HbFe^{3+}$ detection. The concentration of methylene blue and potassium ferricyanide was 0.5 and 100 mM, respectively.

Measured samples which contained only potassium ferricyanide gave higher anodic and cathodic peak current and also they showed a smaller peak-to-peak separation (Figure 68 and Table 16). The highest peak separation was detected for 3.23 mg/mL Hb. Also the oxidation and reduction peaks became less steep. This was caused by electrode fouling with haemoglobin. Additionally, mediators such as potassium ferricyanide and methylene blue must be previously reduced to be able to react with $HbFe^{3+}$. Furthermore reaction between $HbFe^{3+}$ and potassium ferrocyanide occurs to a small extent (Iorio, 1981).

Table 16. Analysis of cyclic voltammgram for sample containing potassium ferricyanide (100 mM) and different concentration of $HbFe^{3+}$.

$HbFe^{3+}$	**E_{pa}**	**E_{pc}**	**ΔE_p**
mg/mL	mV		
0	350	135	215
0.65	460	10	450
1.29	390	10	380
3.23	520	-115	635

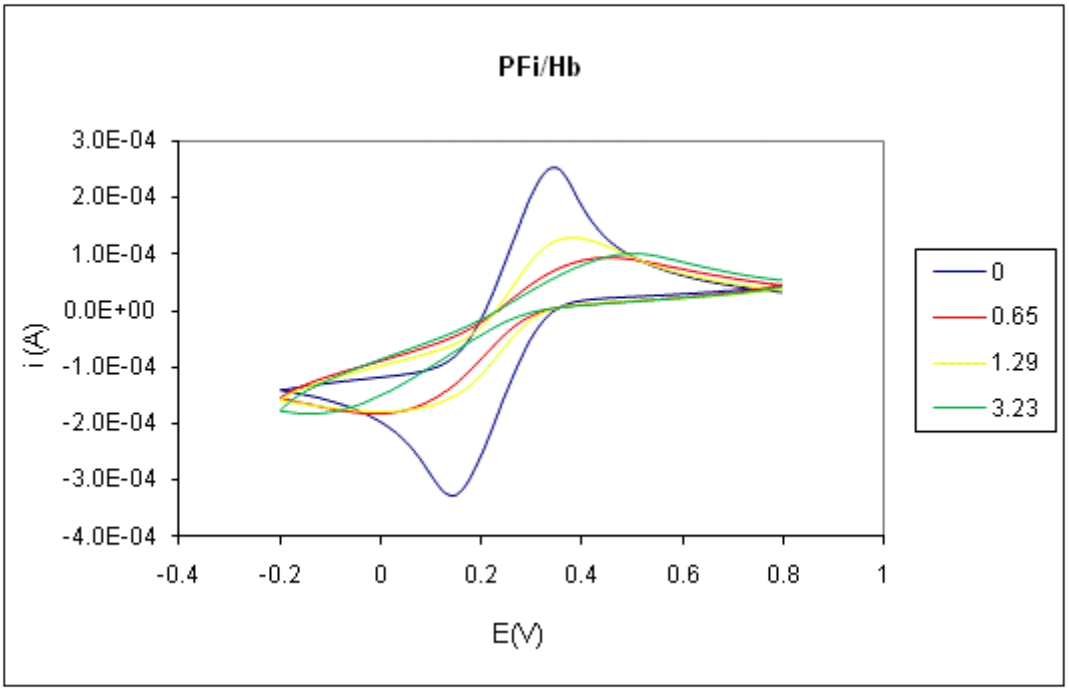

Figure 68. Cyclic voltammetry of potassium ferricyanide (100 mM) with different concentration of $HbFe^{3+}$ (from 0 to 3.23 mg/mL) in PBS at pH 7.4 Electrochemical cell: Au-WE, CE-Pt, calomel-RE. Scan rate 100 mV/s. Second cycle.

Cyclic voltammetry performed with methylene blue and haemoglobin showed anodic and cathodic peaks at the same potential without significant difference from measurements performed only with methylene blue. Leucomethylene blue is recognized to be able to reduce $HbFe^{3+}$ and it is a reversible reaction (Bradberry S.M., 2003). However it was still impossible to detect haemoglobin with cyclic voltammetry, only an insignificant increase in cathodic current response was obtained in the sample $MB/HbFe^{3+}$ (Figure 69).

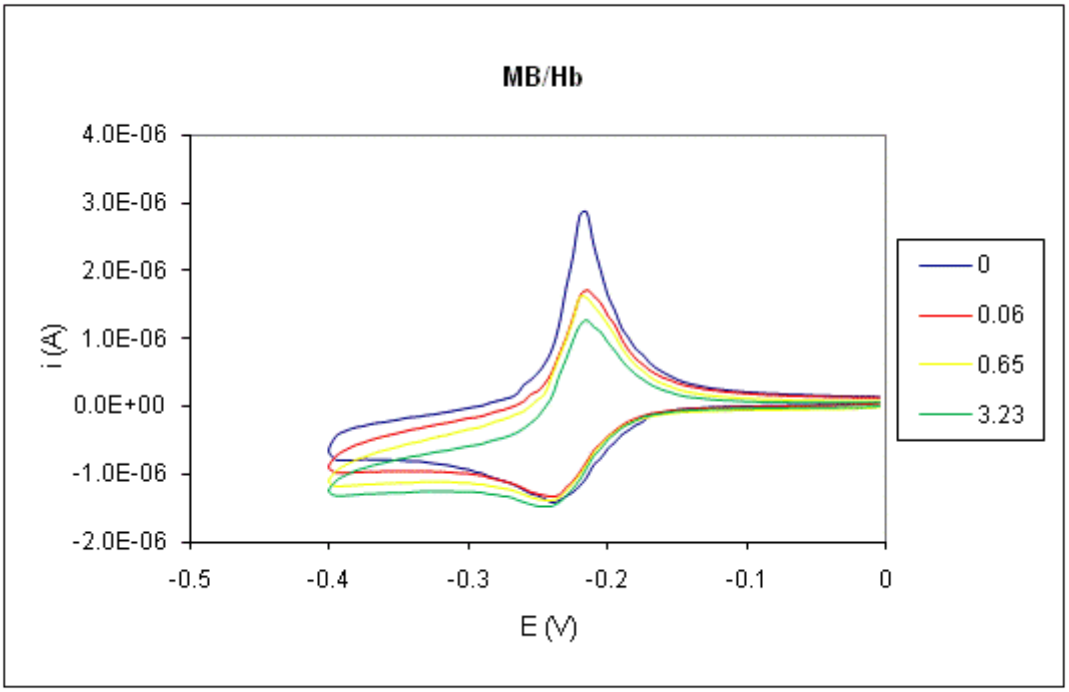

Figure 69. Cyclic voltammetry of MB (0.5 mM) with different concentration of $HbFe^{3+}$ (from 0 to 3.23 mg/mL) in PBS at pH 7.4 Electrochemical cell: Au-WE, CE-Pt, calomel-RE scan rate 50 mV/s.

Further work was focussed on the detection of $HbFe^{2+}$ with potassium ferricyanide using chronoamperometry method. Preliminary studies were performed with cyclic voltammetry to choose electrodes and to select an oxidation potential for potassium ferricyanide. Electrodes which showed good characteristic (Chapter 6.2.2) were tested to verify an effect of haemoglobin and potassium ferricyanide on a sensor response. An attempt was made to decrease a passivation effect and to operate a sensor at the lowest oxidation potential. Cyclic voltammetry was used for the studies with an applied scan rate 50 mV/s. Measurements were performed with 9.35 mg/mL $HbFe^{2+}$ and 50 mM potassium ferricyanide in the sample. Cyclic voltammetry was performed on different electrodes: SPE-D, SPE CNT-D, SPE-P and also with the electrochemical cell. A working electrode surface was modified with CMCNa and compared to an unmodified electrode.

Cathodic and anodic peaks showed lower current in the second and third cycle on SPE DropSens electrodes and the electrochemical cell with and also without CMCNa layer on the gold electrode (Figures 71-76). Samples with potassium ferricyanide and $HbFe^{2+}$ showed significantly higher oxidation and reduction current peaks in the first cycle in

comparison to the second and third cycle in the electrochemical cell (Figure 71, Table 17).

Table 17. Cyclic voltammetry of 50 mM potassium ferricyanide and 9.35 mg/mL $HbFe^{2+}$.

cycle number	i_{pa}	i_{pc}
	i(μA)	
1	94.5	-77.2
2	70.3	-63.3
3	63	-61.2

Peak to peak separation was higher with each cycle on tested electrodes in comparison to the samples with mediators (Figures 71-76). SPE DropSens electrodes showed a different position of anodic and cathodic peaks of samples with potassium ferricyanide and $HbFe^{2+}$ in comparison to the sample containing only mediator (Figures 72-74). Table 18 indicates the position of the oxidation and reduction potential peaks and their separation for samples containing potassium ferricyanide and haemoglobin in the following cycles.

It was suggested by researchers that CMCNa can protect an electrode against fouling by haemoglobin and also can bring haemoglobin closer to the electrode (Jiang *et al*., 2005). However, the research presented in this report showed that the modification of working electrode surface did not have a significant positive effect on cyclic voltammetry of potassium ferricyanide. Electrode modification with CMCNa on the gold electrode showed some protection against passivation in comparison to the SPEs sensors. Cyclic voltammetry exhibited smaller anodic and cathodic peak separation with each cycle compared to the SPE electrodes (Table 18). However, passivation was still noticable during experiments (Figure 71). These results indicate that a polymer layer coated on the electrode may help to avoid passivation, at least partially. Layer CMCNa was not clearly visible on SPEs electrodes and possibly did not form a good quality coating. SPE-P showed the largest peak separation and the highest oxidation potential in each cycle (Table 18, Figures 75-76).

Table 18. Cyclic voltammograms of potassium ferricyanide and $HbFe^{2+}$ on different SPEs in cycles 1-3.

cycle number	1			2			3		
	E_{pa}	E_{pc}	ΔEp	**E_{pa}**	E_{pc}	ΔEp	**E_{pa}**	E_{pc}	ΔEp
	mV								
EC CMCNa	**260**	55	205	**345**	-25	370	**420**	-75	495
SPE-D CMCNa	**105**	-490	595	**185**	-550	735	**245**	-580	825
SPE-D	**130**	-475	605	**190**	-545	735	**250**	-580	830
SPE-D CNT	**115**	-500	615	**180**	-540	720	**225**	-560	785
SPE-P CMCNa	**635**	-645	1280	**675**	-695	1370	**700**	-680	1380
SPE-P	**695**	-635	1330	**705**	-695	1400	**725**	-750	1475

The most convenient oxidation potential (less susceptible for interferences) was obtained with SPE CNT DropSens with haemoglobin (225 mV for the third cycle). Cyclic voltammetry for ferricyanide on these electrodes showed a current increase in the following cycles 1-3 (Figure 74), probably as a result of enhanced electron transfer produced by nanotubes (Yan *et al*, 2005; Zhao *et al*., 2005). The mentioned reaction is not enzymatic and therefore it can be assumed that higher oxidation of haemoglobin will be obtained over a longer period of time. Therefore it is possible to obtain higher response of sensor with time, due to the more efficient reaction and enhanced properties of these electrodes. It was also necessary to perform experiments with $HbFe^{2+}$ in a small sample volume due to difficulties in purifying a large amount of $HbFe^{2+}$. This is especially essential working with HbA1c and also HbA0. Therefore SPE CNT DropSens electrodes were chosen for haemoglobin detection with potassium ferricyanide.

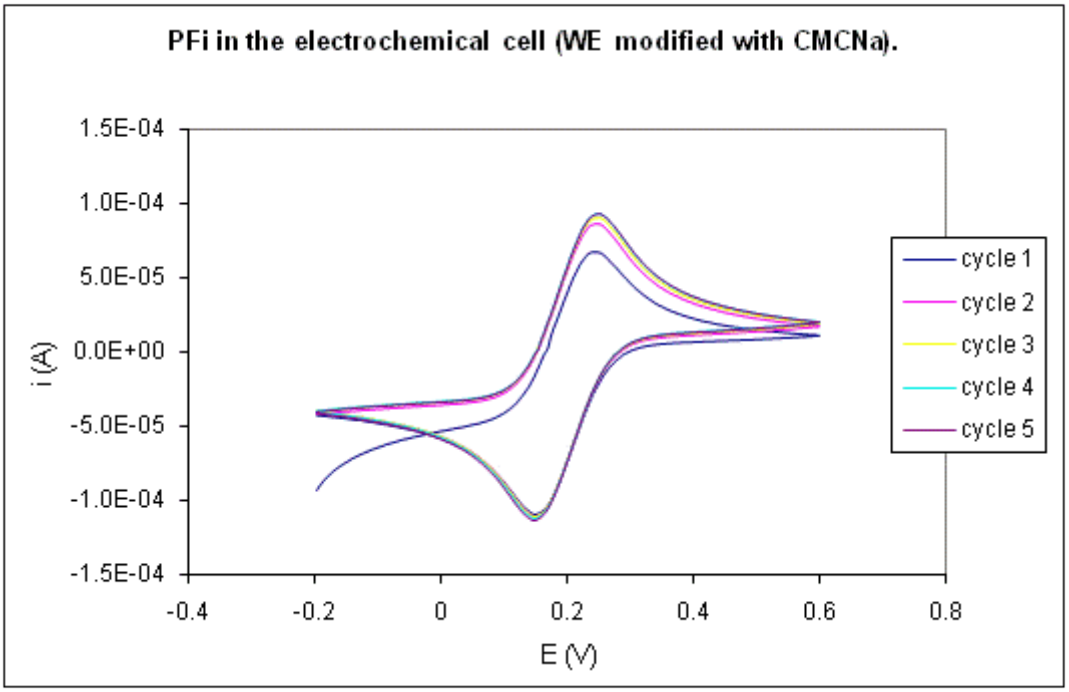

Figure 70. Cyclic voltammetry of potassium ferricyanide (50 mM) in PBS at pH 7.4 Electrochemical cell: Au modified with CMCNa-WE, CE-Pt, calomel-RE. Scan rate 50 mV/s. Cycle1-5.

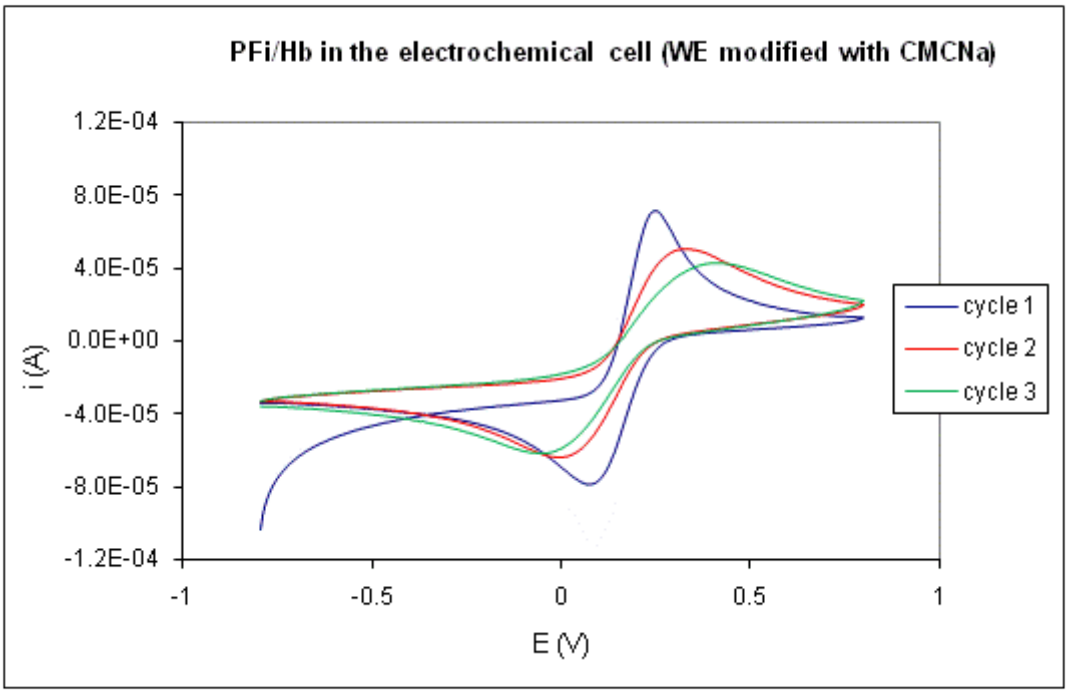

Figure 71. Cyclic voltammetry of potassium ferricyanide (50 mM) in the presence of $HbFe^{2+}$ (9.35 mg/mL) in PBS at pH 7.4 Electrochemical cell: Au modified with CMCNa-WE, CE-Pt, calomel-RE. Scan rate 50 mV/s. Cycle1-3.

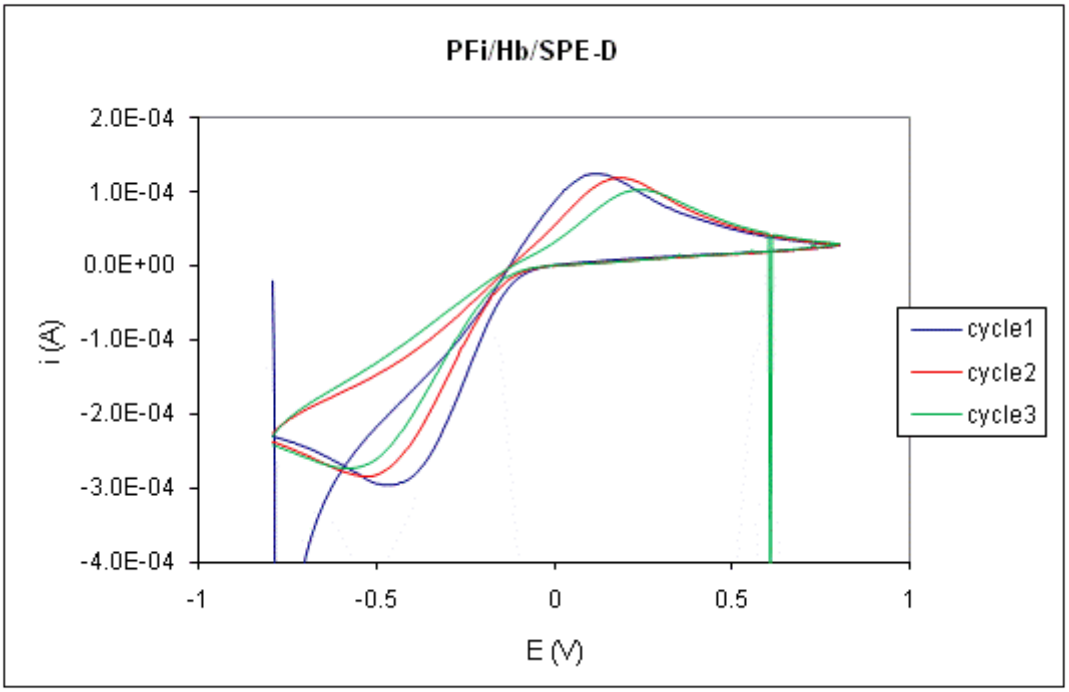

Figure 72. Cyclic voltammetry of potassium ferricyanide (50 mM) in the presence of $HbFe^{2+}$ (9.35 mg/mL) in PBS at pH 7.4 on SPE-D. Scan rate 50 mV/s. Cycle1-3.

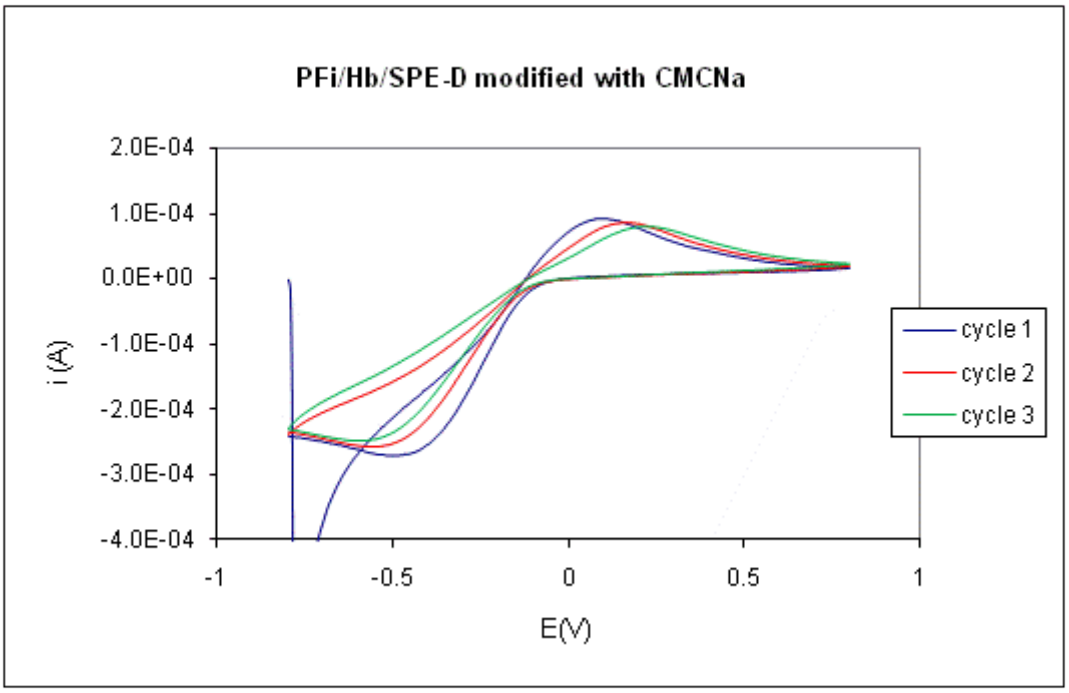

Figure 73. Cyclic voltammetry of potassium ferricyanide (50 mM) in the presence of $HbFe^{2+}$ (9.35 mg/ mL) in PBS at pH 7.4 on SPE-D modified with CMCNa. Scan rate 50 mV/s. Cycle1-3.

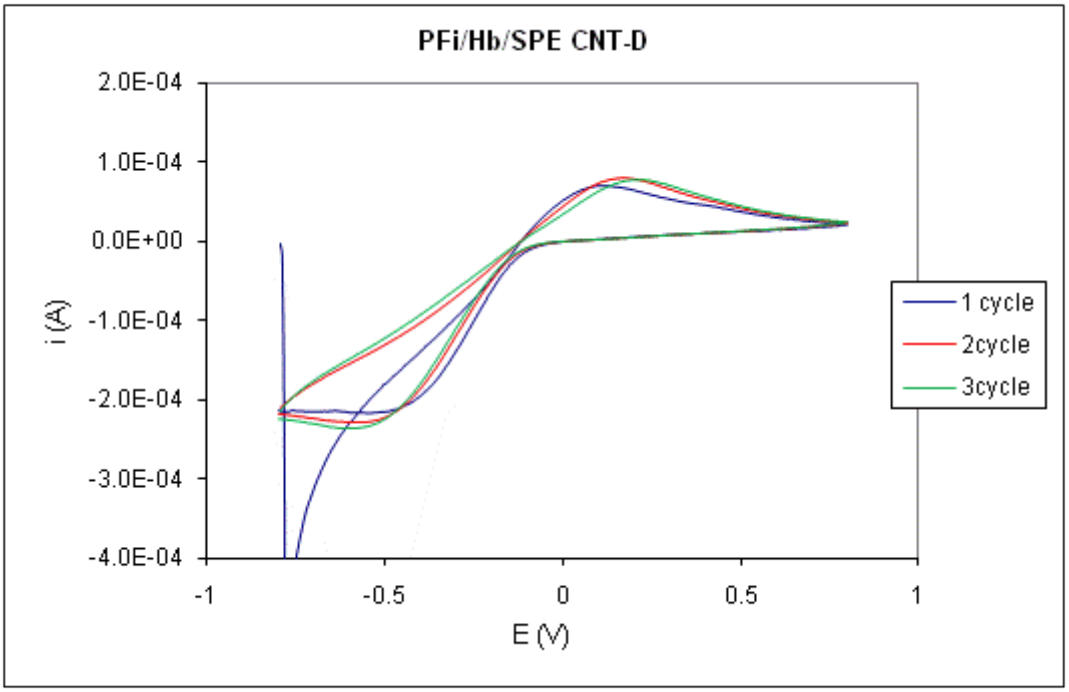

Figure 74. Cyclic voltammetry of potassium ferricyanide (50 mM) in the presence of $HbFe^{2+}$ (9.35 mg/mL) in PBS at pH 7.4 on SPE CNT-D. Scan rate 50 mV/s. Cycle1-3.

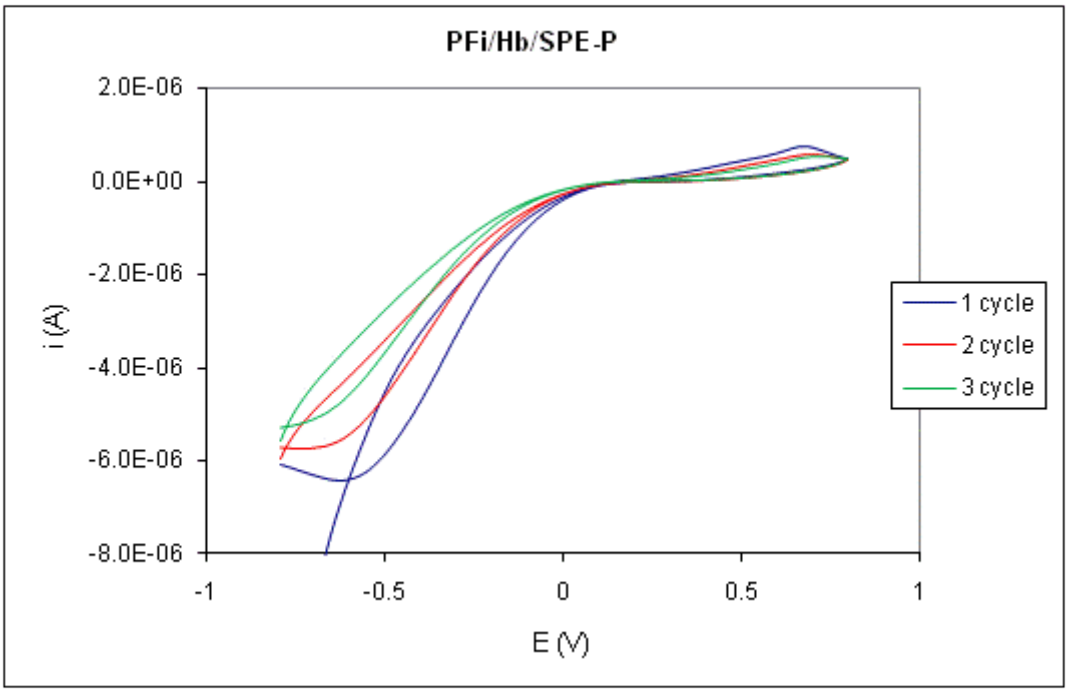

Figure 75. Cyclic voltammetry of potassium ferricyanide (50 mM) in the presence of $HbFe^{2+}$ (9.35 mg/mL) in PBS at pH 7.4 on SPE-P. Scan rate 50 mV/s. Cycle1-3.

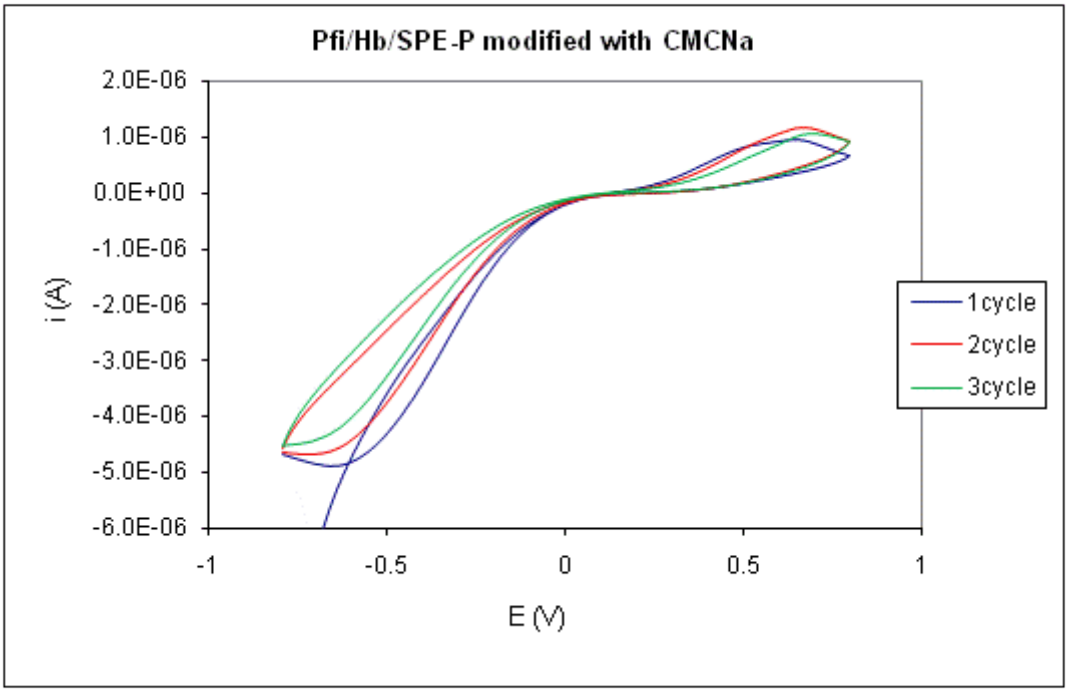

Figure 76. Cyclic voltammetry of potassium ferricyanide (50 mM) in the presence of $HbFe^{2+}$ (9.35 mg/mL) in PBS at pH 7.4 on SPE-P modified with CMCNa. Scan rate 50 mV/s. Cycle1-3.

$HbFe^{2+}$ detection with potassium ferricyanide was performed with a chronoamperometric method on SPE CNT-D electrodes at the oxidation potential of +500 mV (Figure 77). The potassium ferricyanide concentration used was 50 mM in the samples. A calibration curve recorded at 30 seconds was characterised by the equation y = 1.44E-07x – 2.54E-09 and R^2=0.9998. A detection range from 0.83 to 83 mg/mL was obtained. The RSD was calculated to be 3.55 % for 10 electrodes (Figure 78). The linearity and detection range was improved with potassium ferricyanide as compared to the HQ/H_20_2 sensor for haemoglobin detection. However, trials with potassium ferricyanide showed also a lower sensitivity and a higher detection limit (Figures 65 and 78).

Studies performed by Jiang *et al*. suggested that the reaction between potassium ferricyanide and haemoglobin is immediate and the current signal is proportional to the content of haemoglobin. Researchers also showed that detection can be performed in an oxygen environment (Jiang *et al*., 2005). However, the reaction can be stronger in an oxygen free environment (Bradberry, 2003). A larger overpotential +500 mV was applied to enhance electron transfer and reduce passivation. However interferences

resulting from an oxidation of substances such as ascorbic acid and acetaminophen are significant at this potential. Oxidation potential of these substances is around +400 mV vs Ag/AgCl (Wang *et al*., 1998). Furthermore, interferences can be caused by the reaction of potassium ferricyanide and ascorbic acid (Miao *et al*., 2001).

Reaction with other mediators such as ferrocenecarboxylic acid (Chen *et al*., 2003), methylene blue (Brett *et al*., 1999), quinone (Winterbourn, 1985), capable to react with $HbFe^{2+}$ is also a possible route forward. Reaction with quinone may be performed only in a deoxygenated environment due to side reactions with oxygen (Winterbourn, 1985).

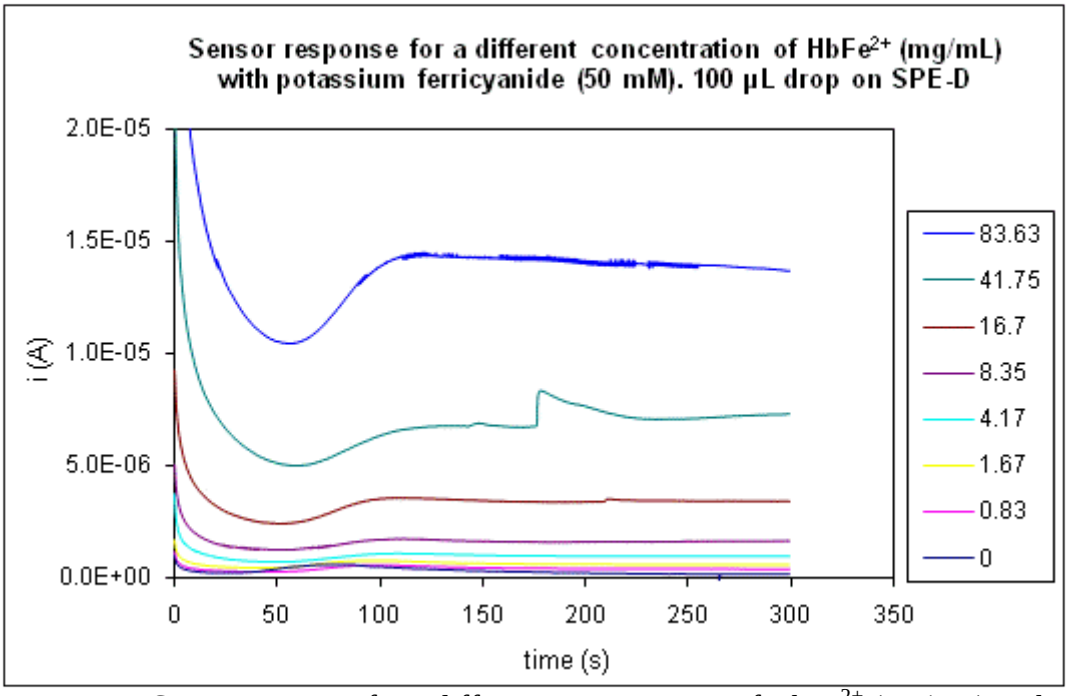

Figure 77. Sensor response for a different concentration of $HbFe^{2+}$ (mg/mL) with potassium ferricyanide (50 mM) in PBS (pH 7.4). Sample was incubated 5 minutes and current recorded at a potential +500 mV.

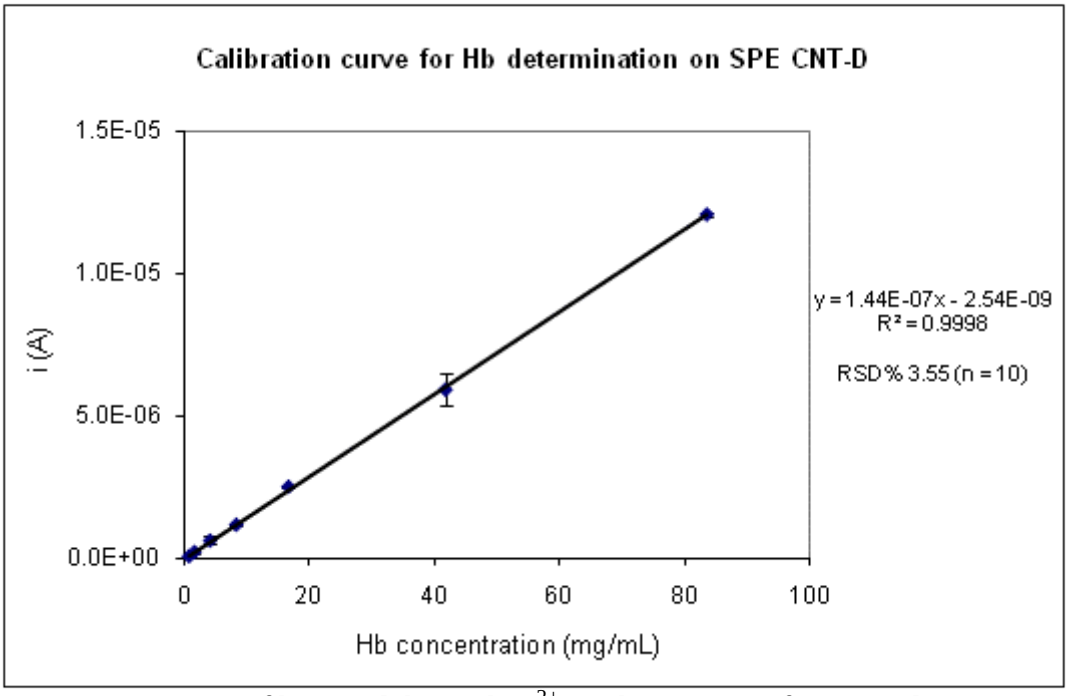

Figure 78. Detection of haemoglobin ($HbFe^{2+}$) with potassium ferricyanide (50 mM) in PBS at pH 7.4 on SPE CNT-D at a potential +500 mV.

8.3 Conclusions

The model system based on horseradish peroxidise, was successfully used for detection of haemoglobin. Reduced or oxidized forms of haemoglobin ($HbFe^{2+}$ or $HbFe^{3+}$) can be detected using its peroxidase activity by chronoamperometry. Haemoglobin ($HbFe^{2+}$) can be also detected by its reaction with potassium ferricyanide. However, a faster response, a higher sensitivity and a lower detection limit were obtained using the enzymatic reaction of Hb. Increased current density resulting from the reaction catalysed by Hb suggested a way to construct a MIPs-based sensor.

Passivation, caused by a high concentration of haemoglobin is overcomed by an application of significant overpotential. Therefore, cyclic voltammetry is not suitable for detection of haemoglobin, even at a very low protein concentration using its peroxidase activity.

Interferences caused by substances such as ascorbic acid, acetaminophen, uric acid and urea can be minimized, but it is impossible to avoid them completely. Therefore, it may be useful to incorporate a purification or separation step in a functional Hb-meter.

Chapter 9: MIP technology for recognition of HbA0 / HbA1c

9.1 Haemoglobin detection on SPE modified with polymer

9.1.1 Materials and methods

9.1.1.1 Reagents

HQ (hydroquinone with CAS No: 123-31-9), hydrogen peroxide 30% (CAS No: 7722-84-1), sodium monobasic phosphate (CAS No: 7558-80-7), sodium dibasic phosphate (CAS No: 7558-79-4), potassium chloride (CAS No: 7447-40-7), APBA (CAS No: 206658-89-1), ammonium persulfate (CAS No: 7727-54-0), sodium hydroxide (CAS No: 1310-73-2) were purchased from Sigma-Aldrich (Dorset, UK), glycated haemoglobin (HbA1c) and non-glycated haemoglobin (HbA0) were purchased from Exocell, Inc. (Philadelphia, USA). Chemicals were used as received.

9.1.1.2 Apparatus

All electrochemical experiments were performed with the Uniscan potentiostat - galvanostat instrument PG580 with UiEChem software (Buxton, UK).

Screen Printed Electrodes (SPE-D) were purchased from DropSens Ltd. (Oviedo, Spain). Three electrodes cell were purchased from BASi Electrochemistry Instruments (Warwickshire, UK). Electrodes were described in Chapter 6.1.2. SPEs were either used without any treatment or the working electrode surface was modified with APBA by chemical polymerisation.

The following other equipment was used in experiments: Flow-Cell for screen-printed electrodes (wall-jet type) from DropSens (Oviedo, Spain); Minipuls3 Peristaltic Pump Gilson (Middleton, USA); injector Valve V-7 was purchased from GE Healthcare (Amersham, UK); centrifuge, Jouan B4i was purchased from Thermo Fisher Scientific (Waltham, USA); spectrophotometer, Shimadzu 1800 (Shimadzu, Kyoto, Japan); pH-meter, Hanna Instruments 8519 was purchased from Hanna Instruments Inc.

(Woonsocket, USA); Amicon ultra-4 centrifuge filter (30kDa cut-off) was provided by Millipore Ltd. (Watford, UK); pump tubes PVC (I.D. 1.42 mm) Anachem Ltd. (Luton, UK); PTFE tubing (I.D. 0.8 mm), 1/4-28 Tube Endfitting (Nut), gripper fitting were purchased from Omnifit Ltd. (Cambridge, UK).

9.1.1.3 Surface grafted polymer on SPE for recognition of HbA0 and HbA1c

Procedure for grafting of APBA, previously developed at Cranfield University was modified and applied with SPE-D electrodes. Non-imprinted polymer (NIP) was grafted on the electrode by means of chemical polymerisation which was carried out by oxidation with ammonium persulfate. Ammonium persulfate was dissolved in distilled water. APBA was dissolved in distilled water by heating at 47°C and stirring for 10 minutes. 15 µL of APBA (100 mM) was placed on the working electrode (WE) and 15 µL of ammonium persulfate (50 mM) was added. Polymerisation was carried out for 30 minutes. Electrodes were thoroughly rinsed with dH_20 and 7 times with buffer. Washing buffer consisted of 3% acetic acid and 0.1% Tween 20. 300 µL of the buffer was pipetted onto the electrode. Electrodes were conditioned in sodium phosphate buffer (50mM) with Tween 20 (0.1%) at pH 7.0 for 30 minutes.

SPE electrodes were coated with a molecularly imprinted polymer layer (MIP) by chemical polymerisation. 15 µL of APBA / Hb solution was placed on the working electrode and 15 µL ammonium persulfate (50 mM) was added. Haemoglobin was dissolved in APBA solution. Concentration of template (Hb) was 1 mg/mL and molar ratio, APBA: ammonium persulfate was 2:1. Polymerisation and washing procedure was the same as described above for preparation of NIP electrodes.

9.1.1.4 Electrochemical analysis

All solutions used for electrochemical analysis were prepared daily. Mediator was prepared in a buffer deoxygenated by purging with nitrogen. Additionally mediator stock solution was deoxygenated by blowing nitrogen over the solution for 5 minutes and used within 3 hours. Hydrogen peroxide was prepared in dH_20 deoxygenated by purging with nitrogen. Mediator and hydrogen peroxide were kept in 4°C during

measurements. Phosphate buffer (40 mM) consisted of sodium monobasic phosphate and sodium dibasic phosphate and potassium chloride (100 mM). pH was adjusted with 1 M sodium hydrooxide. All measurements were performed at room temperature (22^{o}C).

Cyclic voltammetry was used for the qualitative characterisation of electrodes coated with polymer. Experiments were performed on SPE electrodes without modification, SPE modified with non-imprinted polymer (NIP), SPE modified with imprinted polymer (MIP). Electrodes were immersed in a vertical position in a 10 mL beaker. CV was performed under unstirred conditions. All measurements were run in triplicate. Data were transferred to Microsoft Office Excel and average values calculated. Three cycles were performed for each electrode at a scan rate 50 mV/s. All Figures show the second cycle. Voltammograms were run from negative toward positive potentials.

Chronoamperometry was used for detection of haemoglobin. Experiments were performed on SPE electrodes, SPE covered with non-imprinted polymer (NIP), SPE modified with imprinted polymer (MIP), SPE modified with imprinted polymer containing HbA0 as a template (MIP-0), SPE modified with imprinted polymer containing HbA1c as a template (MIP-1c). Experiments were performed in a FIA-system (Flow Injection Analysis) at a flow rate 0.5 ml/min. The FIA system was made up of the peristaltic pump, the injector and the flow cell. Samples containing HQ (hydroquinone), H_20_2 with or without Hb were injected into the loop (50 µL). Carrier solution (PB buffer, pH 6.0; 7.0; 7.5 or 8.0) was pumped by a peristaltic pump through the injector. PTFE tube (I.D. 0.8 mm, length 27 cm) was connected to the outlet of the injector and to the flow cell. Samples were prepared in phosphate buffer and mixed for 20 seconds before injection. Measurements were performed in triplicate. Data were processed in Microsoft Office Excel and average Figures calculated.

9.1.2 Results and Discussion

9.1.2.1 Characetrization of SPEs coated with polymer

Measurements were performed in 40 mM phosphate buffer pH 7.5 with supporting electrolyte 100 mM KCl. Measured samples contained 0.5 or 50 mM HQ, 4 mM H_20_2, 0.3 mg/mL $HbFe^{3+}$.

Cyclic voltammetry was used to characterise SPE electrodes and electrodes covered with APBA polymer. Deposition of polymer is illustrated in Figure 79. The cyclic voltammogram of NIP and MIP electrodes, immersed in PB buffer, showed higher passage of current than SPEs. Anodic and cathodic peaks were not detectable, neither on modified electrodes nor on SPE electrodes. This suggests that the APBA is not a very conductive polymer. Also, different types of substrate may have an influence on polymer properties. A modified gold electrode did not exhibit an increase in passage of current. Previous studies showed that a gold electrode (WE) was more conductive than SPE. Therefore electron transfer may be lowered on a gold electrode covered with polymer in comparison to the bare gold. Also studies carried out by Rick *et al.* (2006) showed a significant difference in APBA polymer, electropolymerised on gold, platinum and platinum coated with polypyrrole. Redox peaks were not detectable on gold electrodes.

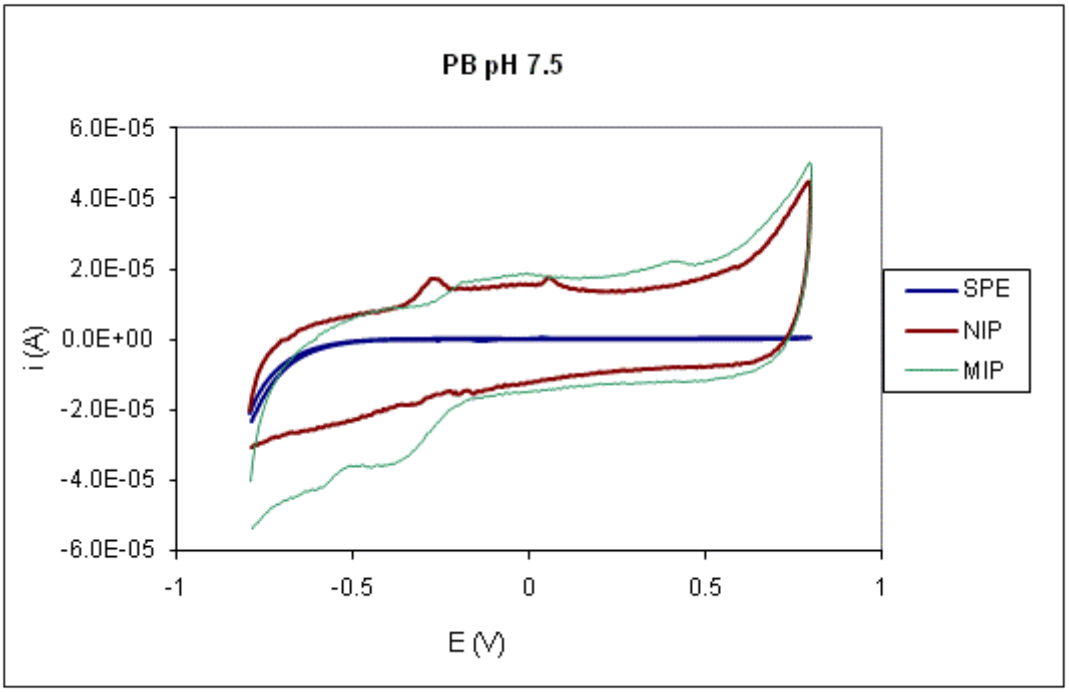

Figure 79. Cyclic voltammetry of SPE and electrodes covered with polymer immersed in PB buffer at pH 7.5. The second cycle is shown and the scan rate was 50 mV/s.

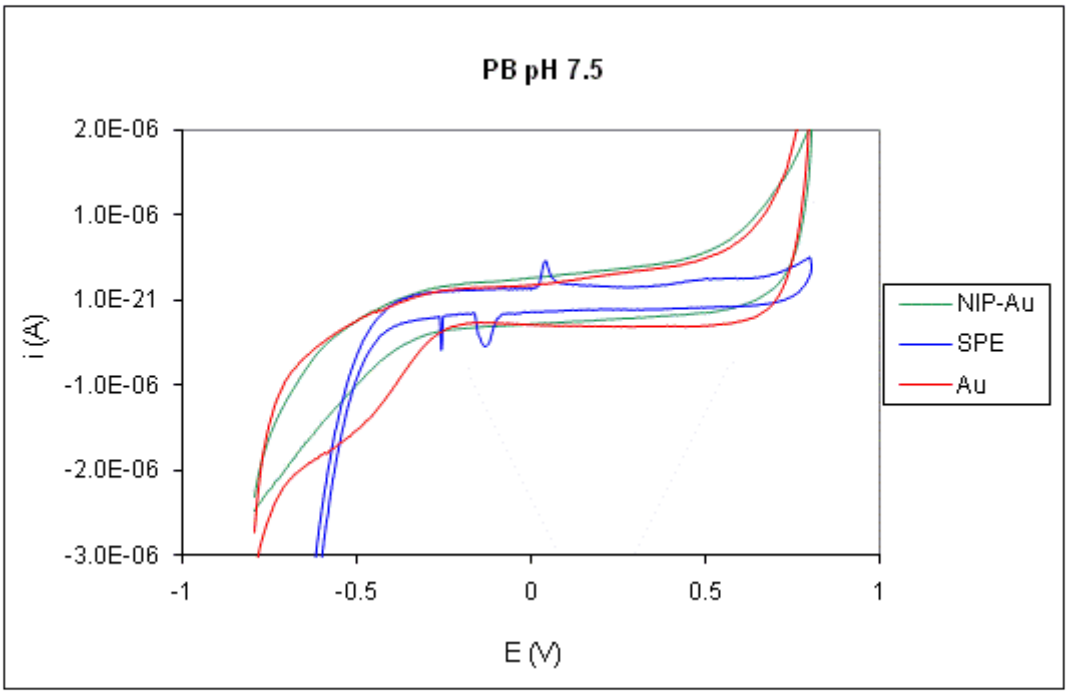

Figure 80. Cyclic voltammetry performed in PB at pH 7.5 on a SPE, Electrochemical cell: Ag/AgCl - RE, Pt-CE, Au-WE or NIP-Au as WE. Second cycle; scan rate 50 mV/s.

The CV of 0.5 mM HQ showed redox peaks at the same potential for SPE and NIP electrodes (Table 19, Figure 81) However NIP showed a broadening of the oxidation peak starting from a potential of +70 mV.

Table 19. Characterisation of electrodes with HQ (0.5 mM).

	Epa	Epc	i_p (forward)	i_p (backward)
	mV		µA	
SPE	160	-135	20	-19
NIP	175	-140	18.3	-16.8

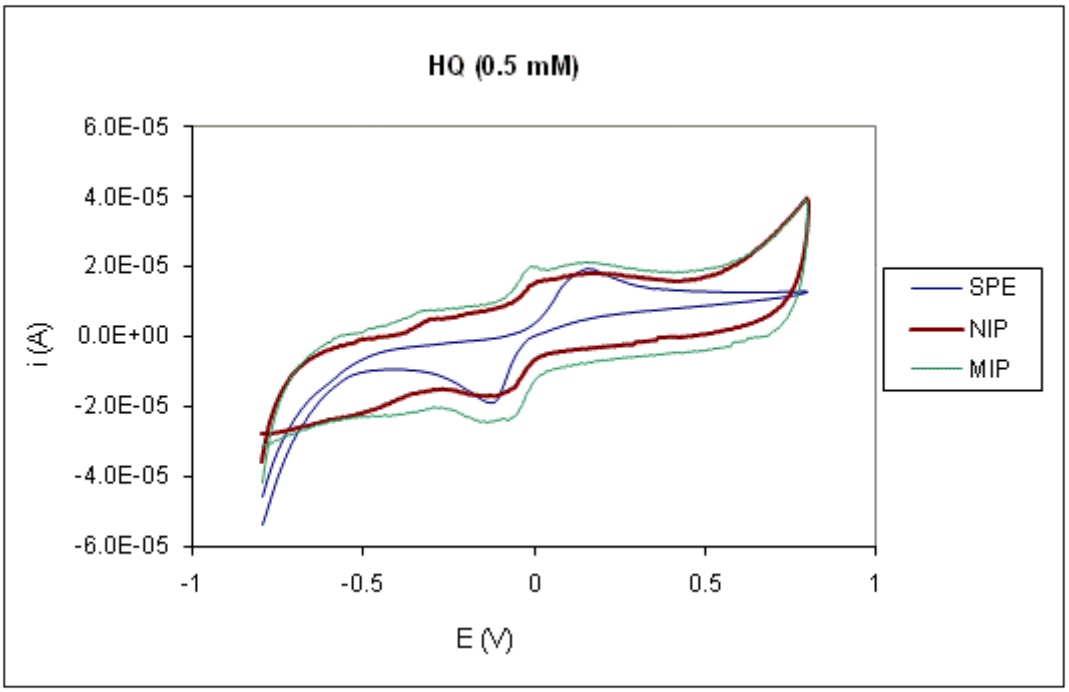

Figure 81. Cyclic voltammetry of HQ (0.5 mM) in PB buffer at pH 7.5 on electrodes. Scan rate 50 mV/s.

The CV of 50 mM HQ showed oxidation peaks at the different potentials for SPE and NIP and the same reduction potential. The higher peak separation on SPE can be explained by a high concentration of mediator which results in higher IR drop (Pletcher, 1991). The difference was also noted in anodic and cathodic current for the electrodes. A lower current was obtained with the NIP sensor (Table 20, Figure 82). The current decrease on the NIP electrode may be due to a larger distance of HQ from the polymer layer. The cyclic voltammogram of 50 mM HQ containing H_20_2 (4 mM) and haemoglobin (0.3 mg/mL) showed a significant decrease in cathodic current, namely

289 µA at a potential -115 mV (Figure 83). Haemoglobin may be in a close proximity to the electrode and may decrease the electron transfer.

Table 20. Characterisation of electrodes with HQ (50 mM).

	Epa	Epc	i_{pa}	i_{pc}
	mV		µA	
SPE	400	-85	518	546
NIP	110	-85	252	482

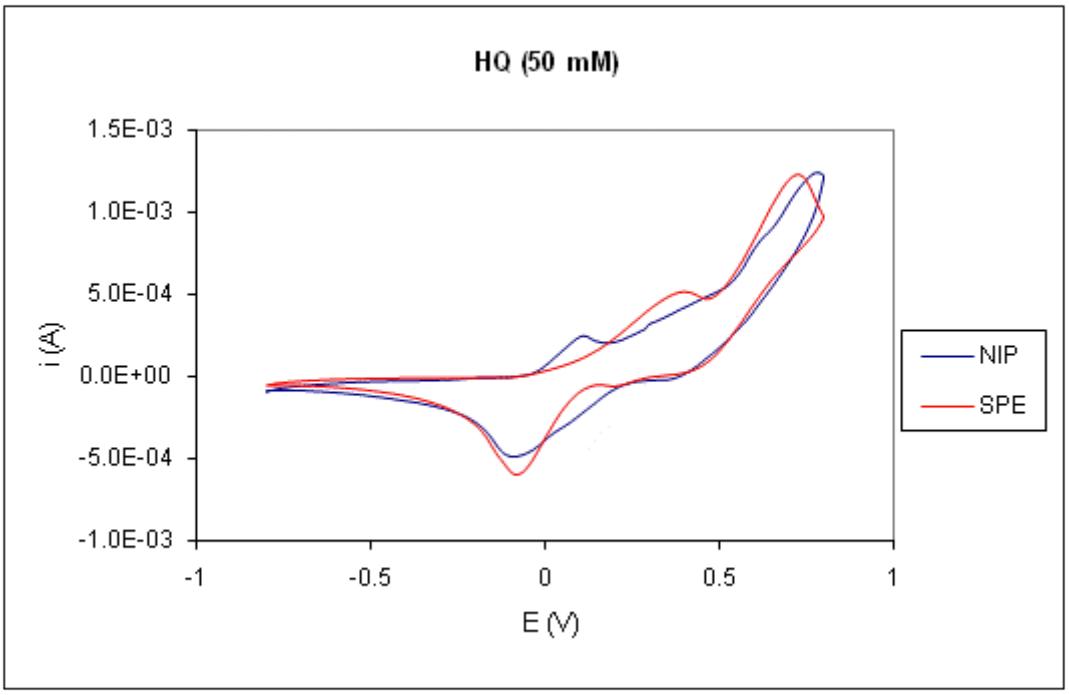

Figure 82. Cyclic voltammetry of HQ (50 mM) in PB buffer at pH 7.5 on SPE-D and NIP electrodes. Second cycle; scan rate 50 mV/s.

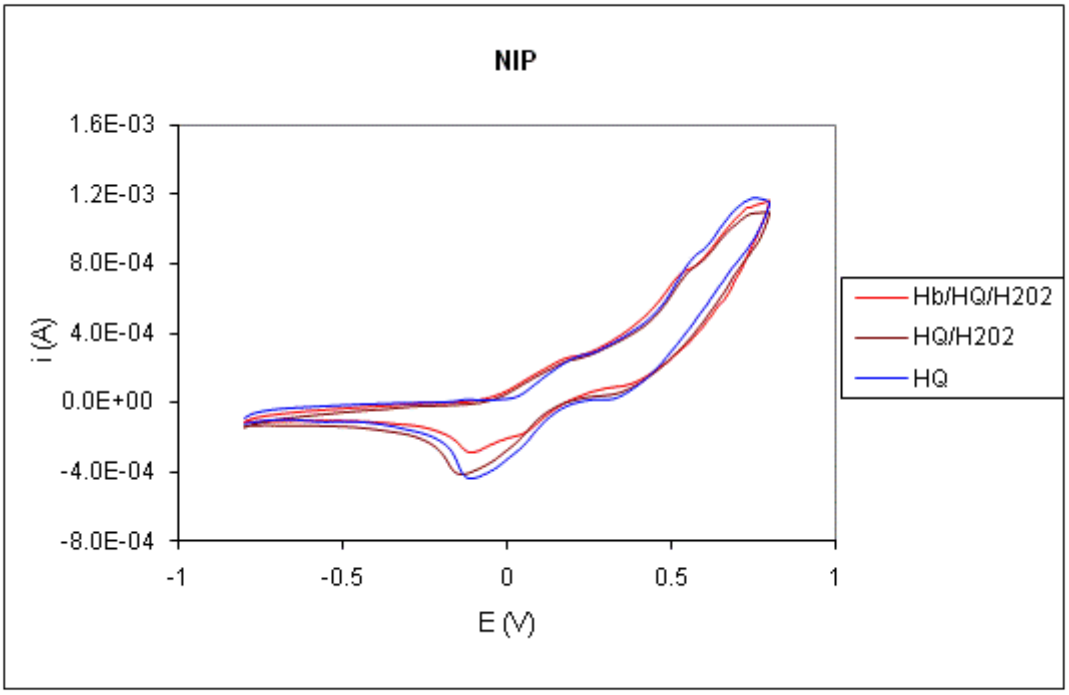

Figure 83. Cyclic voltammetry of HQ in the presence of Hb (0.3 mg/mL) and H_20_2 (4 mM) in PB at pH 7.5 on NIP electrodes. Second cycle; scan rate 50 mV/s.

Also an electrochemical behaviour of HQ was tested at pH 6.0 on NIP electrodes. The cyclic voltammogram of HQ (50 mM) showed a shifted reduction peak at pH 6.0, recorded at -160 mV and cathodic current of -362 μA. The first oxidation peak was not recorded (Figure 84).

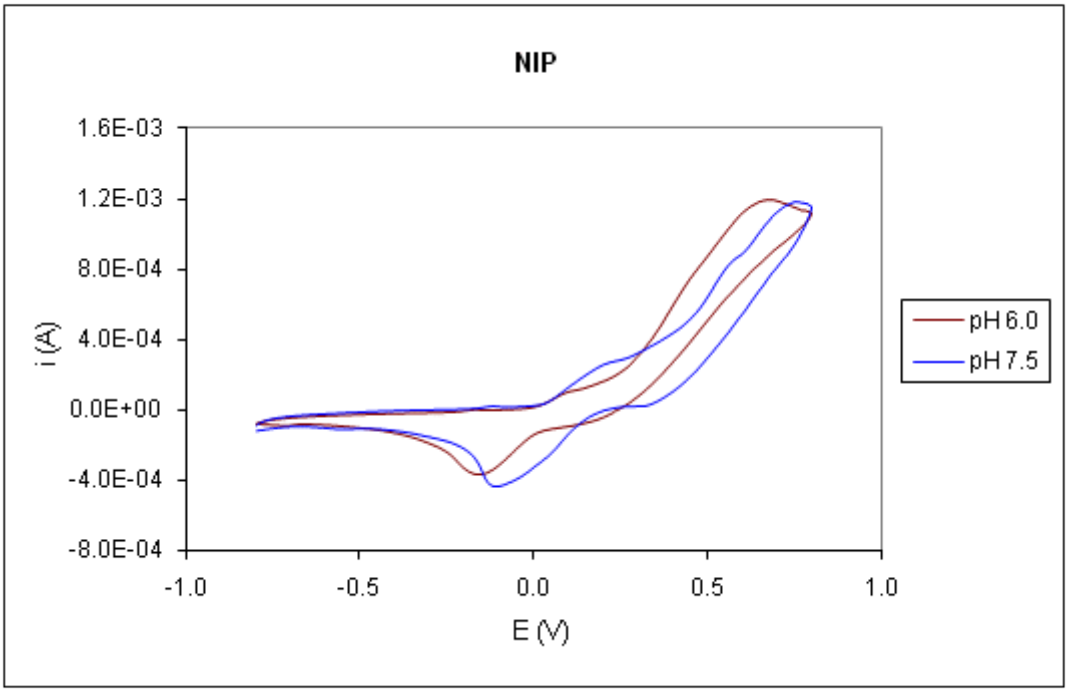

Figure 84. Cyclic voltammetry of HQ (50 mM) in PB buffer at pH 6.0 and 7.5 on NIP electrodes. Second cycle. Scan rate 50 mV/s.

9.1.2.2 Electrochemical detection of haemoglobin using its peroxidase activity

The chronoamperometric signal was measured at a negative reduction potential of -400 mV. SPE, NIP and MIP electrodes were used in these experiments. HQ and $HbFe^{3+}$ stock solutions were prepared in 40 mM phosphate buffer, pH 7.5, with supporting electrolyte of 100 mM KCl, unless otherwise stated. Haemoglobin detection was performed with 50 mM HQ and 4 mM H_20_2.

Detection of $HbFe^{3+}$ was carried out on SPE and NIP electrodes. Studies were focussed on the detection of Hb in the FIA system, which allowed problems with mass transport to be avoided and protein binding to be carried out in real time. A linear calibration curve was obtained for $HbFe^{3+}$ detection in the FIA system over a range of 0.05 to 2 mg/mL. Measured samples gave a calibration curve described by the equations showed in Table 21 (Figures 85-87). Response of those electrodes for samples without haemoglobin was significantly different. Higher current signal was obtained for NIP and MIP in comparison to SPE electrodes: 5.05 µA; 8.16 µA and 2.45 µA, respectively.

Table 21. Calibration curve for Hb detection.

NIPs	y = 1.54E-05x + 4.00E-6	R2 = 0.9877
MIPs	y = 1.44E-05x + 5.86E-07	R2 = 0.9884

Experiments were carried out with deoxygenated and non-deoxygenated carrier solution. Higher background current was recorded with non-deoxygenated carrier solution (PB buffer). A larger background current may cause an increase of noise level, especially at a higher reduction potential, in the presence of oxygen. However, similar detection of $HbFe^{3+}$ was obtained on NIP electrodes experiments with deoxygenated and non-deoxygenated PB buffer (Figure 88).

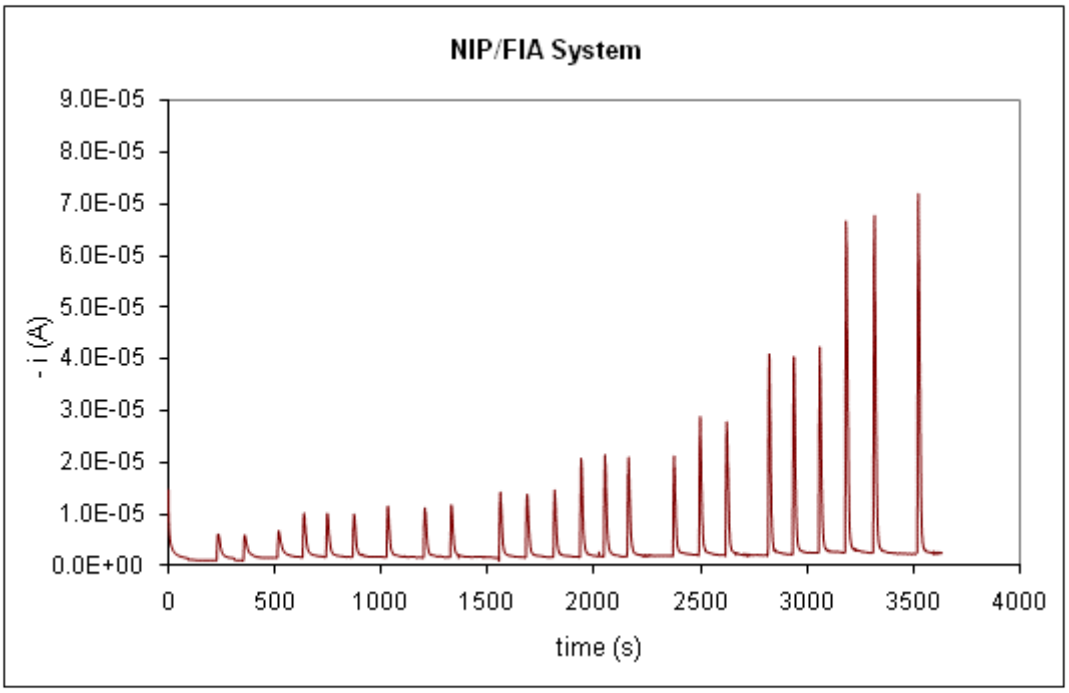

Figure 85. Amperometric response of successive injections of sample (50 µL) containing HQ (50 mM), H_20_2 (4 mM) and $HbFe^{3+}$ (from 0 to 2 mg/mL) on NIP. PB deoxygenated (pH 7.5). Flow rate 0.5 mL/min.

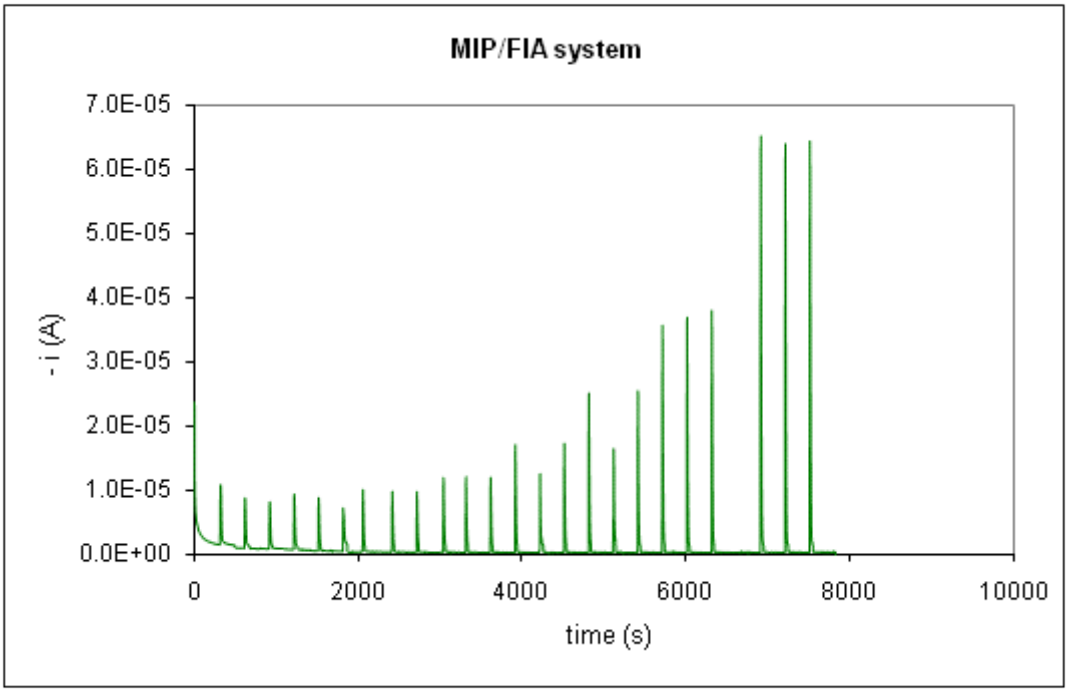

Figure 86. Amperometric response of successive injections of sample (50 µL) containing HQ (50 mM), H_20_2 (4 mM) and $HbFe^{3+}$ (from 0 to 2 mg/mL) on MIP. PB deoxygenated (pH 7.5). Flow rate 0.5 mL/min.

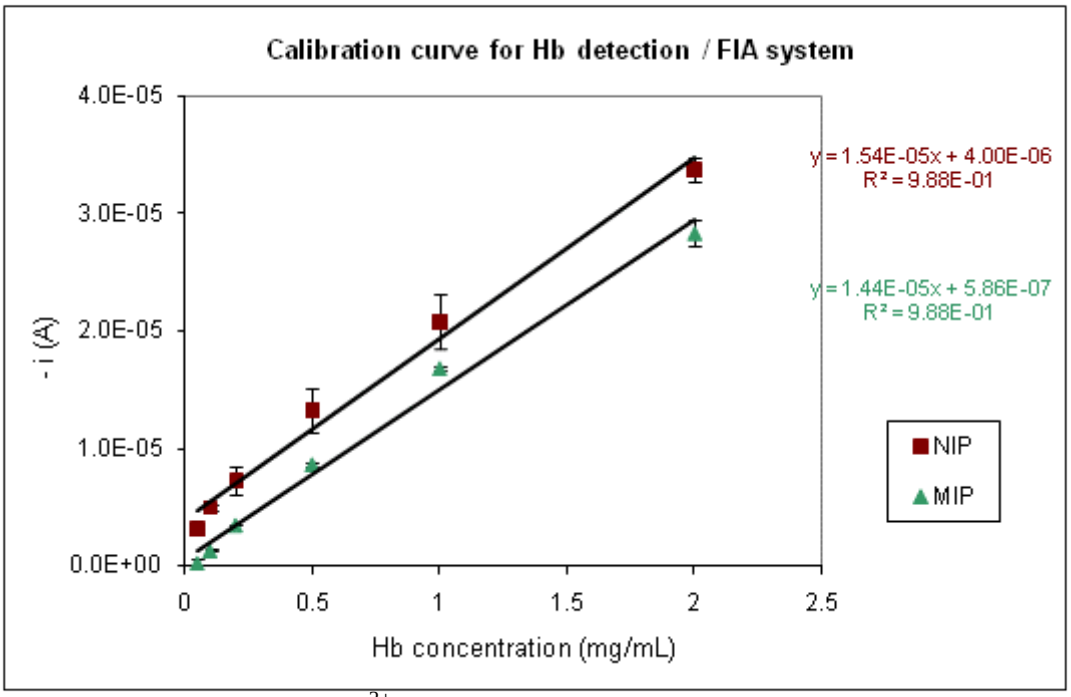

Figure 87. Detection of $HbFe^{3+}$ using its peroxidase activity with HQ (50 mM), H_20_2 (4 mM) in PB at pH 7.5 on NIP and MIP electrodes at a potential -400 mV in the FIA system. Carrier solution deoxygenated.

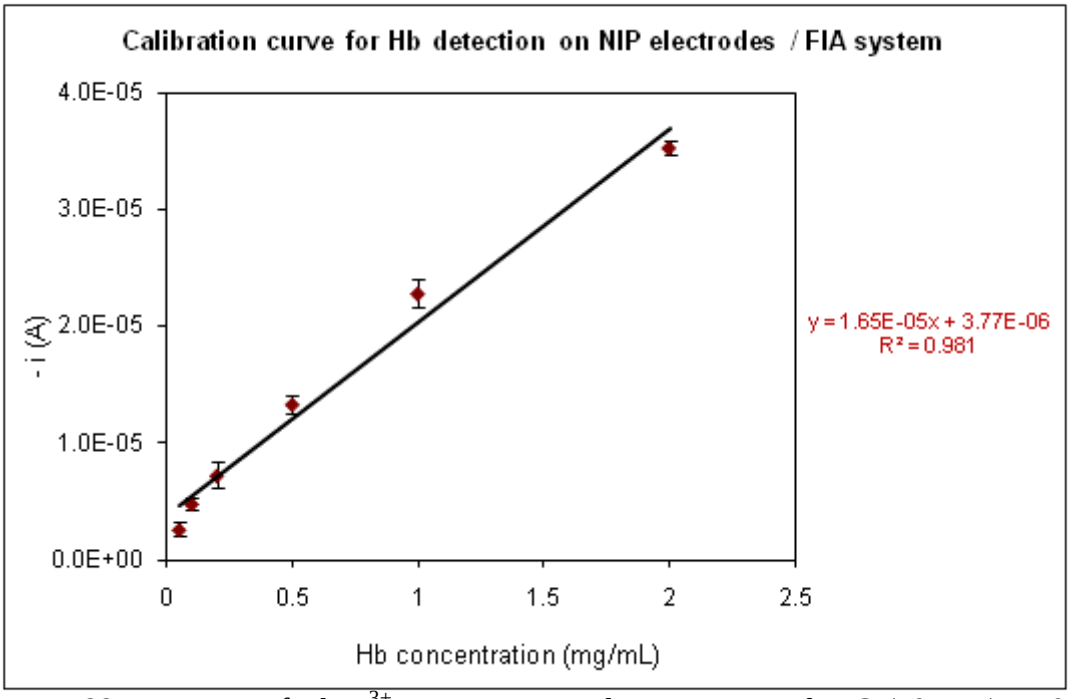

Figure 88. Detection of $HbFe^{3+}$ using its peroxidase activity with HQ (50 mM), H_20_2 (4 mM) in PB at pH 7.5 on NIP electrodes at a potential -400 mV in the FIA system. Carrier solution non-deoxygenated.

Further work was focussed on the detection of HbA0 and HbA1c. HbA0 (0.3 mg/mL) showed a lower electrochemical signal than HbA1c (0.3 mg/mL): 4.61 µA and 6.01 µA, respectively (Figure 89). Lower peroxidase activity of glycated haemoglobin was reported by Khoo *et al*. (1994). However the glycated haemoglobin in presence of sorbitol showed higher peroxidase activity. Non-glycated Hb stabilised with sorbitol did not exhibit a change in enzymatic activity (Khoo *et al.*, 1994). Potassium cyanide may have the same effect on peroxidase activity; it attaches to the heme group and stabilises the protein structure. Decreased consumption of H_20_2 in the presence of sodium cyanide was demonstrated by Nagababu *et al.* (2000). Enzymatic activity of HbA0, HbA1c may be also dependent on different protein concentration and reaction times.

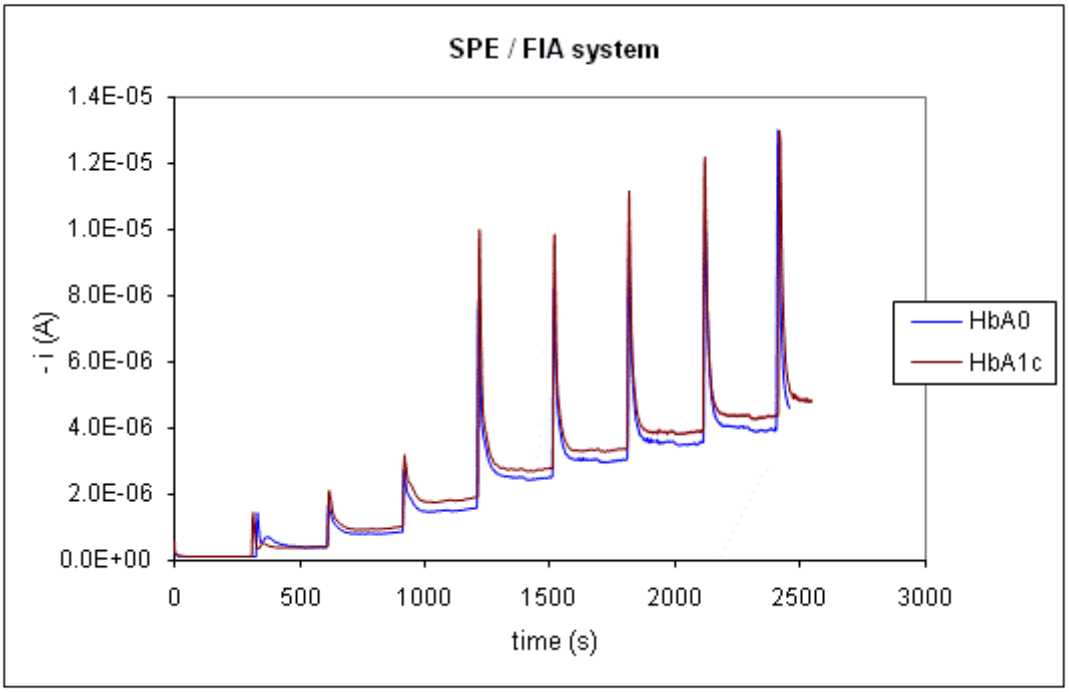

Figure 89. Amperometric response of successive injections of sample (50 µL) containing HQ (50 mM), H_20_2 (4 mM) and 0.3 mg/mL HbA0 or HbA1c on SPE. PB deoxygenated (pH 7.5). Flow rate 0.5 mL/min.

It was reported in previous studies that APBA is able to recognize HbA1c (Bossi *et al*., 2001; Pribyl and Skladal, 2006). It was suggested that the difference in isoelectric point may have a positive effect on variation in recognition (Bossi *et al*., 2001).

Detection of HbA0 and HbA1c was performed on NIP electrodes at pH 6.0; 7.0; 8.0 and also at pH 6.0 in buffer containing 0.5 % Tween 20 as a carrier solution. Injected samples contained HQ (50 mM), H_20_2 (4 mM) with or without haemoglobin (0.3 mg/mL). Samples without haemoglobin were injected in triplicate followed by five injections of samples containing haemoglobin. Figure 90 shows haemoglobin detection at pH 6.0. At pH 6.0 samples without haemoglobin showed a decrease in current in the subsequent injection, the opposite effect was observed at pH 8.0. The current response obtained with samples without haemoglobin was subtracted from the current response of samples with protein. Results are presented in Table 22. The highest difference in signal for HbA1c was obtained at pH 6.0, with or without surfactant. However it is necessary to amplify the difference between HbA1c and HbA0 to be able to work with lower concentration of HbA1c.

Table 22. Influence of pH on NIP sensor response.

NIP electrodes	HbA0		HbA1c		Increase in signal for HbA1c
pH	i (µA)	stdev	i (µA)	stdev	i (µA)
6	**0.91**	0.26	**2.48**	0.59	**2.72**
7	**1.56**	0.17	**3.09**	0.24	**1.98**
8	**2.81**	1.19	**3.99**	1.56	**1.42**
6 with Tween20	**0.82**	0.25	**2.33**	0.64	**2.84**

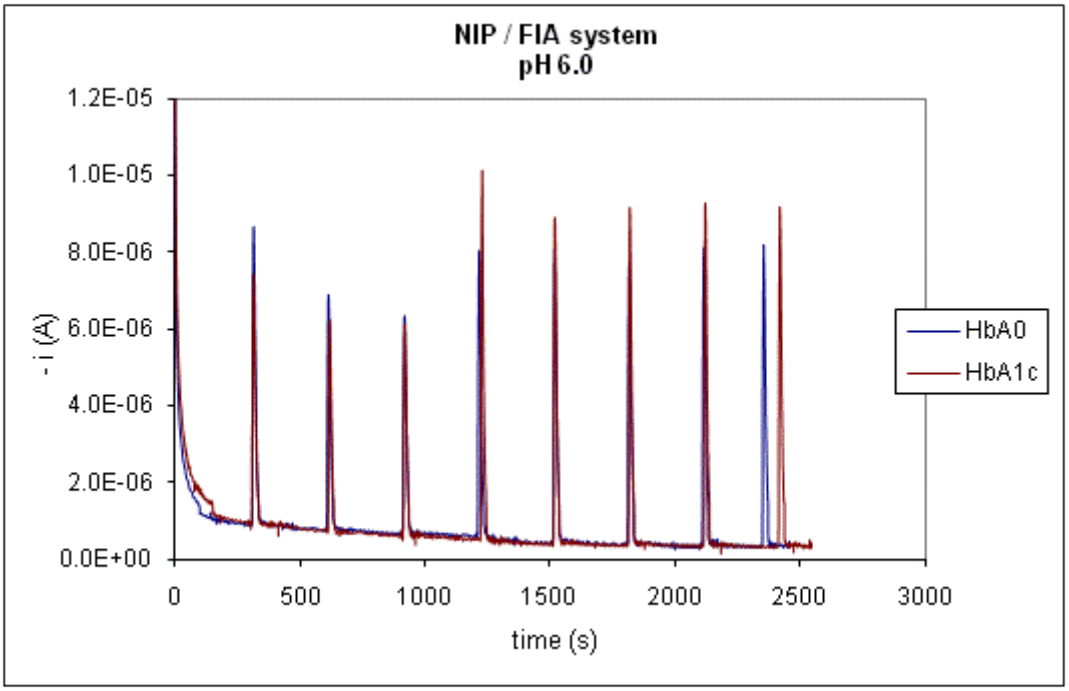

Figure 90. Amperometric response of successive injections of sample (50 µL) containing HQ (50 mM), H_20_2 (4 mM) and 0.3 mg/mL HbA0 or HbA1c on NIP. PB deoxygenated (pH 6.0). Flow rate 0.5 mL/min.

Further work was continued with PB pH 6.0 as a carrier solution on MIP electrodes. A significantly different binding of HbA1c and HbA0 on MIPs electrodes was not obtained (Table 23). The difference in signal was lower than on NIP electrodes. The Hb trapped in polymer matrix might have an influence on the results.

Table 23. HbA0, HbA1c binding on MIP electrodes.

	HbA0		**HbA1c**		signal increase for HbA1c
	i (µA)	stdev	i (µA)	stdev	i (µA)
carrier solution PB pH 6.0					
MIP1c	**1.43**	0.44	**2.57**	0.25	**1.80**
MIP0	**1.53**	0.19	**2**	0.29	**1.31**
carrier solution PB pH 6.0 with Tween 20					
MIP1c	**1.27**	0.26	**1.44**	0.15	**1.13**
MIP0	**1.62**	0.8	**2.37**	0.59	**1.46**

A lack of significant binding of haemoglobin on MIP electrodes may be due to a small surface area and low binding capacity of polymer. The enzymatic reaction of Hb before binding and a short time of binding may have an impact on the results presented. Samples were incubated for 20 seconds before injection and stayed on the electrode for around one minute. There is also a possibility that HQ blocked specific binding sites. Stabilisation of Hb may have a negative influence on a rebinding detection, especially if Hb is detected over a short time. High background current (sample without Hb) on electrodes covered with polymer and electrostatic forces could have a negative effect on the recognition process as well.

9.2 Spectrophotometric analysis of haemoglobin binding

NIP and MIP polymers were grafted on a surface of microplates and tested spectrophotometrically to verify an impact of possible problems related to electrochemical detection described in the previous chapter.

9.2.1 Materials and methods

9.2.1.1 Reagents

Sodium monobasic phosphate (CAS No: 7558-80-7), sodium dibasic phosphate (CAS No: 7558-79-4), sodium hydroxide (CAS No: 1310-73-2), phosphate – citrate buffer tablet, APBA (CAS No: 206658-89-1), ammonium persulfate (CAS No: 7727-54-0) ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, (CAS

No: 30931-67-0), TMB (CAS No: 64285-73-0), were purchased from Sigma-Aldrich (Dorset, UK), glycated haemoglobin (HbA1c) and non-glycated haemoglobin (HbA0) were purchased from Exocell, Inc. (Philadelphia, USA). Chemicals were used as received.

9.2.1.2 Apparatus

The following other equipment was used in experiments: centrifuge, Jouan B4i was purchased from Thermo Fisher Scientific (Waltham, USA); spectrophotometer, Shimadzu 1800 (Shimadzu, Kyoto, Japan); microplate reader, Varioscan Flash version 2.4.3 was purchased from Thermo Fisher Scientific (Hampshire, UK); Microplate Immuno MaxiSorp 96 well flat bottom polystyrene was purchased from Fisher Scientific Ltd. (Loughborough, UK); pH-meter, Hanna Instruments 8519 was purchased from Hanna Instruments Inc. (Woonsocket, USA); Amicon ultra-15 and ultra-4 centrifuge filter (30kDa cut-off) was provided by Millipore Ltd. (Watford, UK);

9.2.1.3 Haemoglobin isolation from human blood

Isolation and purification of haemoglobin was performed at 20^{o}C. 2 mL of human blood was mixed with 6 mL of NaCl (0.9%). Solution was centrifugated for 10 minutes at 1620 g. Supernatant (plasma) was discarded and the washing procedure with NaCl repeated three times. Red blood cells (RBCs) were collected, mixed with 11 mL of distilled water and left for 10 minutes for hemolysis. Solution was centrifugated for 40 minutes at 2880g. Clear, red supernatant containing haemoglobin was collected. The content of Hb was determined by Drabkin's method. Haemoglobin was purified by passing over the PD-10 column packed with Sephadex G-25 fine. 1 mL of Hb (10 mg/mL) sample was loaded on the column and eluted with PB buffer (50 mM) at pH 7.5. Then haemoglobin was concentrated on the amicon ultra-4 centrifugal filter unit 30 kDa cut-off. Firstly the filter unit was rinsed with dH_20 to remove trace amounts of triethylene glycol and then was filled with 4 mL of the sample. Hb solution was centrifugated 10 minutes at 2880 g. The content of Hb was determined by Drabkin's method.

9.2.1.4 Spectrophotometric determination of Hb by Drabkin's method

Drabkin's solution was prepared by reconstitution of one vial of Drabkin's Reagent with 1000 mL of water. Then 0.5 ml of the 30% Brij 35 solution was added. The prepared Drabkin's solution was kept at room temperature 22 ^{o}C, protected from light. Cyanmethemoglobin standard solution was prepared. It contained 50 mg/mL of human haemoglobin (Hb purchased from Sigma, containing mainly $HbFe^{3+}$) in Drabkin's solution. Working standard solutions were prepared. Standards were serially diluted in Drabkin's solution (2-fold dilution). 25 µL per well of standard solution, unknown Hb sample or Drabkin's reagent were pipetted on a microplate. 200 µL of Drabkin's reagent was added per well, plate was shaked and left for 40 min at room temperature (+22 °C). Absorbance was read at 540 nm on a microplate reader and calibration curve of absorbance value versus cyanmethemoglobin concentration was plotted. Total haemoglobin concentration (mg/mL) of each test was determined from the calibration curve.

9.2.1.5 Detection of haemoglobin from different sources with ABTS-P

Enzymatic activity of Hb was carried out with ABTS or TMB substrate for ELISA assay. ABTS and TMB substrates were brought to room temperature before use. All measurements were made in triplicate.

HbA0 and HbA1c in storage buffer 1 mM KCN, pH 7.4; $HbFe^{3+}$ lyophilized form and $HbFe^{2+}$ isolated from blood were tested. An aliquot (10 µL) of Hb (dH_20) was pipetted to the well of a microplate and 90 µL solution of ABTS or TMB per well was added. The ABTS mixture contained 6.7 mg ABTS and 3.3 µL H_20_2 (30%, w/w) in 10 mL citrate buffer (0.1 M, pH 6.0). The TMB solution comprised a TMB tablet dissolved in 10 mL phosphate-citrate buffer containing H_20_2. Phosphate–citrate buffer was prepared by dissolving one tablet in 100 mL dH_20. Absorbance was measured after 10 minutes, 30 minutes and 2 hours at a wavelength of 405 nm for ABTS and of 655 nm for TMB.

9.2.1.6 Preparation of polymer on microplates for recognition HbA0 and HbA1c

Microplates were coated with a polymer layer (NIP) by chemical polymerisation of APBA by means of oxidation with ammonium persulfate. 50 µL of APBA (100 mM) was placed on microplates by single channel pipette and 50 µL of ammonium persulfate (50 mM) was added by multichannel pipette. Polymerisation was carried out 30 minutes at room temperature. Then microplates were washed 2 times with dH_20 and 7 times with a washing buffer (3% acetic acid, containing 0.1% Tween 20) to remove a template. 200 µL of the buffer was put per well by multichannel pipette. Plate was shaken and solution poured out. Plates were conditioned 30 minutes in 50 mM sodium phosphate buffer, containing 0.1% Tween 20. Microplates were left at room temperature and used the same day.

Microplates were also coated with molecularly imprinted polymer. 50 µL of APBA / Hb solution was placed per well and 50 µL ammonium persulfate (50 mM) was added. Haemoglobin was dissolved in APBA solution. Concentration of template (Hb) was 0.5 mg/mL and molar ratio APBA: ammonium persulfate was 2:1. Polymerisation, washing step and conditioning of microplates were performed as described above for NIP microplates.

9.2.1.7 Rebinding of HbA0, HbA1c on microplates coated with APBA polymer

100 µL of haemoglobin in concentration 1; 3; 5; 10; 30; 50; 100 µg/mL was pipetted per well. Haemoglobin was prepared in 50 mM sodium phosphate buffer, containing 0.1% Tween 20, pH 7.0. Rebinding of Hb lasted for 90 minutes and then microplates were washed in sodium phosphate buffer (50 mM) containing 0.1% Tween 20, pH 7.0. 200 µL of solution was pipetted per well, plate was shaken and the solution poured out.

9.2.1.8 Detection of rebound haemoglobin

Rebound haemoglobin was detected with ABTS ready to use substrate. 100 µL solution of ABTS was pipetted per well. Absorbance was measured at 405 nm after 10 minutes, 30 minutes, and 2 hours. Also 50 µL of the solution was transferred to the new plate after 2 hours and absorbance was measured at 405 nm. Background (0 µg/mL of

rebound Hb) which was recorded for NIP, MIP-A0 and MIP-A1c was subtracted from a signal response resulting from rebound Hb (1-100 µg/mL). The dissociation constant (K_d) was calculated from the Scatchard–Rosenthal equation: Bmax/B = Kd 1/C + 1, where B refers to a bound sites, C is a concentration of proteins.

9.2.2 Results and Discussion

9.2.2.1 Detection of haemoglobin from different sources with ABTS-P

Enzymatic activity of commercially available haemoglobin was tested to verify the influence of different Hb preparations. HbA0 and HbA1c in 1mM KCN solution, $HbFe^{3+}$ lyophilised and $HbFe^{2+}$ isolated from blood were studied. Haemoglobin was detected in the range 1 to 30 µg/mL. HbA0 (10 µg/mL) showed the lowest signal after 10 minutes incubation with ABTS-P, but after 2 hours the response was higher in comparison to the other proteins (Figures 91-94).

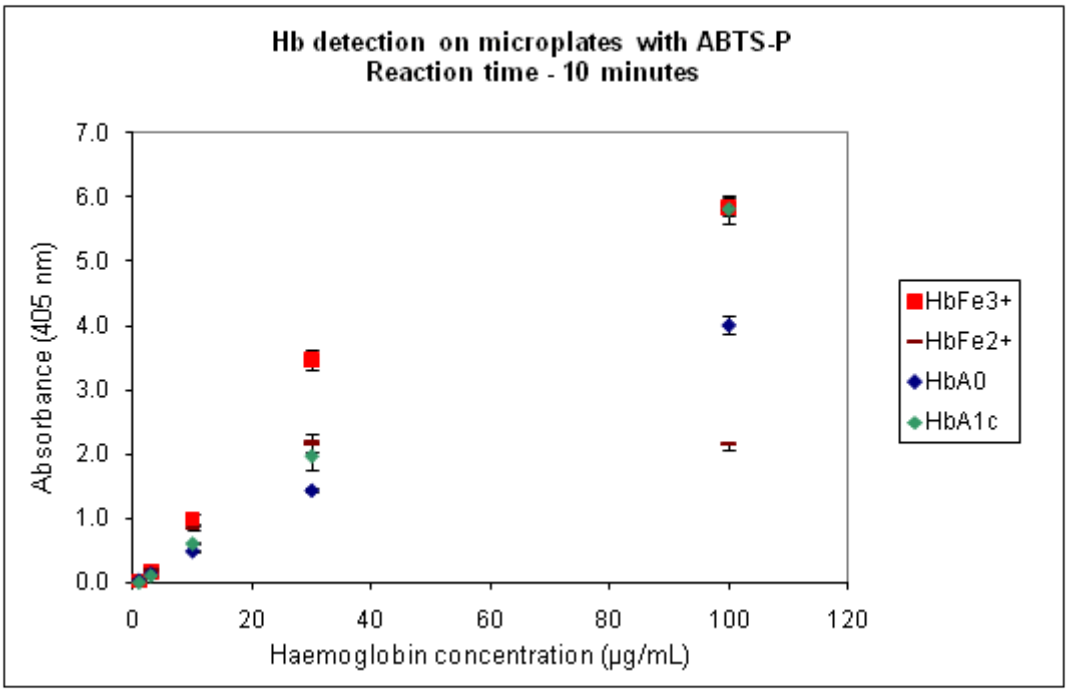

Figure 91. Hb detection on microplates with ABTS-P. Reaction time 10 minutes.

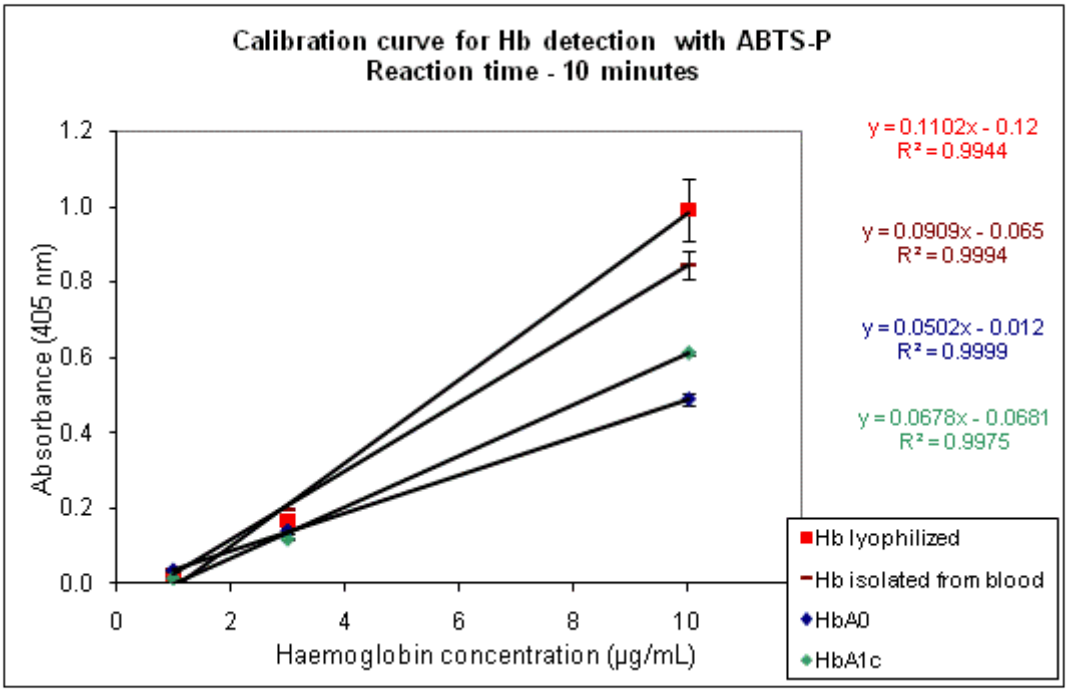

Figure 92. Calibration curve for Hb detection on microplates with ABTS-P. Reaction time 10 minutes.

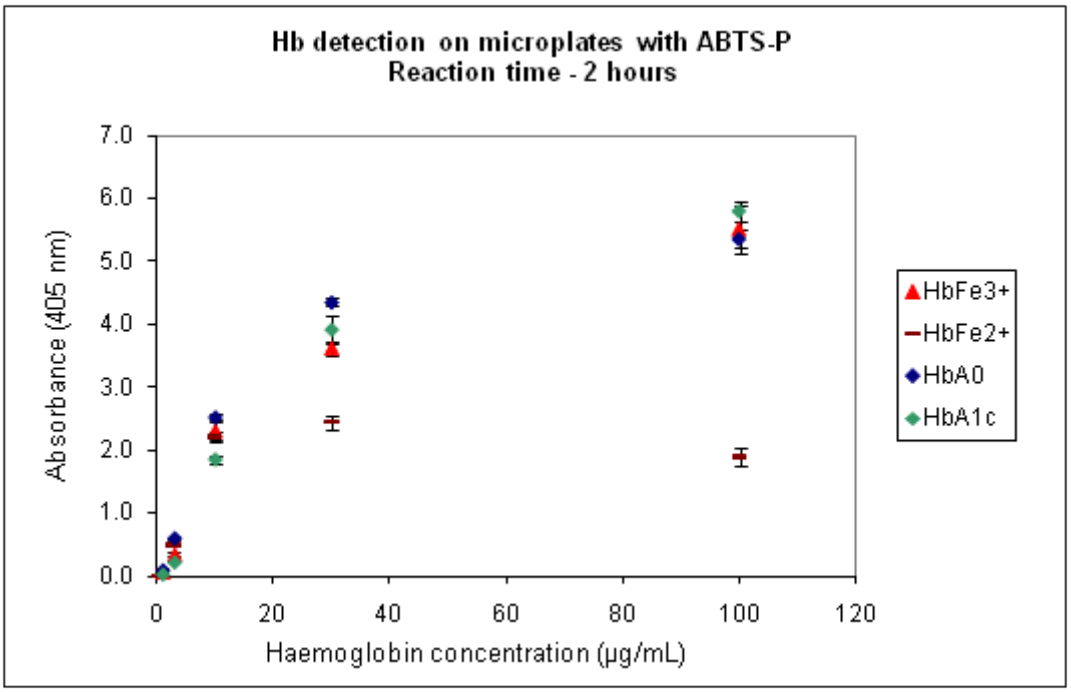

Figure 93. Hb detection on microplates with ABTS-P. Reaction time 2 hours.

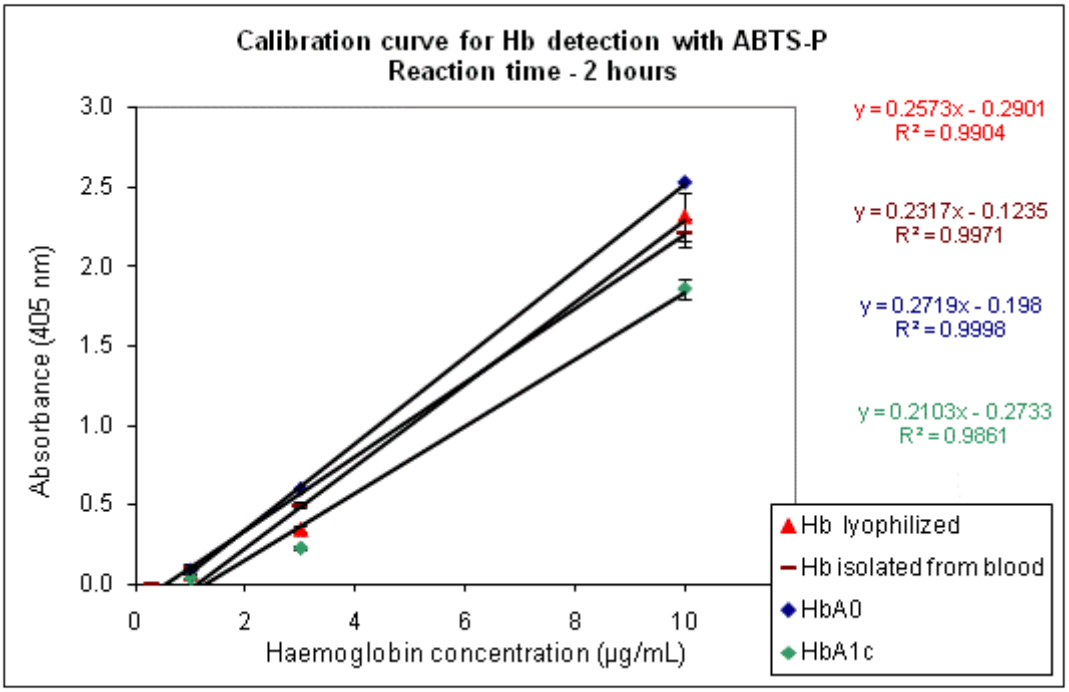

Figure 94. Calibration curve for Hb detection on microplates with ABTS-P. Reaction time 2 hours.

9.2.2.2 Detection of HbA0 with different substrates for ELISA assays

Different substrates for an ELISA assay were tested to choose the compound with the highest sensitivity. The reactivity of ready-to-use ABTS was studied with a chemical enhancer to improve assay sensitivity. The reaction was recorded after 10 minutes, 30 minutes and 2 hours at 405 nm for ABTS and 655 nm for TMB substrate. The best response was obtained with ready-to-use ABTS. Hb was detected in the range 0.1 to 30 µg/mL.

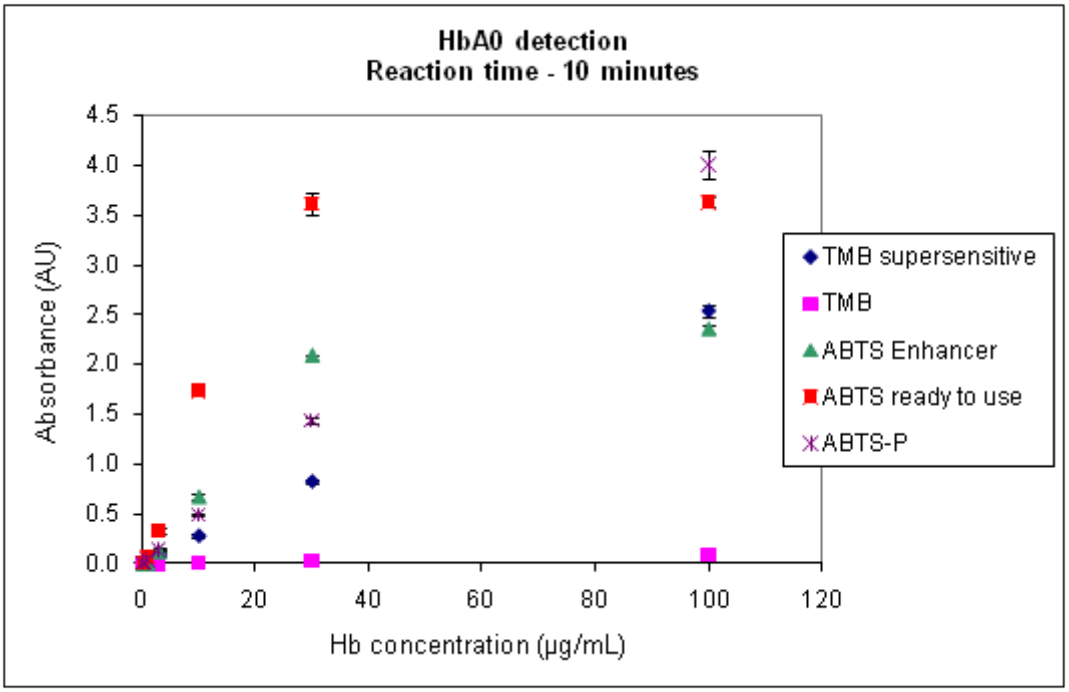

Figure 95. HbA0 detection on microplates with different ELISA substrates. Reaction time 10 minutes.

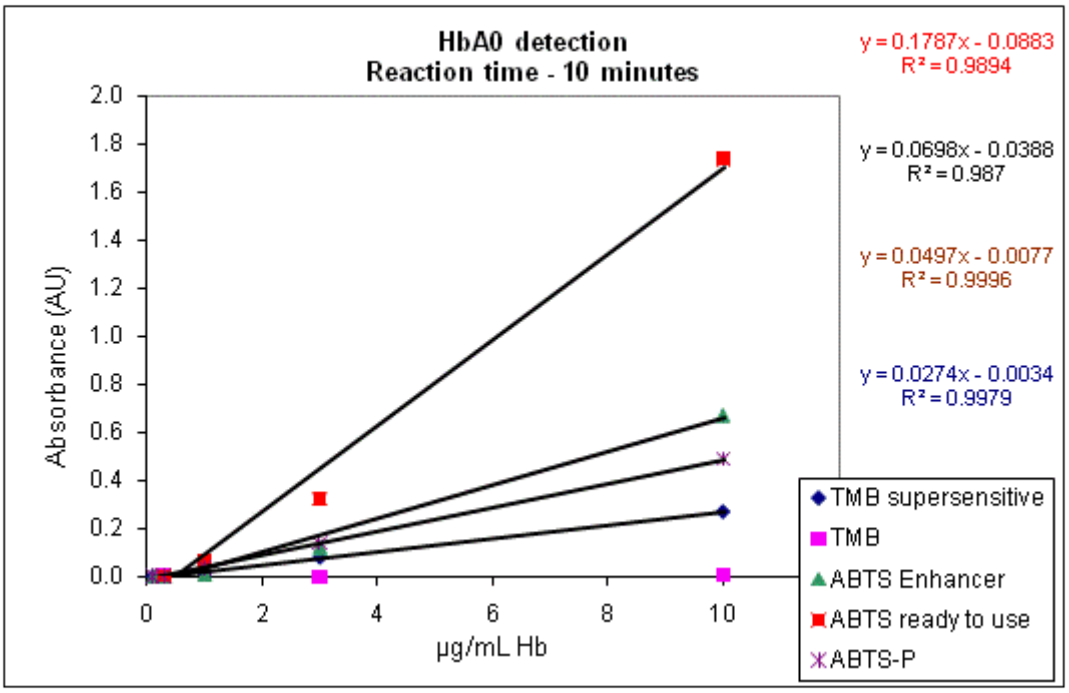

Figure 96. Calibration curve for HbA0 detection on microplates with different ELISA substrates. Reaction time 10 minutes.

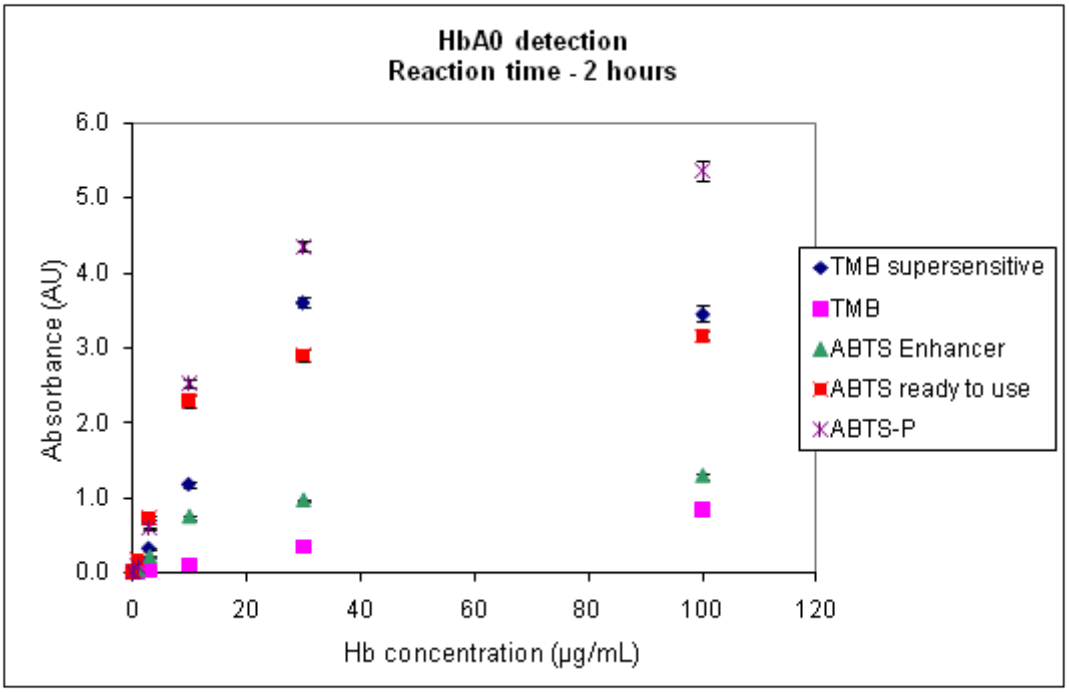

Figure 97. HbA0 detection on microplates with different ELISA substrates. Reaction time 2 hours.

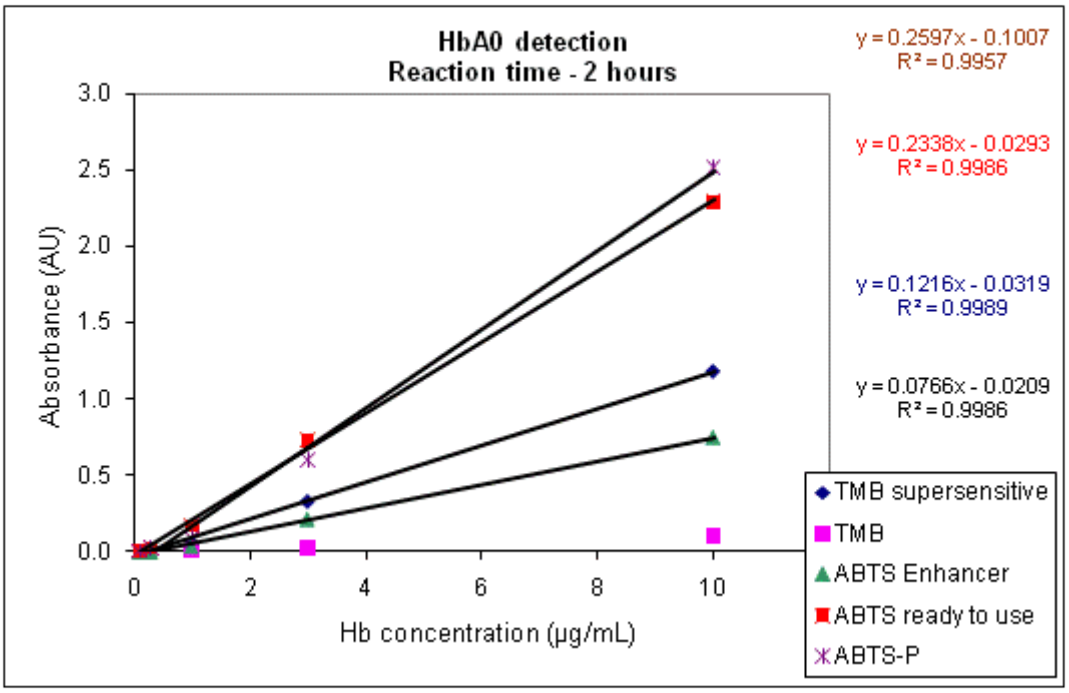

Figure 98. Calibration curve for HbA0 detection on microplates with different ELISA substrates. Reaction time 2 hours.

9.2.2.3 Rebinding of HbA0, HbA1c on microplates coated with APBA polymer

HbA0 and HbA1c binding on the NIP/MIP was studied using the protocols previously developed at Cranfield University (Bossi *et al.*, 2001). Rebinding of Hb was studied in a different range, from 1 to 100 µg/mL. Ready-to-use ABTS was chosen to detect Hb bound to the polymer. Rebinding of Hb on NIP polymer was insignificant. The dissociation constant for rebinding of HbA1c on the MIP-A1c and HbA0 on the MIP-A0 was around 30 – 40 nM. However, rebinding of HbA1c on the MIP-A0 and HbA0 on the MIP-A1c was in the same range, 30 – 40 nM.

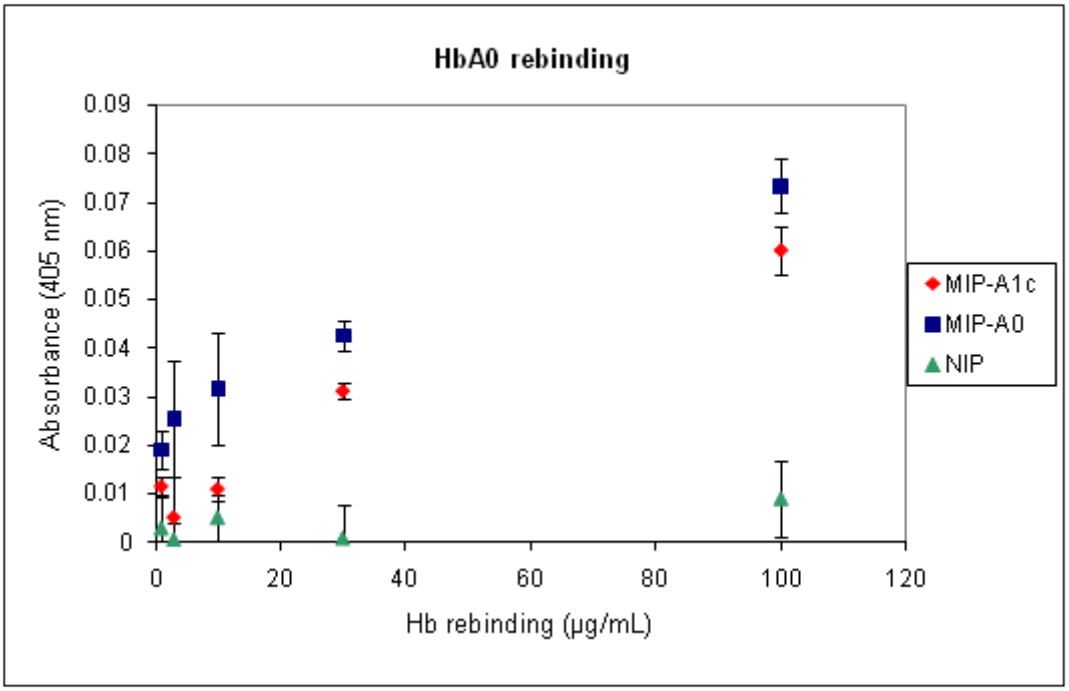

Figure 99. Rebinding of HbA0. Detection with ABTS ready to use. Absorbance read on the same microplate after 2 hours reaction.

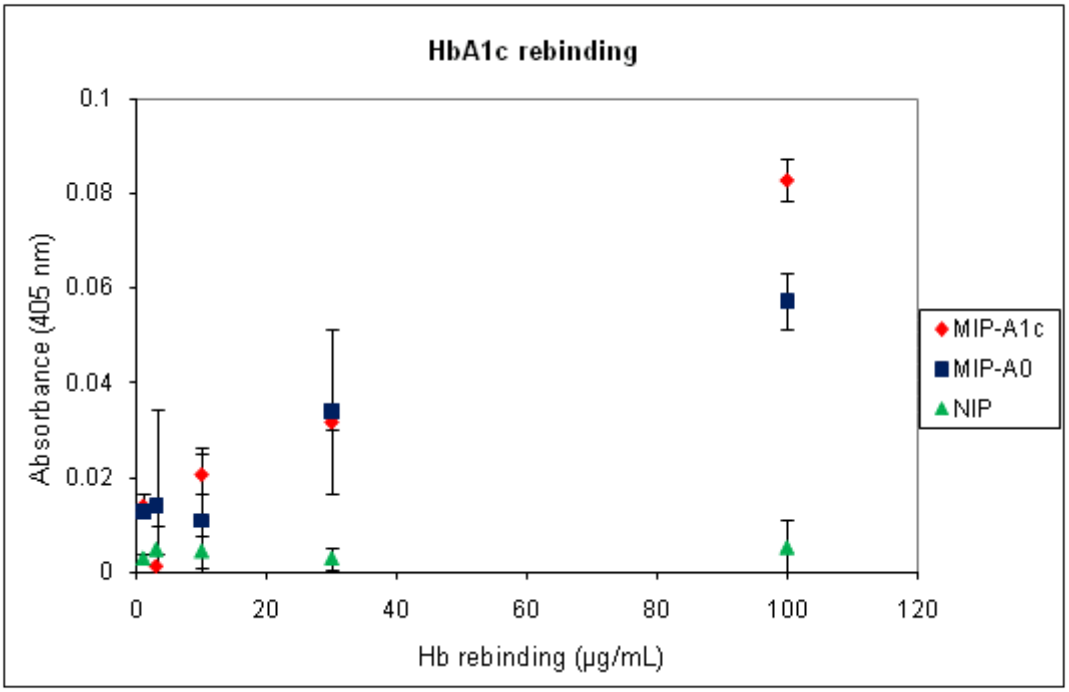

Figure 100. Rebinding of HbA1c. Detection with ABTS ready to use. Absorbance read on the same microplate after 2 hours reaction.

To improve detection of Hb, ABTS solution was transferred to the new plate after 2 hours incubation. A significant difference between results with NIP and MIP was obtained (Figure 101). The K_d for HbA0 rebinded on MIP-A0 was 100 nM. Insignificant binding was detected on NIP polymer for 30 and 50 µg/mL Hb.

Presented research showed binding of HbA1c on MIP-A1c, MIP-A0 and HbA0 on MIP-A0 and MIP-A1c. Previously publish work (Bossi et al., 2001) also showed binding of HbA1c on MIP-A0. The same effect was observed on MIP-A1c, where both haemoglobins, HbA1c and HbA0 were bound. Probably lack of specificity is caused by similar structure of HbA1c and HbA0. Differences in binding of HbA1c on MIP-A1c compared to previous work (Bossi et al., 2001) may be due to different haemoglobins and chemicals which were used for polymer preparation.

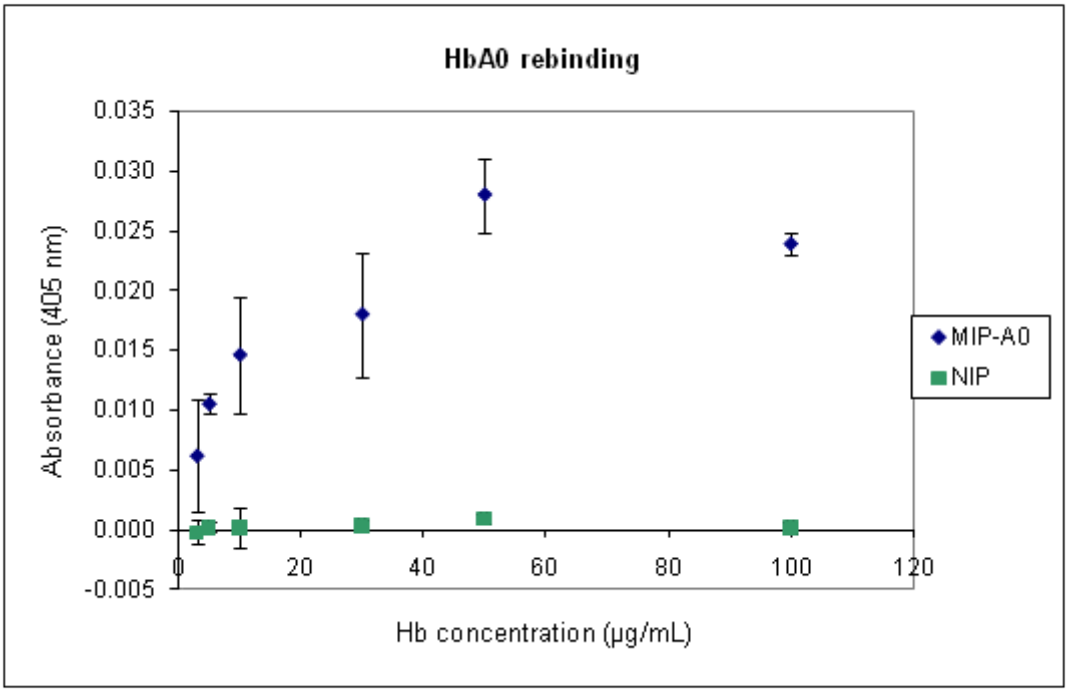

Figure 101. Rebinding of HbA0. Detection with ABTS ready to use. 50 µL solution was transfered to the new microplate after 2 hours and absorbance read at 405 nm.

9.2.3 Conclusions

Stabilised and non-stabilised haemoglobin can be detected using its peroxidase activity. Haemoglobin stabilised in KCN has lower peroxidase activity over the shorter reaction time in comparison to non-stabilised Hb. HbA1c stabilised in KCN has higher peroxidase activity than HbA0 if the enzymatic reaction is carried out over a shorter time. Therefore, response of HbA1c sensor should be validated with haemoglobin without stabilisers.

There was an enhancement of the binding of Hb to MIP as compared to NIP, which indicates presence of an imprinting effect. There is also an indication that MIP binds glycated haemoglobin (HbA1c) better compared with non-glycated haemoglobin (HbA0). This effect however varies and at the moment it can be concluded that the imprinted polymer recognises whole haemoglobin but does not clearly distinguish the glycated part of the protein. Further efforts are required to develop a chromatographic MIP medium to enhance surface area. However, the process of preparing MIP is likely to be unprofitable due to high cost of HbA1c and the amount required for use as a template. Furthermore, recognition of HbA1c on MIP-A1c and HbA0 on MIP-A0 may be not specific enough to conduct measurements in blood samples. Possible difficulties in using MIP technology result from a non-specific binding of HbA0 on MIP-A1c and HbA1c on MIP-A0. It may be problematic to determine level of HbA1c in blood specimen particularly if HbA1c is present in diabetic and non-diabetic patients.

Chapter 10: Separation of GHb from non-glycated Hb by affinity chromatography

Affinity materials, which were previously used for binding of total glycated haemoglobin (GHb), were studied in combination with electrochemical detection of Hb. The purpose of this chapter was to determine HbA1c in blood samples which could potentially lead to construction of a novel device for HbA1c measurement.

10.1 Materials and methods

10.1.1 Reagents

HQ (hydroquinone with CAS No: 123-31-9), hydrogen peroxide 30% (CAS No: 7722-84-1), sodium monobasic phosphate (CAS No: 7558-80-7), sodium dibasic phosphate (CAS No: 7558-79-4), potassium chloride (CAS No: 7447-40-7), sodium chloride (CAS No: 7647-14-5), magnesium chloride (CAS No: 7786-30-3), sodium hydroxide (CAS No: 1310-73-2), HEPES (CAS No: 7365-45-9), phosphate buffer saline tablet, phosphate – citrate buffer tablet, Drabkin's Reagent, 30% Brij 35 solution (9002-92-0), m-aminophenylboronic acid – agarose (aqueous suspension) were purchased from Sigma-Aldrich, product number A8312 (Dorset, UK), glycated haemoglobin (HbA1c) was purchased from Scipac (Sittingbourne, UK). Glyco-Tek Affinity Column Kit and control samples for HbA1c: Normal and Abnormal level were purchased from Helena BioSciences Europe, ClinChek Control (Whole Blood Diabetes Control lyophilised), Level I and II were provided by KBWingrove Ltd. (Northampton, UK). Human blood was collected into tubes containing EDTA as an anticoagulant. Human blood was kept at 4^{o}C and used within two weeks. Chemicals were used as received.

10.1.2 Apparatus

All electrochemical experiments were performed with the Uniscan potentiostat - galvanostat instrument PG580 with UiEChem software (Buxton, UK). Screen Printed Electrodes (SPE-D) were purchased from DropSens Ltd. (Oviedo, Spain). Electrodes were described in Chapter 6.1.2.

The following other equipment was used in experiments: Flow-Cell for screen-printed electrodes (wall-jet type) from DropSens (Oviedo, Spain); injector Valve V-7 was purchased from GE Healthcare (Amersham, UK); centrifuge, Jouan B4i was purchased from Thermo Fisher Scientific (Waltham, USA); spectrophotometer, Shimadzu 1800 (Shimadzu, Kyoto, Japan); microplate reader, Varioscan Flash version 2.4.3 was purchased from Thermo Fisher Scientific (Hampshire, UK); Microplate Immuno MaxiSorp 96 well flat bottom polystyrene was purchased from Fisher Scientific Ltd. (Loughborough, UK); pH-meter, Hanna Instruments 8519 was purchased from Hanna Instruments Inc. (Woonsocket, USA); Amicon ultra-15 and ultra-4 centrifuge filter (30kDa cut-off) was provided by Millipore Ltd. (Watford, UK); Slide-A-Lyzer Dialysis Cassettes, 10K MWCO, 0.1 - 0.5 ml capacity were purchased from Thermo Fisher Scientific Inc. (Rockford, USA); column assembly 3mm dia x 100mm long, End Piece Fixed with 25um PTFE Frit; 3-way single key valve were purchased from Omnifit Ltd. (Cambridge, UK), mixing Tee-Valve, multisyringe pump (two syringes), syringe pump KDS 100 CE Model from KD Scientific Inc. (Holliston, USA), syringe pump Cat 78-9100C, Cole Palmer Instruments Corporation (Illinois, USA), syringe 60 mL BD Plastipak (Drogheda, Ireland).

10.1.3 Separation of GHb from non-glycated Hb by affinity chromatography combined with spectrophotometric detection

m-Aminophenylboronic acid agarose gel was tested as an affinity material for binding total glycated haemoglobin (GHb). 0.5 mL of agarose was transfered to 1 mL catridge and equilibrated with 5 mL of a binding buffer containing HEPES (0.1 M) and $MgCl_2$ (0.05 M) at pH 8.0. 150 µL HbA0 (3.4 mg/mL) was placed on the column and eluted without an incubation step. HbA0 was eluted with 20 mL of binding buffer and further elution was perform with 3 mL of HEPES buffer (0.1 M) containing sorbitol (0.1 M), pH 8.0. 1 mL fractions were collected. The same concentration of HbA0 was also analysed on Helena Glyco-Tek Affinity Column with a binding buffer containing HEPES (0.1 M) and $MgCl_2$ (0.05 M) at pH 8.0.

HbA0, blood samples, Helena Normal and Abnormal Controls, ClinCheck Controls, level I and level II were analysed on Helena Glyco-Tek Affinity Column Kit. The

HbA1c and GHb values and method of analysis of control samples are shown in the Table 24. Data were provided by the producer. HbA1c value of ClinCheck controls of the different methods is attached in Appendix 3.

Table 24. Content of HbA1c and GHb (%) in control samples. Data provided by the producer.

		Helena Lab controls		ClinCheck controls			
		Method of Analysis					
		Helena Glyco-Tek Affinity Column Method		RECIPE (HPLC)		Bayer /Siemens (immunologic)	
		Level					
		Normal	Abnormal	I	II	I	II
%GHb	Mean	5.7	18.8				
	Range	4.7-6.7	17.3-20.3				
%HbA1c	Mean			4.8	8.7	5.7	10
	Range			4.3-5.3	7.9-9.5	5.2-6.2	9.2-10.8

Catridges contained cellulose resin with covalently bound dihydroxyboryl groups and were saturated with a preservative solution containing sodium azide (0.1 %). Control or blood samples were incubated for 10 minutes in a hemolysate reagent in the ratio 1:9. The hemolysate reagent comprised glycine (0.1 M), $MgCl_2$ (0.05 M), Triton X-100 (2%) and sodium azide. The column was shaken twice to resuspend the resin adhered to the top closure, and solution was eluted until matrix was settled to a level 1.1 cm. The rest of the liquid was removed to the top of the resin bed. The column was equilibrated with 3 mL of a Glyco-Tek Developer A containing glycine (0.1 M), $MgCl_2$ (0.05 M), and sodium azide, pH 8.1-8.6. 100 uL of hemolysate sample was placed on the column and left for 8 minutes. Non-glycated haemoglobin was eluted with 4.5 mL of the Developer A. Total glycated haemoglobin was eluted with 3 mL of the Developer B, buffer containing sorbitol and sodium azide (0.1 %), pH 8.0. Non-glycated Hb solution was diluted with dH_20 (10.4 mL).

Separation of GHb from non-glycated Hb was also studied with different buffers. Control and blood samples were hemolysed in HEPES (0.1 M) buffer containing $MgCl_2$ (0.05 M) and Triton X100 (1%), pH 8.0 in the ratio 1:9. Samples were hemolysed for 10 minutes. The Glyco-Tek Affinity Column was equlibrated with 3 mL of a binding

buffer containing HEPES (0.1 M) and $MgCl_2$ (0.05 M) at pH 8.0. 100 µL of the hemolysate sample was placed on the column and incubated for 8 minutes. Non-glycated haemoglobin was eluted with 4.5 mL of a binding buffer, firstly 0.5 mL buffer was placed on the column. Total glycated haemoglobin (GHb) was eluted with 3 mL of HEPES buffer (0.1 M) containing sorbitol (0.1 M), pH 8.0. Separation of GHb was also performed on the Omnifit glass column packed with affinity matrix (ID 3 mm; volume 0.701 mL). The bottom of the column was connected to the end piece fixed with 25 um PE frit. Sleeve (3 mL) was connected with the column and filled with a matrix. The column was packed with an aqueous solution of Glyco-Tek affinity matrix previously degassed. The column was packed in the flow of buffer. Once the slurry was settled, the column was connected to the pump with flow rate adjusted to 0.14 mL/min. The column was again completely filled up with the matrix and connected to the injector. The column was equilibrated with 7 mL of degassed binding buffer: HEPES (0.1 M) containing $MgCl_2$ (0.05 M) at pH 8.0 at the flow rate adjusted to 0.14 mL/min during equilibration of the column. ClinCheck control sample, level I was analysed. The flow rate was adjusted to 0.07 mL/min and 50 uL of the hemolysate was injected on the column. Non-glycated Hb solution was diluted with dH_20 (10.4 mL).

Absorbance was read at 415 nm for GHb and non-glycated Hb solution immediately. The content of GHb (%) was calculated. Percentage of HbA1c was calculated according the algorithm: HbA1c = 0.6846 x %GHb + 0.973258

10.1.4 Effect of buffer composition on electrochemical response of HQ and Hb detection on SPE

Cyclic voltammetry was used for the qualitative characterisation of redox behaviour of HQ in different buffer solutions. Three cycles were performed for each electrode at a scan rate 50 mV/s. Voltammograms were run from negative toward positive potentials.

Glycine buffer (pH8.6), HEPES and phosphate buffer (PB) at pH 8.0 were studied. HEPES buffer consisted of HEPES (100 mM), $MgCl_2$ (50 mM); Glycine buffer consisted of glycine (100 mM), $MgCl_2$ (50 mM); PB consisted of sodium monobasic phosphate and sodium dibasic phosphate (100 mM). Electrodes were immersed in a

vertical position in a 10 mL beaker containing 5 mL of HQ solution and 5 mL of a tested buffer. HQ stock solutions (100 mM) used for electrochemical analysis were prepared daily in a deoxygenated solution of KCl (200 mM). Mediator was prepared in a solution deoxygenated by purging with nitrogen. Additionally mediator stock solution was deoxygenated by blowing nitrogen over the solution for 5 minutes. Mediator was kept in 4^{o}C during measurements. CV was performed under unstirred conditions.

The same buffers were used to plot a calibration curve for Hb detection by a chronoamperometric method. Samples consisted of HQ (50 mM), H_20_2 (4 mM) and haemoglobin. 100 µL of the solution under study was placed on the electrode. Samples comprised 50 µL of the HQ/H_20_2 solution and 50 µL of the studied buffer. Experiments were performed on SPE-D electrodes at room temperature 22^{o}C. Measurements were performed in triplicate. Data were processed in Microsoft Office Excel and average values calculated.

10.1.5 Separation of GHb from non-glycated Hb by affinity chromatography combined with electrochemical detection

Total glycated haemoglobin (GHb) was separated from non-glycated haemoglobin on the Glyco-Tek Affinity Column. It contained cellulose matrix with covalently bound dihydroxyboryl group. Blood samples, Helena Normal and Abnormal Controls, ClinCheck Controls, level I and level II were analysed. Haemoglobin concentration in control and blood samples was determined by Drabkin's method described in Chapter 9.2.1.4. Blood samples were hemolysed in HEPES (0.1 M) buffer containing $MgCl_2$ (0.05 M) and Triton X100 (1%), pH 8.0 in the ratio 1:9. The samples were hemolysed for 10 minutes. The affinity column was shaken twice to resuspend the resin adhered to the top closure, and solution was eluted until matrix was settled to a level 1.1 cm. The rest of the liquid was removed to the top of the resin bed. The column was equlibrated with 3 mL of a binding buffer: HEPES (0.1 M) containing $MgCl_2$ (0.05 M) at pH 8.0. 100 µL of hemolysate sample was placed on the column and left for 8 minutes. Non-glycated haemoglobin was eluted with 4.5 mL of binding buffer. Total glycated haemoglobin was eluted with 3 mL of HEPES buffer (0.1 M) containing sorbitol (0.1 M), pH 8.0.

Chronoamperometry was used to analyse eluted samples on SPE-D electrodes at a reduction potential of -400 mV. Measured samples comprised 50 µL of the HQ/H_20_2 solution and 50 µL of eluted haemoglobin. Samples contained HQ (50 mM), H_20_2 (4 mM) and eluted non-glycated Hb or GHb. 100 µL of the solution under study was incubated 20 seconds then placed on the SPE electrode and the amperometric signal was recorded over 500 seconds. HQ and H_20_2 stock solutions were prepared in a deoxygenated solution of KCl (200 mM) daily. Solutions were deoxygenated by purging with nitrogen. Additionally mediator stock solution was deoxygenated by blowing nitrogen over the solution for 5 minutes. Mediator was kept in room temperature (22^oC) during measurements. Experiments were performed on SPE-D electrodes at room temperature. Measurements were performed in triplicate. Data were processed in Microsoft Office Excel and average values calculated. The content of GHb and non-glycated Hb was read from the calibration curve and GHb (%) calculated. GHb (%) was also determined directly from the values of current signal for GHb, non-glycated Hb and total Hb.

Separation of GHb was also performed on the Omnifit glass column which was packed with affinity material (ID 3 mm; volume 0.701 mL). Column was prepared in the same manner as previously described in Chapter 10.1.3. HQ (200 mM) and H_20_2 (16 mM) were pumped in a separated stream with a multisyring pump. Substrates were mixed in the Micro Static Mixing Tee and further mixed in the PTFE tube (I.D. 0.8 mm, length 54 cm), at a combined flow rate 0.07 mL/min. Omnifit Column (I.D. 3 mm; volume 0.701 mL) was connected to the injector (Valve V-7, loop 50 uL) with PTFE tube (I.D. 0.8 mm, length 13.5 cm). The bottom of the column was connected to the PTFE tube (I.D. 0.8 mm, length 13.5 cm). HQ/H_20_2 substrates were mixed with a stream passing through the affinity column, in the second Micro Static Mixing Tee and further mixed in the PTFE tube (I.D. 0.8 mm, length 54 cm) which was connected to the flow cell. Either the binding buffer or the elution buffer was run through the injector and the column. For that purpose two syringe pumps were used. PTFE tubes from the syringes (I.D. 0.8 mm, length 27 cm) were connected with a stream changing valve. The flow rate was adjusted to 0.07 mL/min through the column. Chronoamperometry was used to determine the

content of non-glycated Hb and GHb in the sample. Eluted fractions of haemoglobin were detected on SPE-D electrodes at a potential -400 mV.

Percentage of HbA1c was calculated according the algorithm: HbA1c = 0.6846 x %GHb + 0.973258

10.2 Results and Discussion

10.2.1 Spectrophotometric detection of haemoglobin

m-Aminophenylboronic acid agarose was tested as an affinity material for HbA1c determination. Non-specific binding was observed for HbA0 since only 43.5 % and 59 % of total HbA0 was eluted with 10 and 20 mL of binding buffer, respectively. These results suggest a poor quality of the affinity material. It is possible that pores were too small for haemoglobin to enter. Polysaccharide chains of agarose that consist of galactose units, without covalent cross-linking can lead to shrinkage and lower stability of the matrix. The procedure of preparation and factors, such as amount of stabiliser, stirrer speed and storage condition may lead to damage of agarose beads (Dean *et al*., 1985). An activation process of matrix has also an impact on non-specific binding caused by ion-exchange interferences (Mallia *et al*., 1981). Elution of HbA0 was also verified on Helena Glyco-Tek Affinity Column Kit and 96 % of haemoglobin was recovered. Therefore this affinity resin was used for further research.

Analysis of blood samples was performed on Helena Glyco-Tek Affinity Column Kit. Catridges contained cellulose resin with covalently bound dihydroxyboryl groups. HEPES buffer was tested with Glyco-Tek and Omnifit column containing the same affinity resin. Control samples containing normal, abnormal level of total glycated haemoglobin (GHb) and blood samples were analysed. Results are summarized in Table 25.

Table 25. Content of HbA1c and GHb (%) in control samples.

APBA cellulose								
	Catridges – commercial kit					Catridges HEPES buffer		Omnifit Column HEPES buffer
	Diabetes Controls				Blood	Diabetes Controls		
	Helena		ClinCheck			ClinCheck		
	Normal	Abnormal	I	II		I	II	I
% GHb	4.9	17.2	7.5	12.1	4.6	6.7	10.1	6
% HA1c	4.3	12.7	6.1	9.3	4.1	5.6	7.9	5.1

Percentage of HbA1c was comparable with data provided by the producer of control samples. Small differences between results obtained with HEPES buffer and glycine buffer (Catridges – commercial kit) could be due to the lower pH of HEPES buffer (pH 8.0) or because of some variation between columns. Blood samples contained lower concentration of HbA1c compared to controls with normal level of HbA1c.

10.2.2 Electrochemical detection of haemoglobin

10.2.2.1 Effect of buffer composition on electrochemical response of HQ and Hb detection SPE

Measurements were performed in HEPES, phosphate (pH 8.0) or glycine buffer (pH 8.6). All measured samples contained HQ (50 mM).

Cyclic voltammetry was used to obtain fast information about the redox behaviour of HQ in different buffer formulations. Similar voltammograms of HQ were recorded on SPE-D electrodes (Figures 102, 103 and 104). Three reduction peaks were recorded for phosphate and HEPES buffer but two peaks were obtained with glycine buffer. The difference may be caused by different pH of buffer since the mediator is pH dependent. The cyclic voltammogram of HQ in HEPES buffer was shifted towards more negative potentials. Third reduction peak was recorded at a negative potential of -240 mV. Summary of the cyclic voltammograms is shown in the Table 26.

Table 26. Effect of buffer composition on electrochemical response of HQ (50 mM) on SPE-D electrodes.

	E_{pc}			i_p (reverse peak)		
	mV			µA		
buffer/peak	1	2	3	1	2	3
HEPES	110	-115	-240	-170	-440	-490
Glycine	110	-85		-494	-382	
Phosphate	215	-25	-135	-132	-393	-448

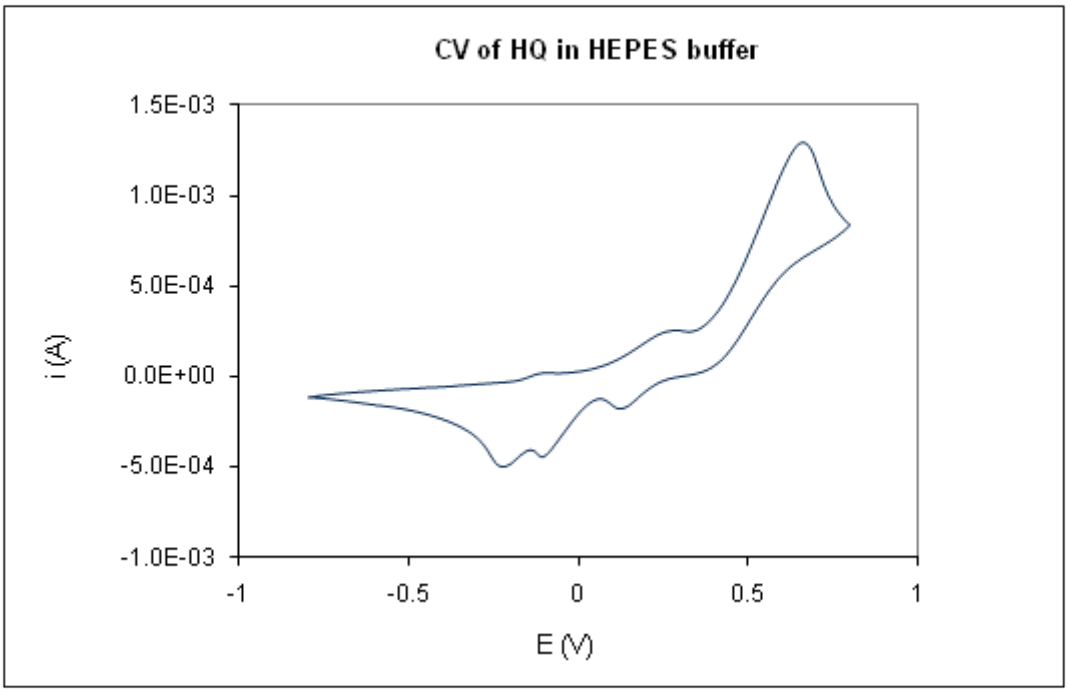

Figure 102. Cyclic voltammetry of HQ (50 mM) in HEPES buffer at pH 8.0 on SPE-D electrodes. Second cycle; scan rate 50 mV/s.

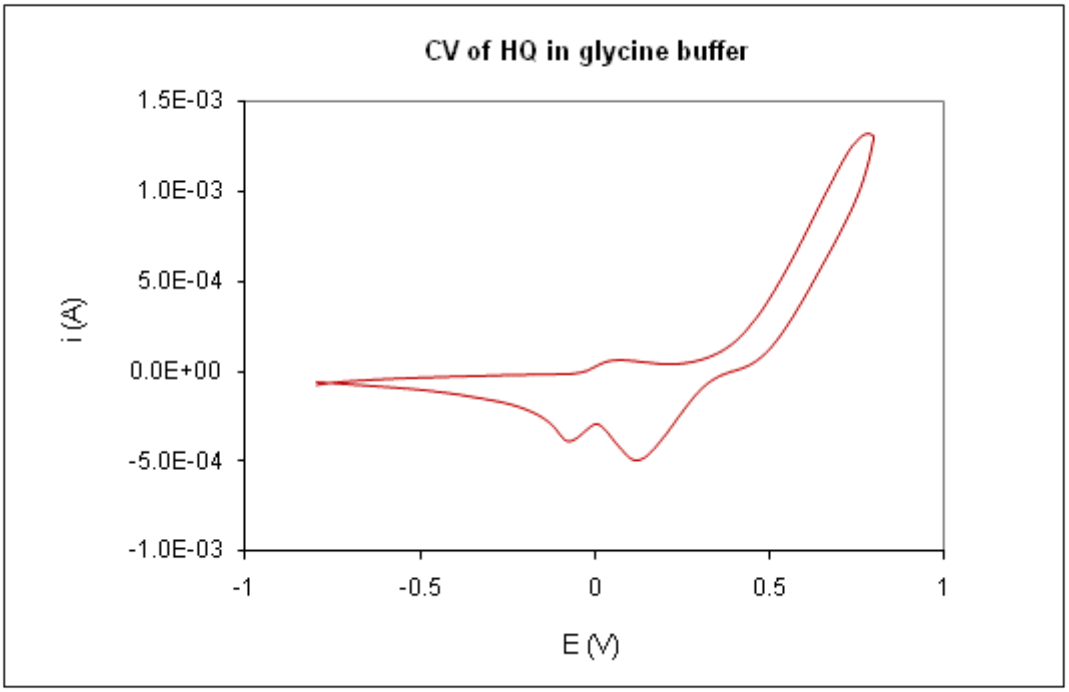

Figure 103. Cyclic voltammetry of HQ (50 mM) in glycine buffer at pH 8.6 on SPE-D electrodes. Second cycle; scan rate 50 mV/s.

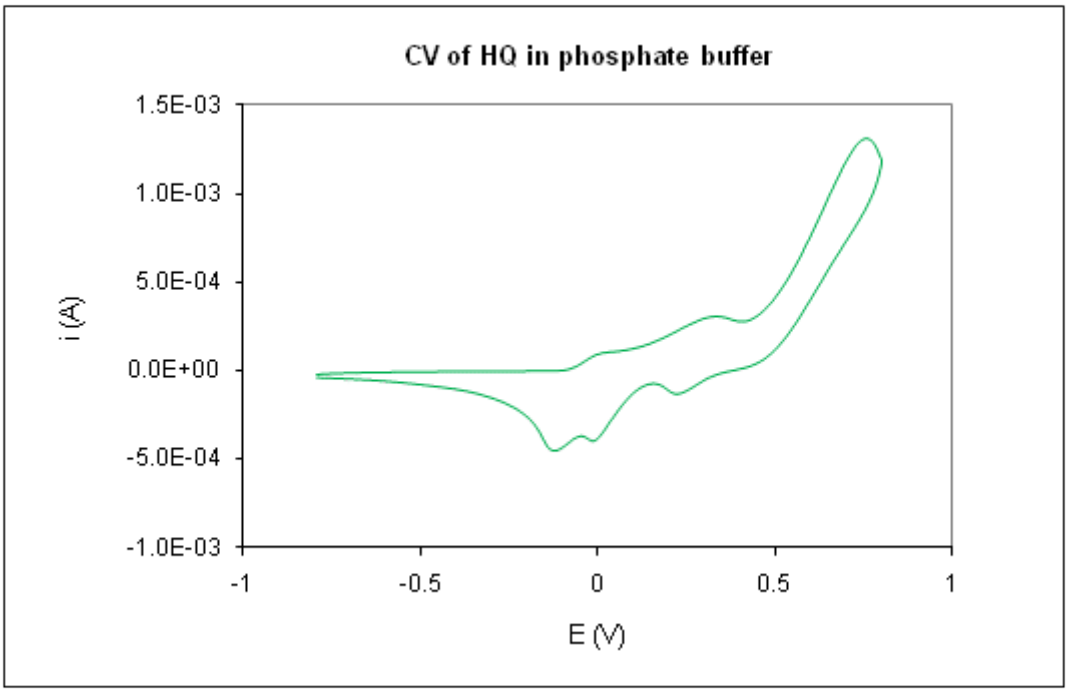

Figure 104. Cyclic voltammetry of HQ (50 mM) in phosphate buffer at pH 8.0 on SPE-D electrodes. Second cycle; scan rate 50 mV/s.

The chronoamperometric signal was measured at a reduction potential of -400 mV. Haemoglobin detection was performed with HQ (50 mM) and H_20_2 (4 mM). HEPES,

phosphate and glycine buffer were tested (Figures 105-107). HEPES and glycine buffer are well recognized in separation of glycated haemoglobin from non-glycated one.

100 µL of the solution under study was incubated 20 seconds, placed on the SPE-D electrode and the amperometric signal was recorded. The lowest detection limit (0.008 mg/mL Hb) was obtained with HEPES buffer at 500 seconds (Figures 107-108). Increased incubation time to 1 and 5 minutes, prior to electrochemical detection, did not improve a detection limit. A calibration curve for detection of Hb was not obtained in glycine buffer. A higher current signal was obtained with blank samples. Measurements with Hb solution did not show a significantly different response (Figure 106).

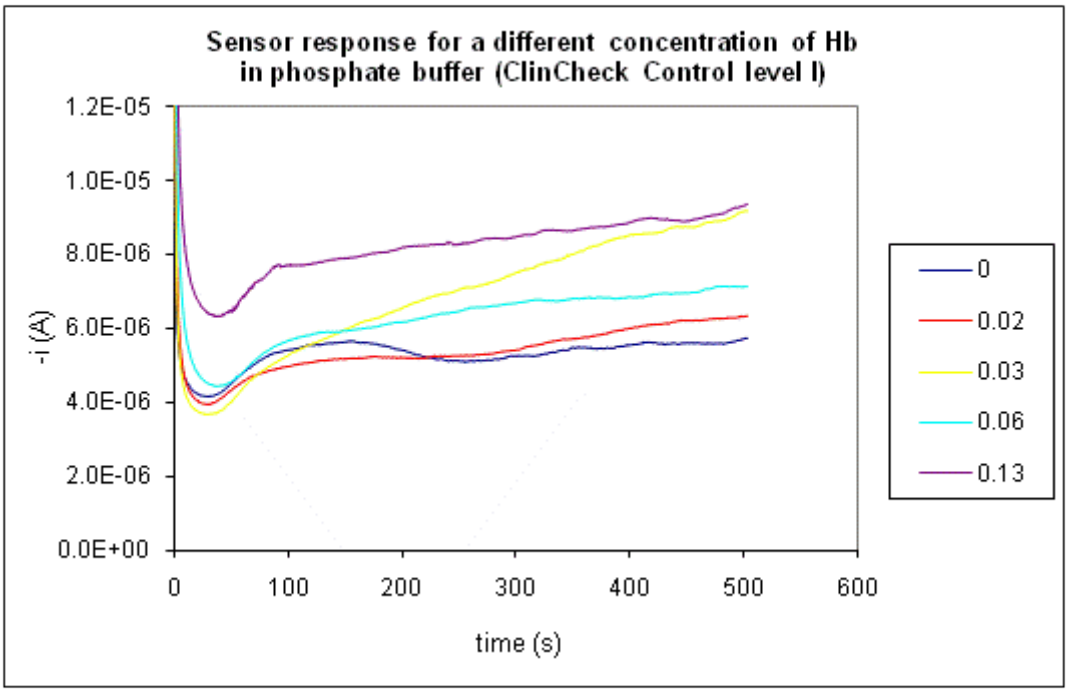

Figure 105. Amerometric response for different concentration of Hb (from 0 to 0.13 mg/mL) in phosphate buffer, pH 8.0 on SPE-D at a potential -400 mV. Sample contained ClinCheck Control (level I), HQ (50 mM) and H_20_2 (4 mM).

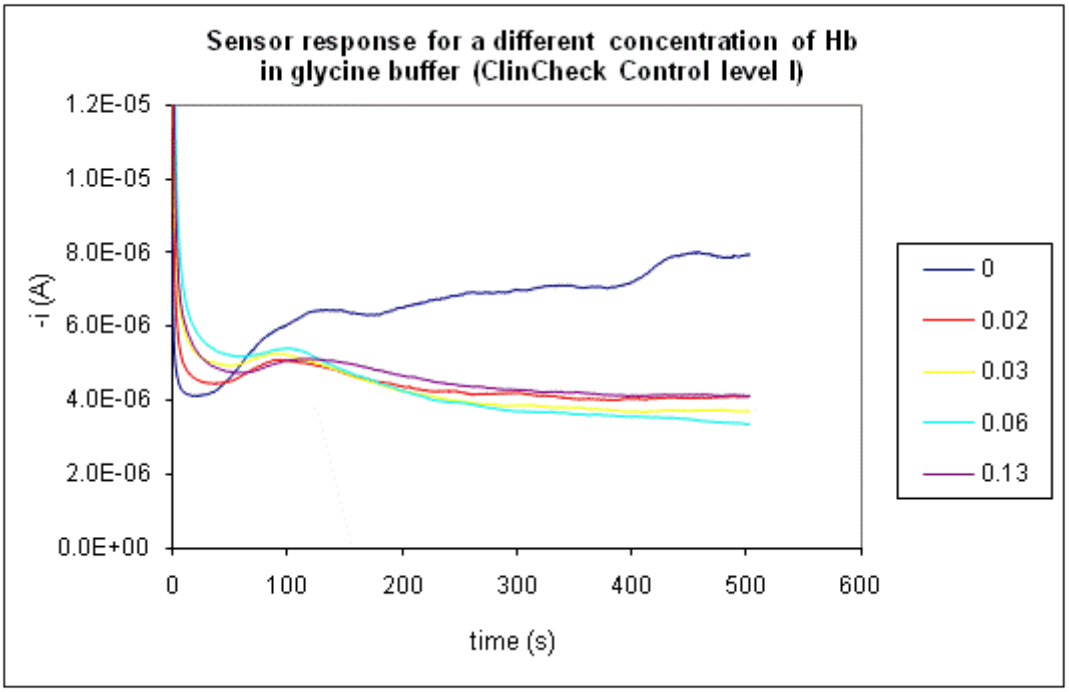

Figure 106. Amerometric response for different concentration of Hb (from 0 to 0.13 mg/mL) in glycine buffer, pH 8.6 on SPE-D at a potential -400 mV. Sample contained ClinCheck Control (level I), HQ (50 mM) and H_20_2 (4 mM).

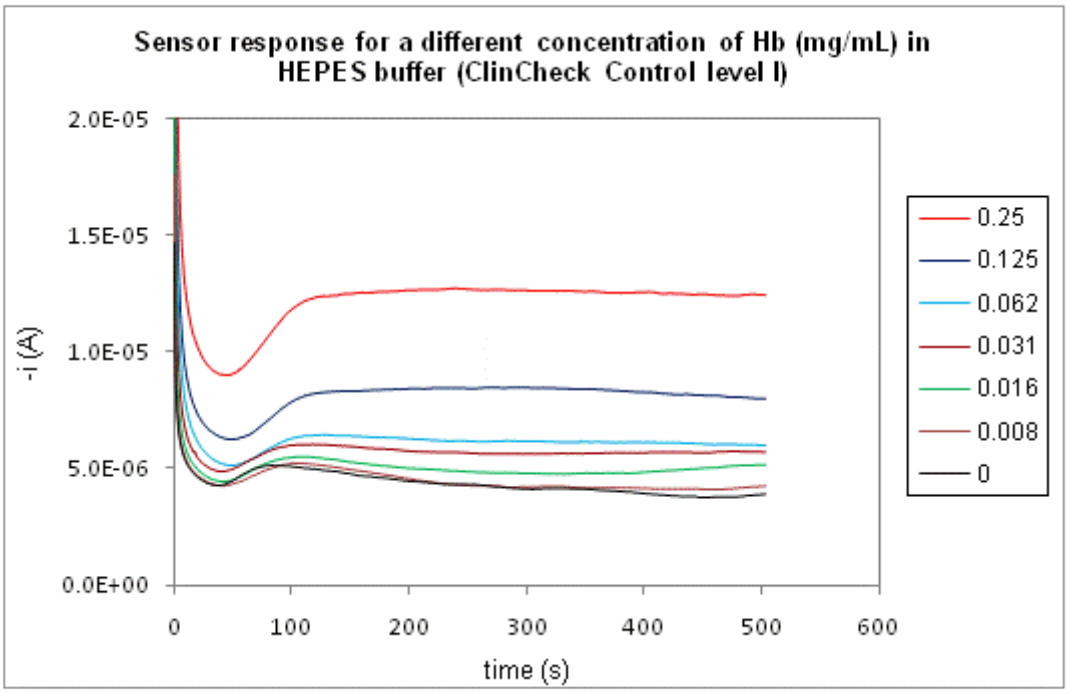

Figure 107. Amerometric response for different concentration of Hb (from 0 to 0.25 mg/mL) in HEPES buffer, pH 8.0 on SPE-D at a potential -400 mV. Sample contained ClinCheck Control (level I), HQ (50 mM) and H_20_2 (4 mM).

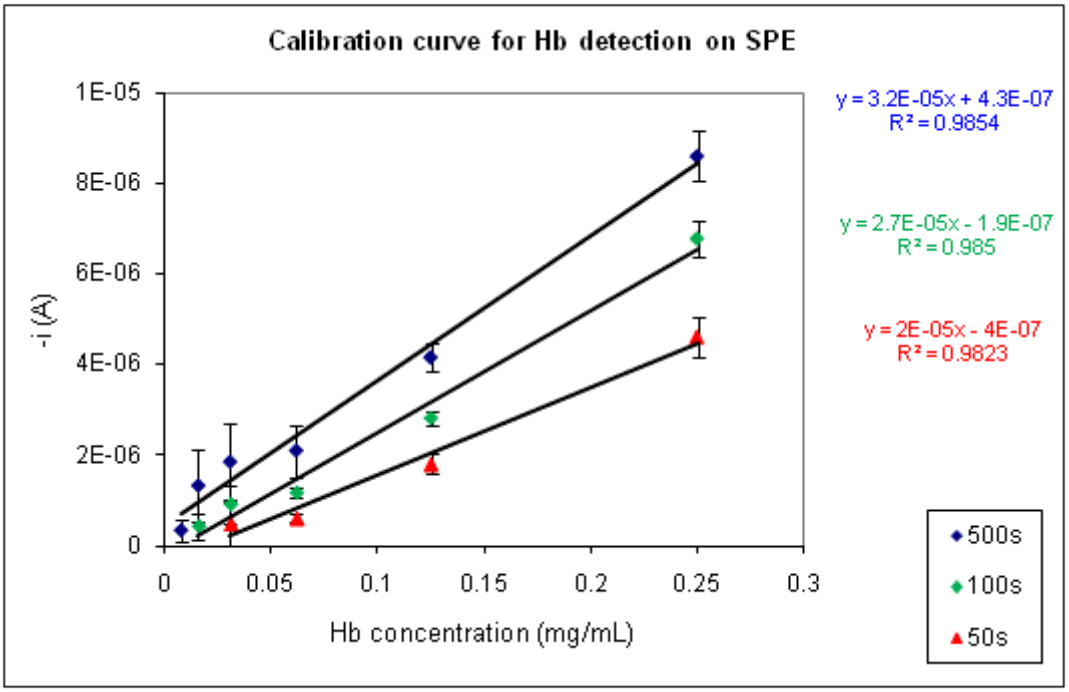

Figure 108. Detection of Hb (ClinCheck Control, level I) using its peroxidase activity with HQ (50 mM), H_20_2 (4 mM) in HEPES buffer, pH 8.0 on SPE-D at a potential -400 mV.

10.2.2.2 Separation of GHb from non-glycated Hb by affinity chromatography – catridge setup

The chronoamperometric signal was measured at a negative reduction potential of -400 mV. Haemoglobin detection was performed with HQ (50 mM) and H_20_2 (4 mM) on SPE-D electrodes. HEPES buffer was used for total Hb detection.

Table 27 shows the content of GHb and non-glycated Hb in control samples. The concentration of measured samples on the electrode was calculated on the basis of total Hb concentration (determined by Drabkin's method) and HbA1c (%) provided by the producer. The concentration of GHb and non-glycated Hb was in a detection range after elution from the column and dilution on the electrode (Figures 107-108).

Table 27. Concentration of non-glycated Hb and GHb in control samples based on data provided by the producer

		ClinCheck Controls		Glyco-Tek Controls	
		Level I	**Level II**	**Normal**	**Abnormal**
Hb concentration in the sample					
GHb	%	5.6	11.3	**5.7**	**18.8**
HbA1c	%	**4.8**	**8.7**	4.9	13.8
HbA1c	mg/mL	6.96	12.69	8.13	22.23
GHb	mg/mL	8.10	16.47	9.51	30.19
non-glycated Hb		136.83	129.43	157.34	130.41
Total Hb		144.93	145.9	166.85	160.6
Hb concentration on SPE					
GHb	mg/mL	0.015	0.030	0.018	0.056
non-glycated Hb		0.165	0.156	0.190	0.157
Total Hb		0.175	0.176	0.202	0.194

ClinCheck control samples, level I and II were analysed. Total glycated haemoglobin (GHb) was separated on the column containing the cellulose resin with covalently bound dihydroxyboryl group. The concentration of non-glycated and total glycated haemoglobin after elution from the column was determined electrochemically. The total content of Hb was determined without passing a sample through the column and was also calculated from the signal of the eluted samples, non-glycated and glycated fractions. Percentage of HbA1c was determined on the basis of amperometric response of total Hb, GHb and non-glycated Hb recorded at 500 seconds (Figures 109-110). The concentration of Hb was also calculated from the calibration curve that was prepared for ClinCheck Control, level I. Data were analysed at 500 seconds (Figure 108) and percentage of HbA1c was calculated. Analysis of electrochemical data of non-glycated Hb and GHb is shown in the Tables 27, 28 and 29. GHb (%) was determined from the calibration curve and HbA1c (%) calculated. Also analysis of electrochemical signal of GHb and non-glycated Hb gave similar results. Analysis of control sample, level II showed percentage of HbA1c similar to data provided by producer (around 10 %, Table 28). However, the same percentage of HbA1c was obtained for the control sample, level I. Analysis of samples from a second affinity column showed the increased content of HbA1c. In that case the difference between controls, level I and II was around 2% (non-glycated Hb and GHb fractions, Table 29). It was possible to detect GHb and non-glycated Hb in normal and elevated level of HbA1c in control samples, but it was problematic to detect small difference between normal and abnormal levels of HbA1c.

The problem with repeatability was observed at 500 seconds (Figures 109, 110). The same measurements were analysed at shorter time (50 seconds). The current signal showed decreased relative standard deviation but GHb was not detected at 50 seconds (Figures 111, 112).

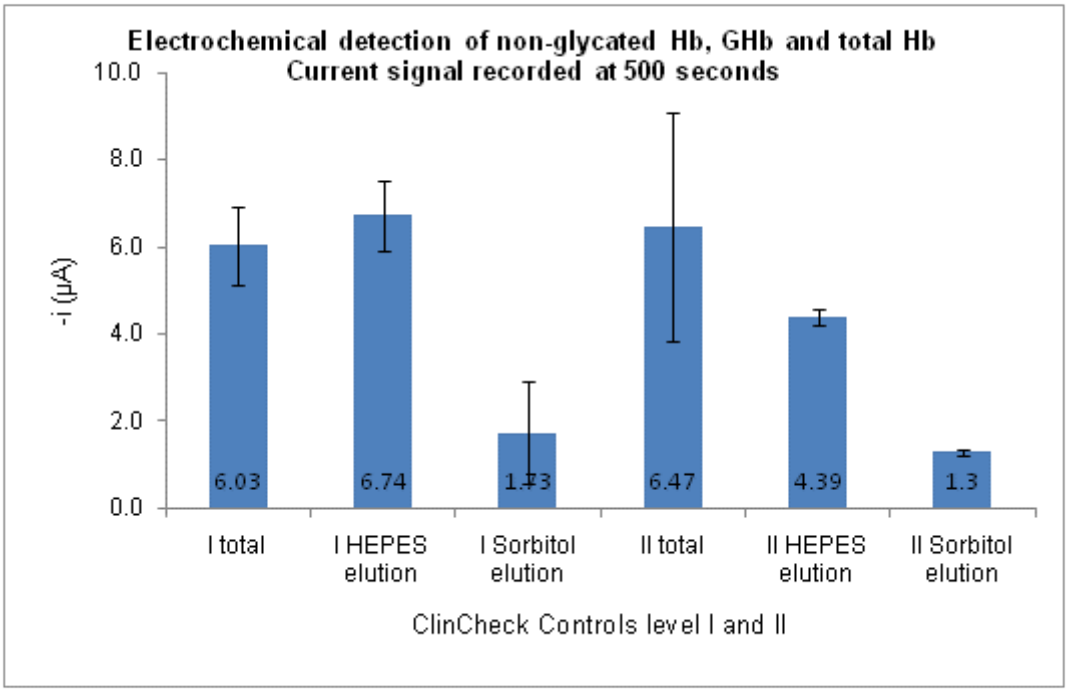

Figure 109. Electrochemical determination of non-glycated Hb and GHb after elution from column 1. Data analysis after 500 seconds. A background current was subtracted from a signal current.

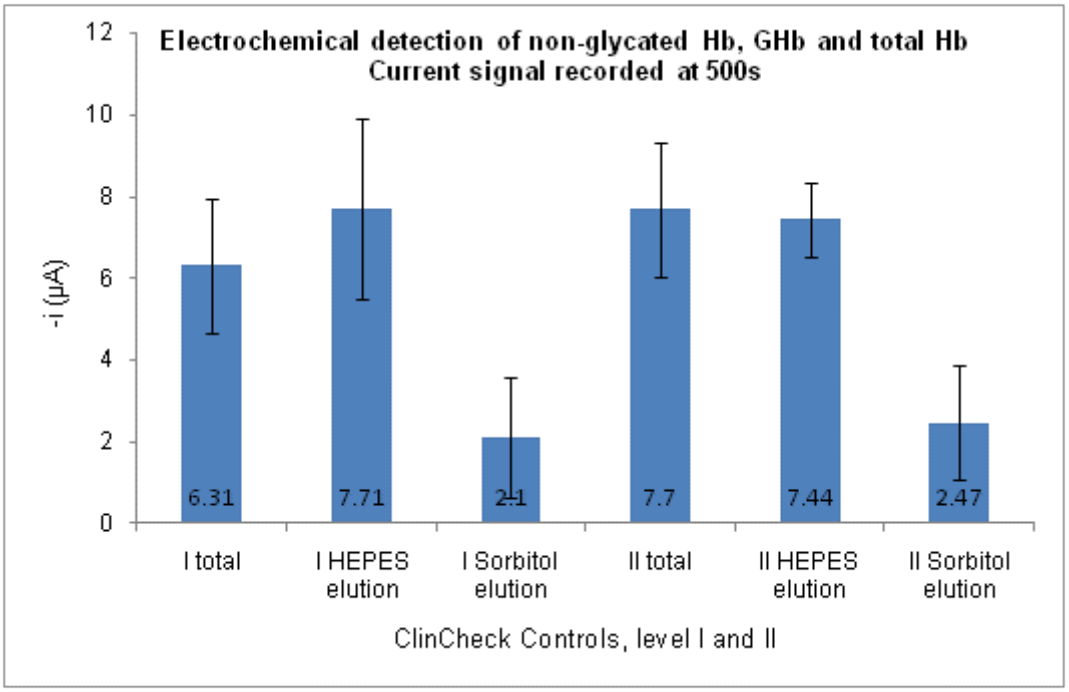

Figure 110. Electrochemical determination of non-glycated Hb and GHb after elution from column 2. Data analysis after 500 seconds. A background current was subtracted from a signal current.

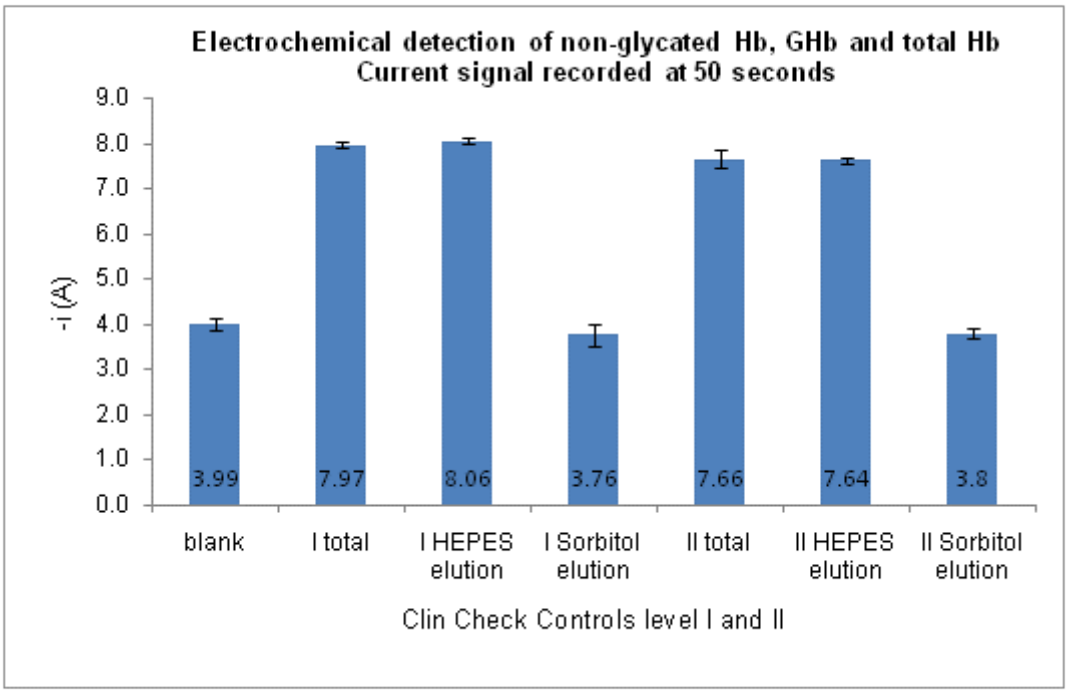

Figure 111. Electrochemical determination of non-glycated Hb and GHb after elution from column 1. Data analysis after 50 seconds. A background current was not subtracted from a signal current.

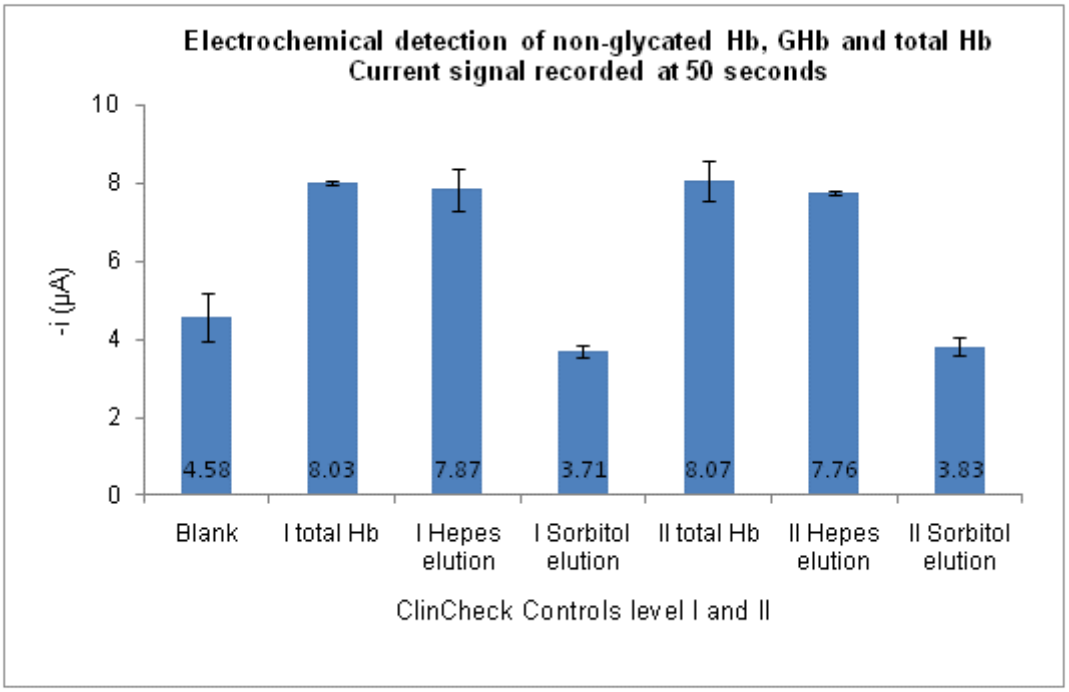

Figure 112. Electrochemical determination of non-glycated Hb and GHb after elution from column 2. Data analysis after 50 seconds. A background current was not subtracted from a signal current.

Table 28. HbA1c (%) in ClinCheck control, level I and II determined by electrochemical detection. Separation of GHb on Column1.

	HbA1c (%) in ClinCheck control, level I		
Analysis of electrochemical signal	GHb, Total Hb	non-glycated Hb, GHb	non-glycated Hb, Total Hb
mg/mL (calibration curve)	11.3	9.1	
i (A)	13.8	10.8	
	HbA1c (%) in ClinCheck control, level II		
mg/mL (calibration curve)	7.4	9.5	24.6
i (A)	9.9	12.0	23.0

Table 29. HbA1c (%) in ClinCheck control, level I and II determined by electrochemical detection. Separation of GHb on Column2.

	HbA1c (%) in ClinCheck control, level I		
Analysis of electrochemical signal	GHb, Total Hb	non-glycated Hb, GHb	non-glycated Hb, Total Hb
mg/mL (calibration curve)	13.6	9.9	-
i (A)	15.8	11.3	-
	HbA1c (%) in ClinCheck control, level II		
mg/mL (calibration curve)	13.5	11.9	3.4
i (A)	15.3	13.1	3.3

The increased level of HbA1c (Level I) in comparison to the data provided by the supplier might results from uncontrolled processes running on screen printed electrodes over a longer time. Theoretically, concentration of Hb in samples was within a detection limit. However, a calibration curve should be of hight quality to detect a very small variation. Detection should be improved to determine very small differences between normal and abnormal level of HbA1c in that configuration.

Longer pre-incubation time (1 and 5 minutes) was also tested. However it did not improve sensitivity. Detection of Hb was also conducted in the FIA system. Injected samples containing HQ, H_20_2 and total Hb or non-glycated Hb were analysed. Results showed no difference between these Hb samples.

10.2.2.3 Separation of GHb from non-glycated Hb by affinity chromatography – FIA system

Further work was focussed on separation of GHb from non-glycated Hb performed on a chromatographic column combined with electrochemical detection in the flow system. The GHb (%) was determined on the basis of peak area of non-glycated Hb and GHb. HbA1c (%) was calculated from the algorithm. Clincheck, Helena control samples and blood specimens were analysed.

The HbA1c (%) in ClinCheck control samples, level I and II amounted to 10.3 and 17.2 %, respectively (Figure 113). 7.4% HbA1c was detected in blood samples (Figure 114).

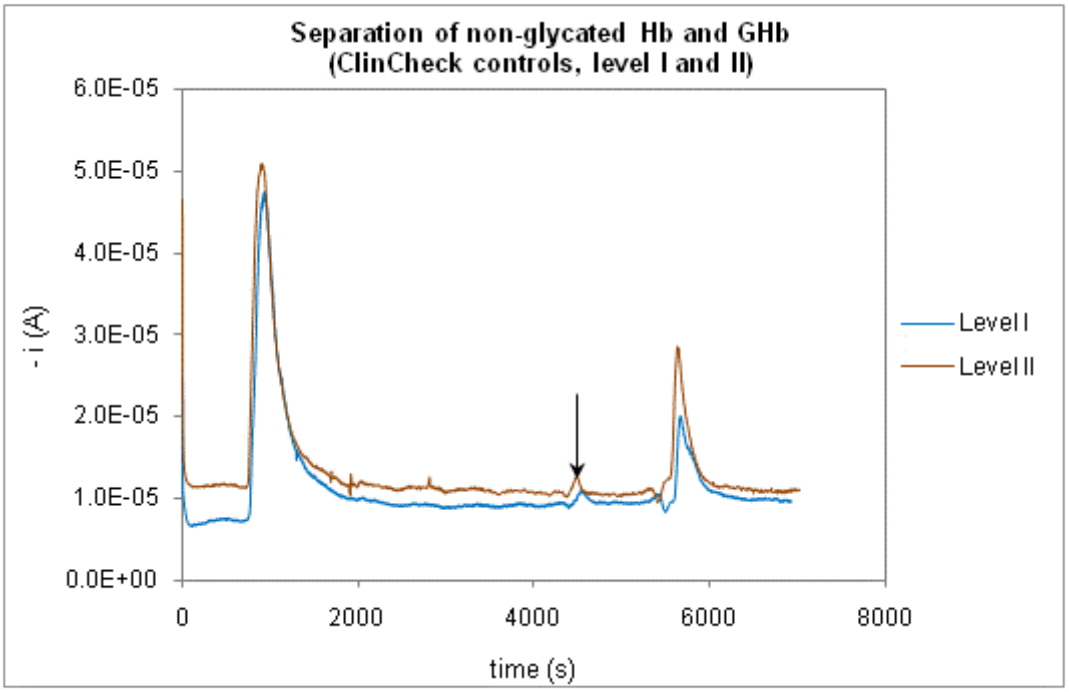

Figure 113. Chromatogram of ClinCheck control samples and electrochemical detection of non-glycated Hb (first peak) and GHb (second peak) fractions. The arrow indicates a change of buffer.

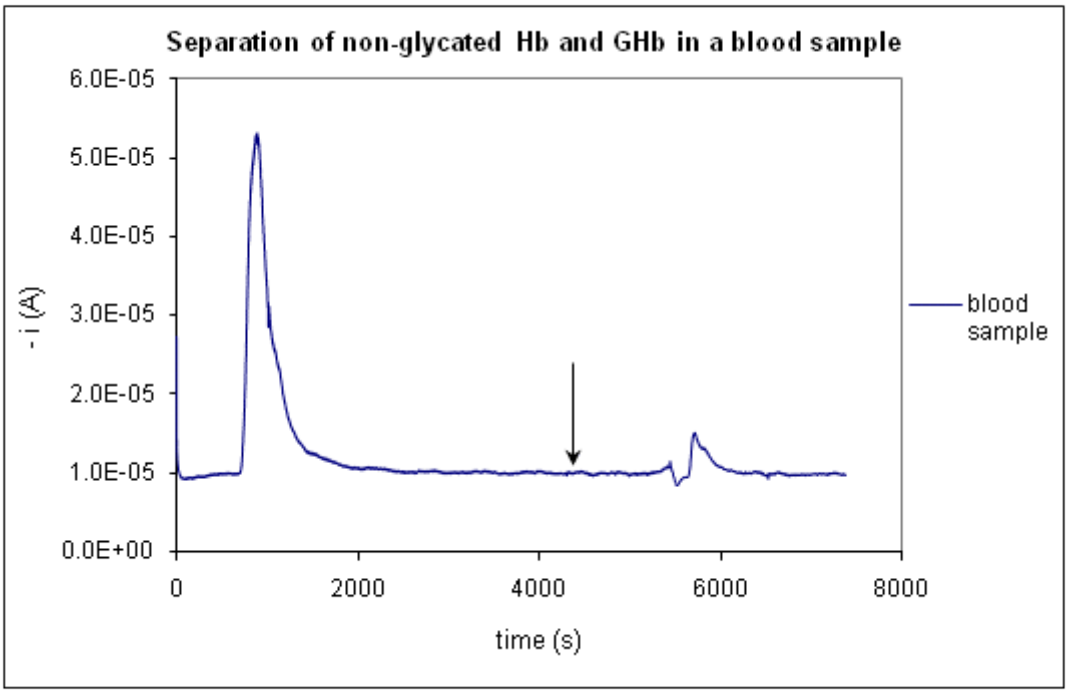

Figure 114. Chromatogram of a blood sample and electrochemical detection of non-glycated Hb (first peak) and GHb (second peak) fractions. The arrow indicates a change of buffer.

The Glyco-Tek Abnormal Control was used to determine different concentration of Hb (Figures 115-118). Samples contained the same percentage of HbA1c. Results for Glyco-Tek Normal and Abnormal Controls are shown in the Table 30. Non-glycated Hb was detected in a range from 1.83 to 11 mg/mL and GHb from 0.4 to 2.4 mg/mL (Figures 116-118).

Table 30. HbA1c (%) determined by affinity chromatography combined with electrochemical detection in the flow system.

	non-glycated Hb	GHb	HbA1c	HbA1c (average)
	mg/mL		%	
Glyco-Tek Abnormal Control	1.83	0.4	27.1	
	3.66	0.8	21.4	
	7.32	1.6	22.9	
	10.98	2.4	19.9	
	14.64	3.2	24.7	**23.2**
Glyco-Tek Normal	analysis of GHb, non-glycated Hb (electrochemical signal)		**6.5**	
	GHb, non-glycated Hb (mg/mL) read from calibration curve		**1.7**	

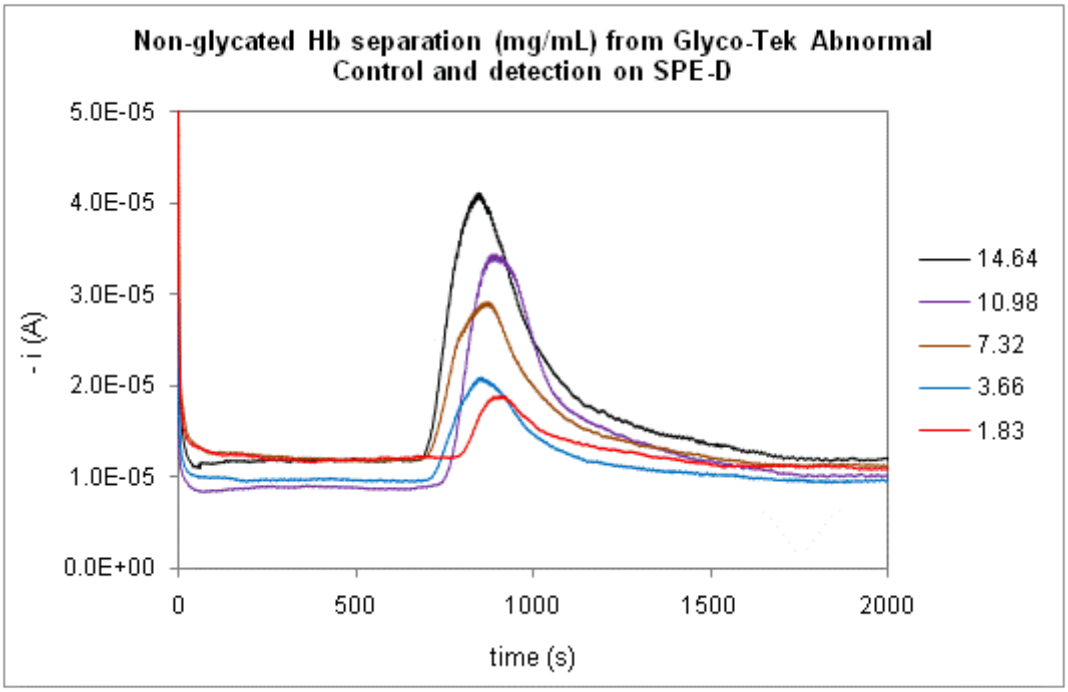

Figure 115. Separation of non-glycated Hb from GHb (Glyco-Tek Abnormal Control) on the affinity column and its electrochemical detection at -400 mV on SPE-D1

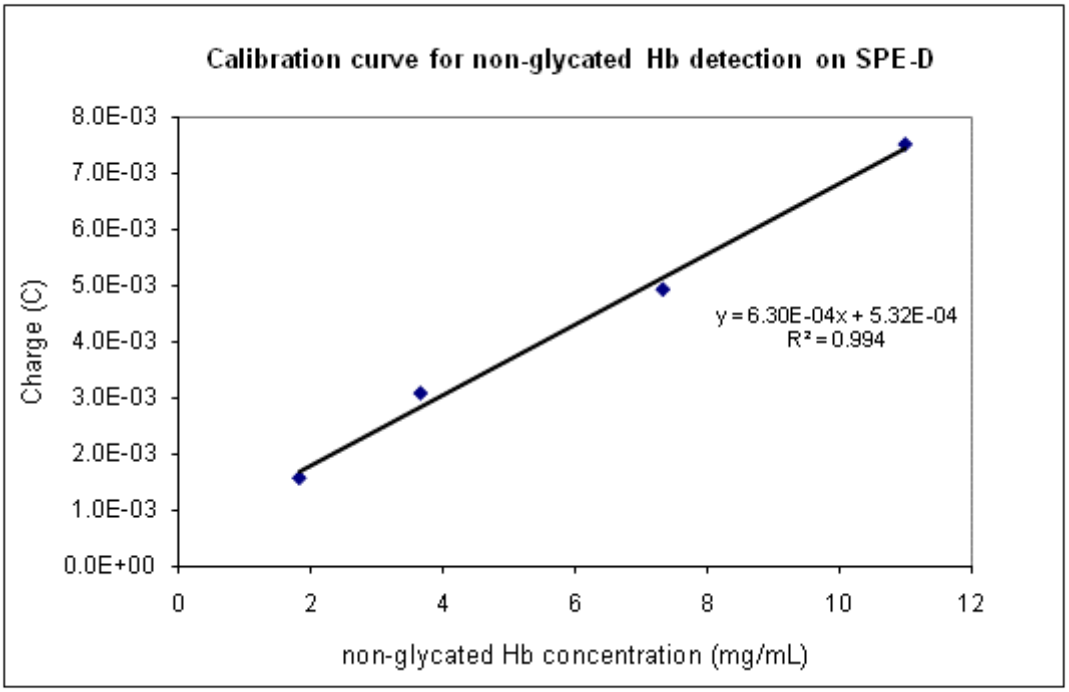

Figure 116. Detection of non-glycated Hb separated from GHb (Helena Abnormal Control) by affinity chromatography combined with electrochemical detection on SPE-D1 at a potential -400 mV in the FIA system.

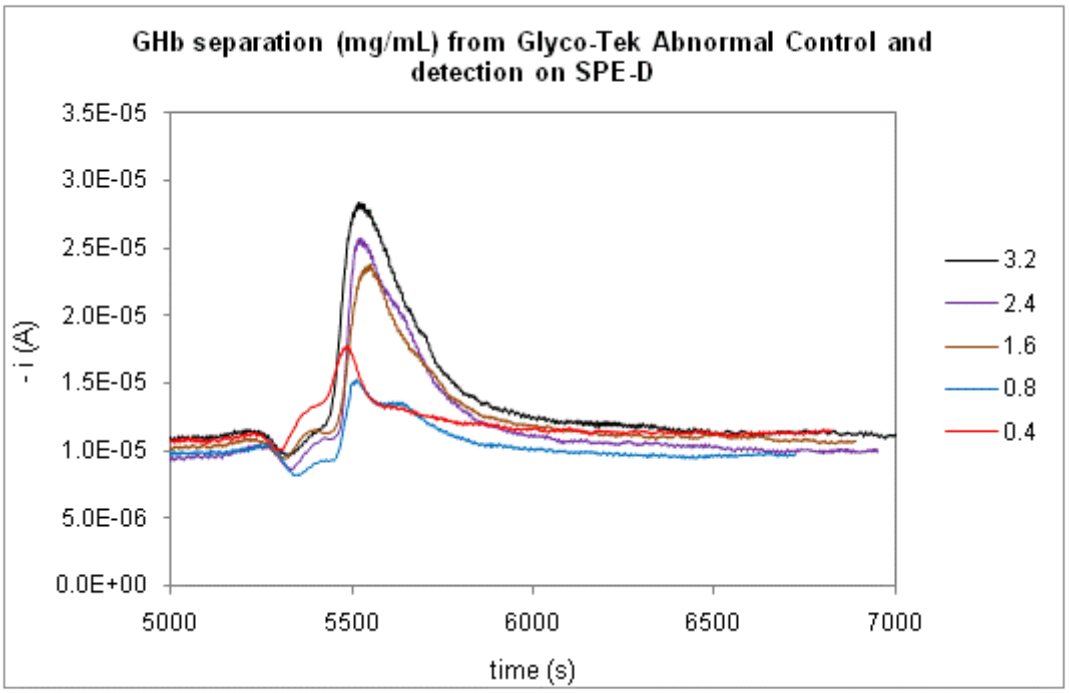

Figure 117. Separation of GHb from non-glycated Hb (Glyco-Tek Abnormal Control) on the affinity column and its electrochemical detection at -400 mV on SPE-D1.

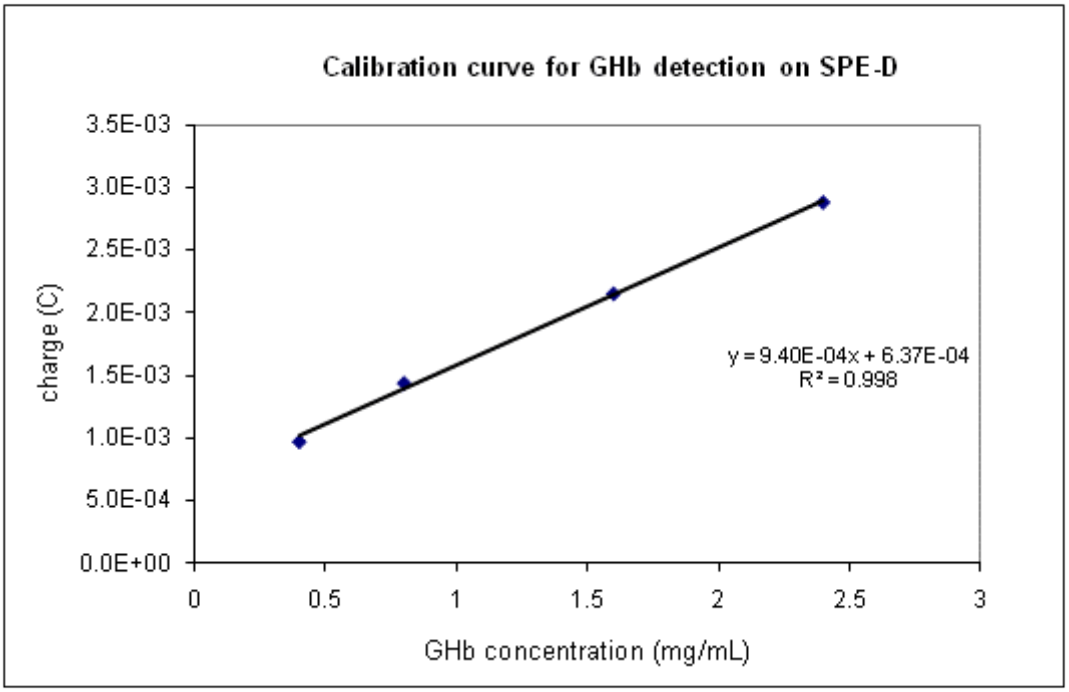

Figure 118. Detection of GHb separated from non-glycated Hb (Helena Abnormal Control) by affinity chromatography combined with electrochemical detection on SPE-D1 at a potential -400 mV in the FIA system.

The chromatogram of blood samples spiked with a known concentration of HbA1c showed a lower current signal compared to the chromatograph of control samples (Figures 118 and 121). It may be attributed to a stabiliser that was presented in Glyco-Tek controls. That could explain a much higher amount of HbA1c (%) in Glyco-Tek Abnormal control samples compared to data provided by the producer (Table 30). The presence of interferences caused by substances such as ascorbic acid, acethaminophen and uric acid could also decrease response of Hb. 5.2 % of HbA1c was determined in blood samples by the multiple standard addition method (Figure 121).

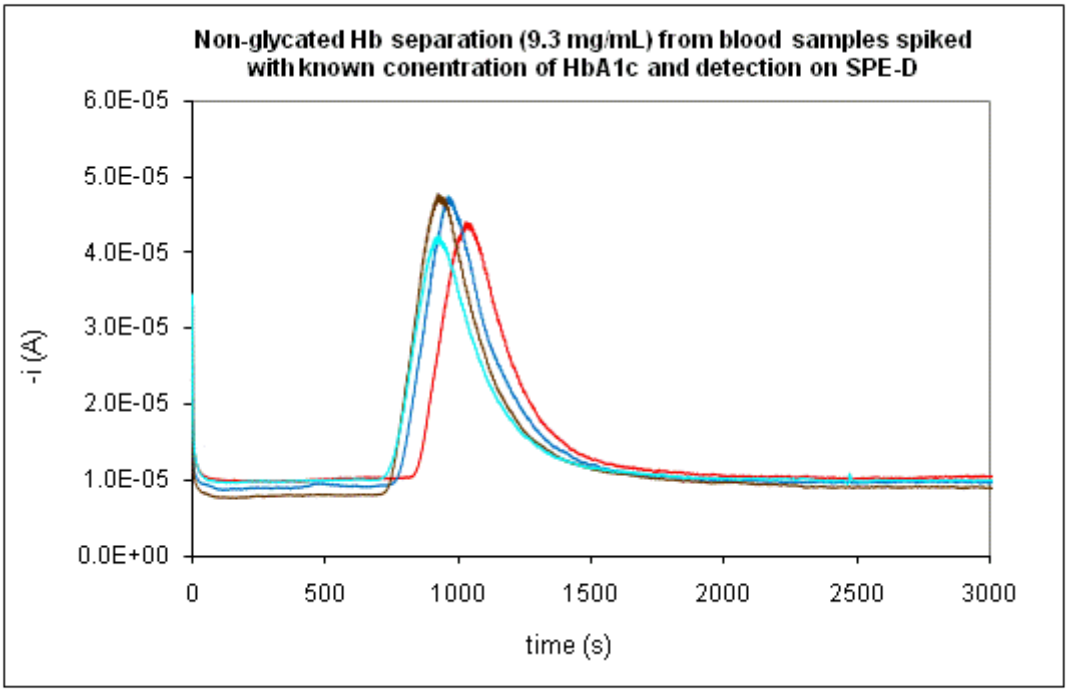

Figure 119**.** Separation of GHb from non-glycated Hb on the affinity column and its electrochemical detection at -400 mV on SPE-D2. Blood samples were spiked with a known concentration of HbA1c.

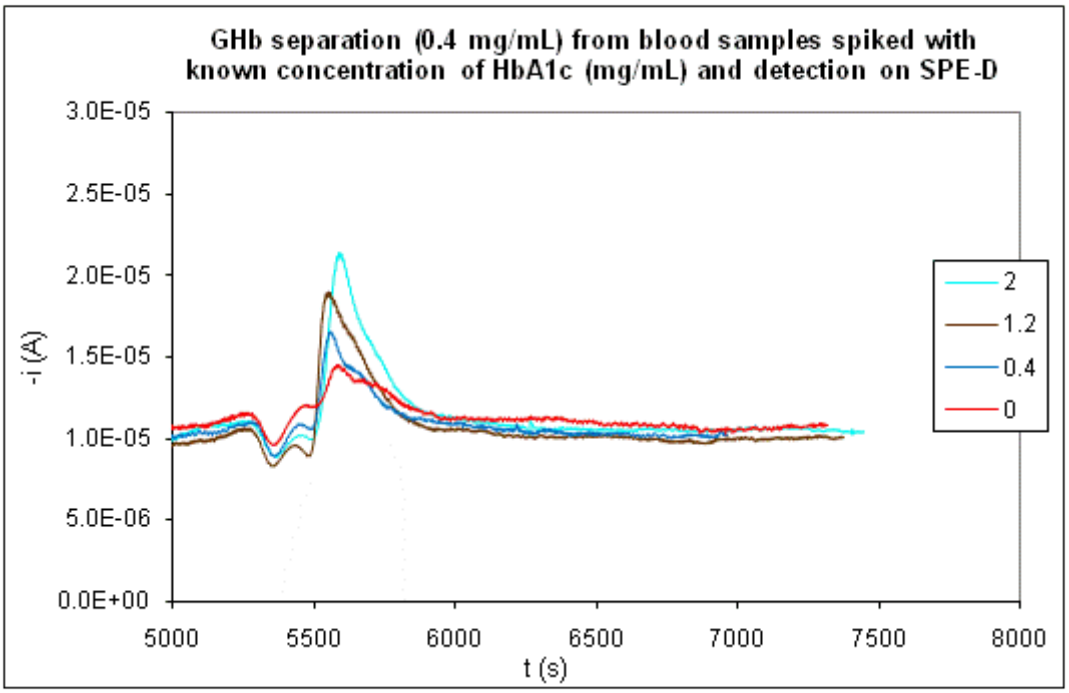

Figure 120. Separation of GHb from non-glycated Hb on the affinity column and its electrochemical detection at -400 mV on SPE-D2. Blood samples were spiked with a known concentration of HbA1c.

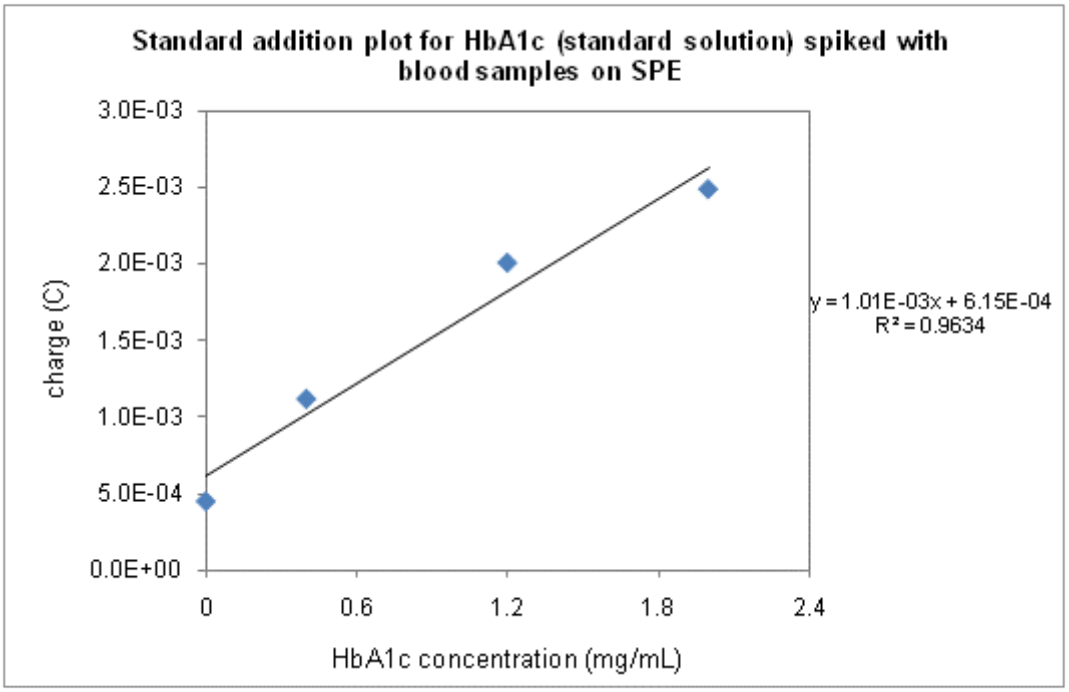

Figure 121. Detection of GHb separated from non-glycated Hb by affinity chromatography combined with electrochemical detection on SPE-D2 at a potential -400 mV in the FIA system. Blood samples containing GHb (0.4 mg/mL) spiked with HbA1c (standard solution).

Chromatographic separations and sensor response was verified against the Glyco-Tek Affinity Column Method and the correlation was obtained with R^2 = 0.9945 (Figure 122). Electrochemical detection was also verified against data provided by the producer of control samples. Helena Normal and Abnormal level analysed by the Glyco-Tek Affinity Column Method and ClinCheck controls verified by HPLC (HPLC RECIPE) or immunologic method (Bayer / Siemens) gave the correlation R^2 = 0.9254 and R^2 = 0.981, respectively (Figures 123 and 124).

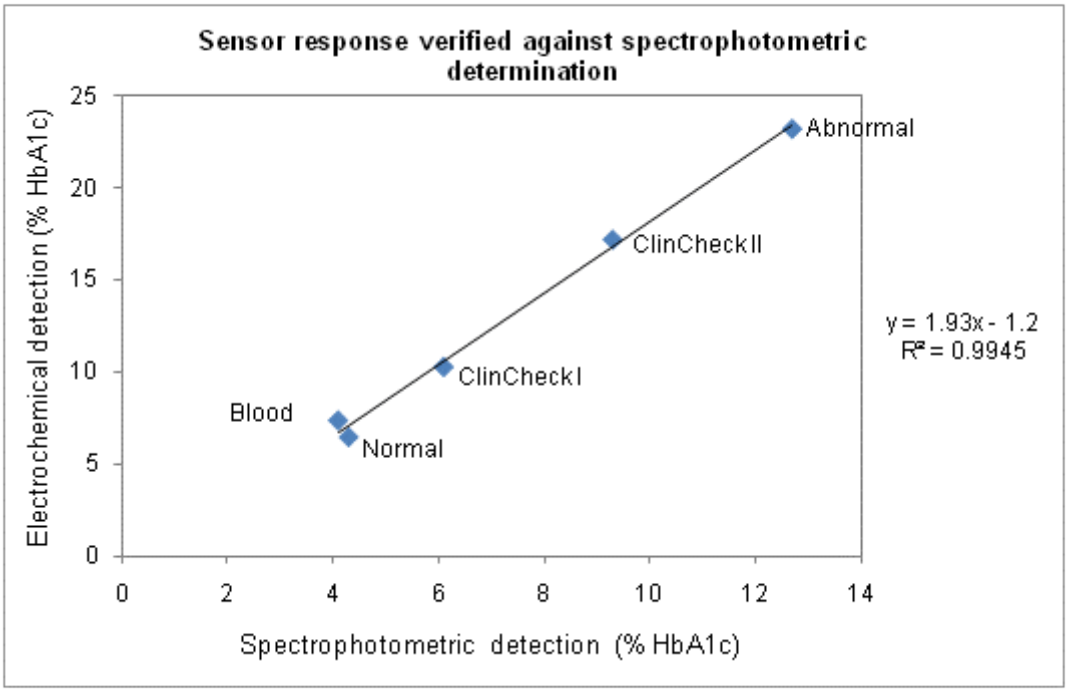

Figure 122. Electrochemical detection of HbA1c verified against spectrophotometric determination. Control samples: Helena Normal and Abnormal level of HbA1c, ClinCheck controls – level I and II and blood samples were analysed.

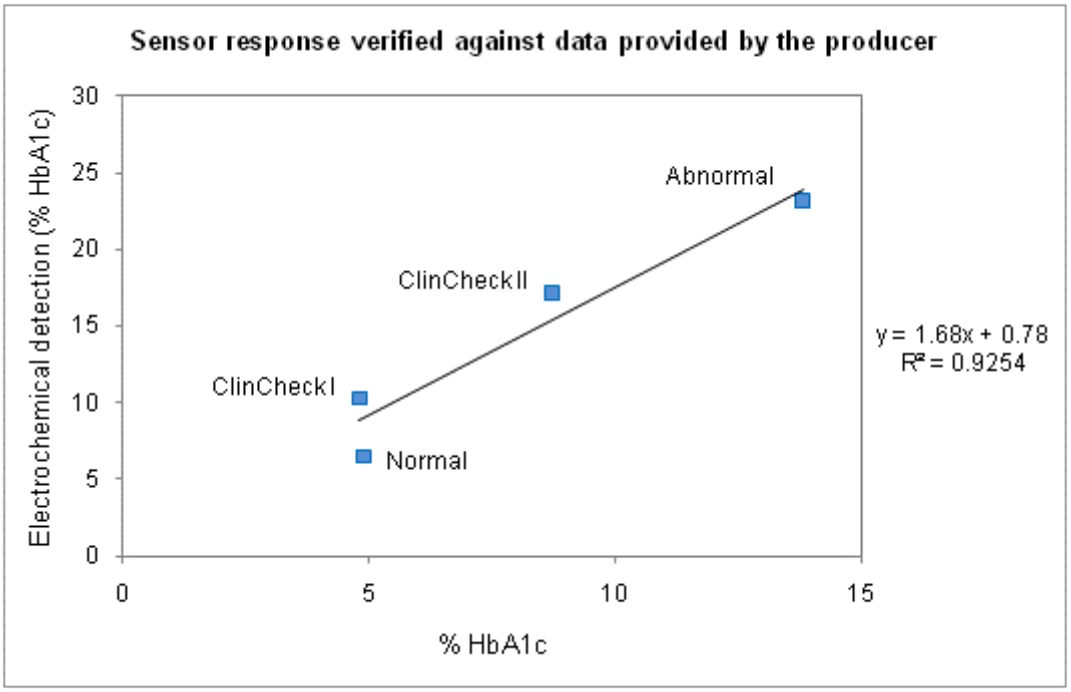

Figure 123. Electrochemical detection of HbA1c verified against data provided by the producer. Control samples: Helena Normal and Abnormal level of HbA1c (GLYCO-Tek Affinity Column Method), ClinCheck controls – level I and II (HPLC RECIPE).

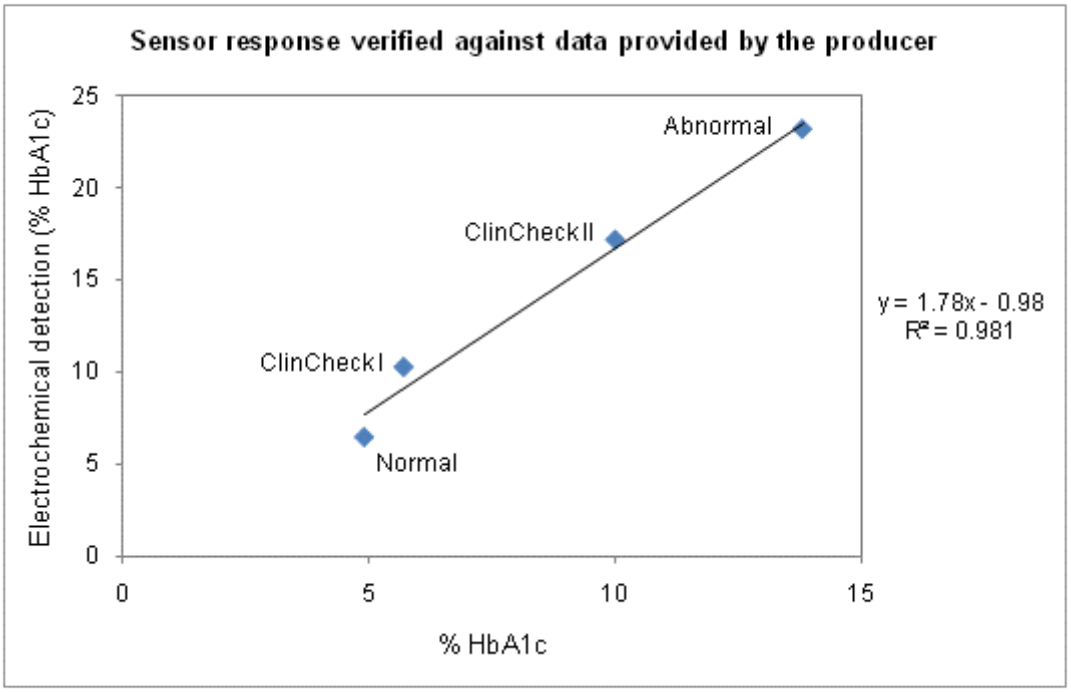

Figure 124. Electrochemical detection of HbA1c verified against data provided by the producer. Control samples: Helena Normal and Abnormal level of HbA1c (GLYCO-Tek Affinity Column Method), ClinCheck controls – level I and II (Bayer / Siemens – immunologic method).

10.3 Conclusions

There is still a necessity to perform more laboratory trials for HbA1c determination, with different level of HbA1c (blood samples) and compare them with validated laboratory reference method that is certified by NGSP (Gallagher *et al*., 2009, http://www.ngsp.org).

However, promising results and a good correlation of studied system as compared with other methods suggest a possibility to construct a novel device for HbA1c determination. So far there is no successful HbA1c sensor on the market based on electrochemical detection. The reason may be a complicated procedure necessary to follow and a high cost of a device. This chapter demonstrated development of a relatively simple system. Comparison of non-glycated haemoglobin and glycated haemoglobin (one injected sample) without requirement of precise collection, dilution and injection of sample may facilitate procedure of HbA1c determination. A device should be easy to use by unqualified person to avoid errors caused by manual operations. This is one of the major problems encountered when point of care devices are used in a busy clinics, for example A1C Now (Metrica) device generated an abnormal value of HbA1c as a result of reduced volume (50%) of blood samples or diluted samples, NycoCard (Axis-Shield) produced inaccurate results for increased volume (50%) of blood samples (John *et al*., 2006). An attempt could be made in the future to use results obtained in this work to design a point-of-care device, a benchtop or a microfluidic device.

Chapter 11: Final conclusions

The main objectives of this project were achieved during research on development of HbA1c sensor. Two approaches for electrochemical detection of haemoglobin were successfully developed. Novel approach based on enzymatic activity of haemoglobin was applied for studies focused on recognition of HbA1c.

Binding of haemoglobin was substantially higher on MIP in comparison to NIP polymer (both prepared on the microplate surface). However, preparation of efficient MIP - based sensor is still a challenge, especially to detect a large, similar protein in a complex medium, such as blood. Therefore boronic affinity materials, which are commonly used in diagnostic of HbA1c, were studied to evaluate a plausible integration with an electrochemical device capable of determining HbA1c in blood samples.

It was possible to distinguish normal and abnormal levels of HbA1c by boronic acid affinity chromatography combined with electrochemical detection in a FIA system. Sensor response showed a good correlation with spectrophotometric detection, and with data provided by a producer of control blood samples which were analyzed by methods, such as HPLC and immunochemistry. The system is relatively cheap, fast and simple, which is required to introduce a device on the market. Determination of HbA1c (%) on the basis of the electrochemical signal of non-glycated haemoglobin and total glycated haemoglobin (GHb) may facilitate the construction of an easier-to-use point of care device. Precise collection of a blood specimen may be not required and the protocol will be easier to follow by non-medical personnel. Point of care devices, currently available on the market are difficult to operate and there is a need to improve sensitivity and repeatability to detect very small variations in HbA1c in blood specimens. In order to yield good results, the affinity column and electrodes must be of high quality.

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APPENDICES

Appendix 1: Cranfield Health Partnership Poster

Cranfield UNIVERSITY

Biosensor / Biomimetic Sensor for Diabetes (HbA1c Nanosensor)

A.A. **Biela**[1], S.A. **Piletsky**[1], D. **Freeman**[2], N. **Bartetzko**[2] and A.P.F. **Turner**[1]
1 Cranfield Health, Cranfield University, Silsoe, Bedfordshire, MK45 4DT, UK
2 Pelikan Technologies Inc., 1072 East Meadow Circle, Palo Alto, CA 94303, USA

Introduction

Diabetes mellitus is growing health problem worldwide. The disease is characterised by insulin deficiency, insulin resistance and hyperglycemia, which can be associated with serious complications leading to premature death. Suitable long-term control and management of the disease are enabled by determination of glycosylated haemoglobin (HbA1c) in blood. The results are given as %HbA1c of total haemoglobin. Presently available tests vary in cost and convenience and there is an identified need to introduce improved equipment for self-monitoring. Therefore, production of an HbA1c sensor or its integration with a glucometer suitable for home use would be highly desirable on the market. This project focuses on developing highly innovative Molecularly Imprinted Polymer (MIP) technology and other selective receptors for the recognition of HbA1c coupled with electrochemical detection methods.

Methods/Materials

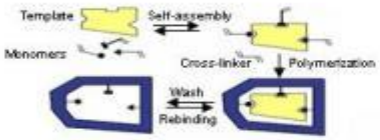


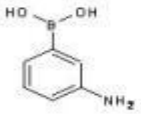
Fig. Schematic process of MIPs[2]

SYNTHETIC RECEPTORS

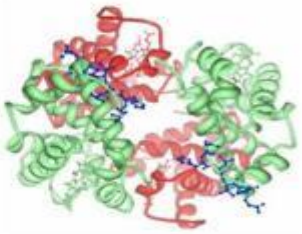
- Molecularly Imprinted Polymers
- Boronic acid derivatives

POLYMER SYNTHESIS – surface grafting[1] / electropolymerization

Monomer – aminophenyl boronic acid (APBA)



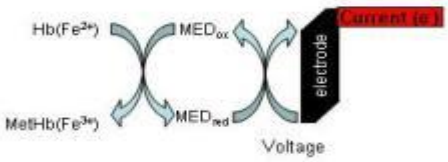
Template – HbA1c
HbA_0
Crosslinker



ELECTROCHEMICAL DETECTION – redox properties / catalytic activity of Hb

MODIFICATION OF ELECTRODE SURFACE

Mediator – TMPD, FcCOOH, ferricyanide
Polymers – polypyrrole, polyaniline
Nanoparticles



DETECTION

- Various forms of voltammetry

REFERENCE METHOD

- HPLC

Conclusions

Initial experiments are focussed on functional monomers suitable for HbA1c recognition. PBA has been reported to have good affinity to HbA1c and a three-dimensional MIP has already been successfully prepared at Cranfield University. Its integration with an electrochemical transducer should lead to a fast and simple analytical device.

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2. www.miptechnologies.se
3. www.rcsb.org./pdb/explore.do?structureId=1GZX

www.cranfield.ac.uk

a.biela.s06@cranfield.ac.uk

Appendix 2: Ethical Approval Forms

Cranfield UNIVERSITY

Cranfield Health Research Ethics Committee
Approval Form Frontispiece

CHREC Ref: 08/07 .

Principal Investigator: Anna Biela
Title of Study: Biosensor / Biomimetic sensor for diabetes (HbA1c nanosensor).

Before submitting the ethics approval form, please ensure you have completed all sections and provided all supporting documents.

The following list should be checked, completed and submitted with your application:

Document	Enclosed? Delete as applicable	Version/date	Checked by CHREC
Completed Approval Form	Yes	Version 1 August 2006	
Full Study Protocol	Yes	Version 1 September 2007	
Volunteer Information Sheet*	Yes	Version 1 September 2007	
Volunteer Consent Form*	Yes	Version 1 September 2007	
Invitational Letters/E-mails*	Yes	Version 1 September 2007	
Investigator Signatures	Yes	Version 1 September 2007	

* - must be presented on headed paper

Checked by:

Principal Investigator

Signature  Date 04.09.2007

Administrator

Signature_____ Date_____

Cranfield UNIVERSITY

Cranfield Health Research Ethics Committee Approval Form

(Please read the guidance document 'CHREC Application Process and Requirements for Approval' carefully before completing this form)

Project Reference No [ ] (To be filled in by committee)

Title of Project

Biosensor / Biomimetic sensor for diabetes (HbA1c nanosensor).

Principal Investigator Student
Name Anna Biela

Department Cranfield Health

E-mail a.biela.s06@cranfield.ac.uk Telephone +44(0)1525 863771

Investigator 2 Staff

Name Prof. S. Piletsky

Department Cranfield Health

E-mail s.piletsky@cranfield.ac.uk Telephone +44(0)1525 863584

Investigator 3 Staff

Name Prof. A.P.F Turner

Department Cranfield Health

E-mail a.p.turner@cranfield.ac.uk Telephone +44(0)1525 863006

External Collaborator / Investigator

Name

Address

E-mail Telephone

NOTE: In case of student projects, please indicate which of the above are the project supervisor(s)

What is the proposed start date and duration of this project?

Start date: September 2007

Project duration:.....months.....3.....years

Section 2 - Recruitment

Volunteer population

Where are volunteers going to be recruited for this study?

Internally / Externally (highlight in bold as appropriate)

If samples are to be collected internally, please state which departments are to be approached and whether staff, students or both will be approached.

Department(s) involved: Cranfield Health

Staff Students **Staff and students** (highlight in bold as appropriate)

If samples are to be collected externally, please state who is involved and, in the case of NHS, which ethics committee will be approached for approval.

External bodies involved:

NHS REC for ethical approval:

Cranfield UNIVERSITY

Recruitment methods

Please describe how recruitment will be undertaken, naming those who will be responsible for each step involved.

An e-mail will be sent to all staff and students of Cranfield Health requesting volunteers for this study. The specific email will contain information about the principal investigator and her research, as well as some basic information about the specific study. Moreover, Anna Biela will give a verbal explanation to the interested volunteers, including the fact that the samples will be anonymous. The volunteers will be kindly asked if they are interested to contact Anna Biela.

Will volunteers be paid for taking part in this study? No

If yes, state how much and when volunteers will be informed of payment:

NOTE: You must include a copy of invitations (e-mails / letters) to be used for recruiting within this study along with your volunteer information sheet

Consent process

Who will be responsible for taking consent?
Anna Biela

How will consent be taken?
Consent for participation in the study will be taken prior to any of the procedures being undertaken – this will be written consent. All participants will always have at least 24 hours between being invited to participate and being asked for consent.

If there will be less than 24 hours between inviting people to participate and asking for consent, please justify:

NOTE: You must include a copy of your consent form

Section 3 – Sample / volunteer requirements

This study involves:

Sample collection (*e.g.* blood, urine) [Complete sub-section (ii)]

Volunteer population

State any specific inclusion or exclusion criteria for this study:

Inclusion criteria: Adults aged over 18

Exclusion criteria: NONE

Interventions / sample requirements / other

Sub-section (i): Intervention / activity

What will the volunteers be asked to do? Include all measures for minimising risk in this activity.

N/A

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Sub-section (ii): Sample collection

Sample requirements

List the sample types required, including the number of samples. Indicate whether single or multiple samples will be taken from volunteers.

Sample type	Total number	Single	Multiple (+ number)
blood	10 samples (of 20mL each)	Y	

If multiple samples are required, how frequently will they be taken?

Sample use

How will the samples be collected?

Collected by a medical professional at the Medical Centre, At Cranfield University, in Silsoe

Will the samples be anonymised? Yes
If no, please state why.

How and where will the samples be stored?

The blood samples will be kept in freezers at -20 C, in the laboratory of Molecular Biology.

How will the samples be used?

The samples will be used to investigate the electrochemical behaviour of haemoglobin. As a consequence, an isolation and purification of haemoglobin from the provided samples will be done. Haemoglobin will be determined by spectrophotometry.

What will happen to any samples remaining at the end of the study? Who will be responsible for destruction of the samples?

All samples will be destroyed at the end of the study by the principal investigator

NOTE: You must include in your protocol all measurements etc. which will be undertaken with the samples, including any external measurements

Sub-section (iii): Other

What will volunteers be required to do?

N/A

NOTE: You must include copies of any questionnaires etc to be used

How long will patient involvement be within the study as a whole?
Patients will be involved for a single one time sample donation

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Section 4

Data Protection

Who will be responsible for storage of consent forms and all personal data relating to this study?

Anna Biela
Consent forms and all personal data will be stored in locked cabinet.

If data is to be stored electronically, where will it be stored (please remember electronic data must be stored in a password protected file)?

Data will be stored electronically on the personal computer in a password protected file.

Training requirements

Do you think that you have any specific training requirements before undertaking this study?

None

Special Issues

Are there any specific ethical issues relating to this study?

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N/A

Signatures

Principal Investigator..... Date 04.09.2007

Investigator 2..... Date 5.09.2007

Investigator 3..... Date.....

External Investigator..... Date.....

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Volunteer Information Sheet

1. Study Title

Biosensor / Biomimetic sensor for diabetes (HbA1c nanosensor).

2. Invitation paragraph

You are being invited to take part in a research study. Before you decide whether to take part it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. There are two sections to this information:

- Part 1 tells you the purpose of this study and what will happen to you if you take part.
- Part 2 gives you more detailed information about the conduct of the study.

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this.

3. What is the purpose of the study?

The purpose of this study is to design and optimise a sensor for a fast and straightforward determination of glycosylated haemoglobin.

4. Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time, without giving a reason and any samples collected will be immediately destroyed. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

5. What will happen to me if I take part?

If you decide to take part you will be asked to sign a consent form for this study and provide a blood sample of 20 mL

6. What do I have to do?

We ask you to read this information and take your time in deciding if you want to be part of this study. If you agree to take part you will be asked to sign a consent form. Then a blood sample will be taken. All information given is anonymised, only linked to your sample and not to any personal details.

7. What are the side effects of taking part?

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Taking a blood sample may cause slight discomfort and sometimes you can get localised bruising around the area from which the sample was obtained.

8. What are the other possible disadvantages and risks of taking part?
There are no possible disadvantages or risks associated with taking part in this study.

9. What are the possible benefits of taking part?
There is no direct benefit to you.

10. What happens when the research study stops?
All samples allocated for this study will be destroyed and the results will form the basis of a scientific thesis and may generate scientific publications.

11. What if there is a problem?
Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information of this is given in Part 2.

12. Will my taking part in this study be kept confidential?
Yes. All the information about your participation in this study will be kept confidential. The details are included in Part 2.

13. Contact Details:
Anna Biela, a.biela.s06@cranfield.ac.uk

Part 2

14. What if there is a problem?
If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain about any aspects of the way you have been approached or treated during the course of this study, please contact Prof. Joe Lunec, Head of Cranfield Health.

15. Will my taking part in this study be kept confidential?
All information that is collected about you during the course of this research will be kept strictly confidential. All the samples will be analysed anonymously.

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All procedures for handling, processing, storage and destruction of your data are compliant with the Data Protection Act 1998.

16. What will happen to any samples I give?
Any samples provided will be stored in one of the laboratories of Cranfield University, Silsoe, until used. Haemoglobin will be isolated from samples and used for the study.

17. Will any genetic tests be done?
No genetic testing will be undertaken with the samples collected for this study.

18. What will happen to the results of the research study?
All results obtained during this study will form the basis of a scientific thesis, reports for the EU and may generate scientific publications

19. Who is organising and funding the research?
The research is being organised Cranfield Health and funded by Pelikan Technologies

20. Who has reviewed the study?
The study has been reviewed by: Cranfield Health Research Ethics Committee

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CONSENT FORM

Title of Project: Biosensor / Biomimetic sensor for diabetes (HbA1c nanosensor).

Name of Researcher:

Please initial box

1. I confirm that I have read and understand the information sheet dated September 2007 (version 1) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my legal rights being affected. ☐
3. I agree to take part in the above study. ☐

Name of Volunteer	Date	Signature
Name of Person taking consent	Date	Signature

When completed, 1 for volunteer; 1 for researcher site file

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Full Study Protocol

Invitation e-mail sent out.

⇩

Information provided to volunteers and explained by the principal investigator

⇩

Arrange date for sample collection

⇩

Consent given on day of sample collection by the principle investigator

⇩

Blood sample collected by the medical professional

⇩

A total volume of 200mL blood sample will be collected. (20mL from each volunteer)

⇩

Blood sample transferred and stored at -20C, in the Molecular biology lab of Cranfield Health

⇩

Initially haemoglobin isolation and purification from blood samples

⇩

Haemoglobin determination by electrochemical and spectrophotometrical measurements

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Dear All,

My name is Anna Biela and I am one of Sergey Piletsky's PhD students, working on sensors for haemoglobin detection. In one of my future experiments, it will be necessary to use haemoglobin from the human blood and to test this form in comparison to the form of haemoglobin commercially available. Therefore, an assay with 10 different samples from 10 volunteers would be ideal. It is worthy to note that all samples will be anonymous. Experiment will include isolation of haemoglobin and its purification. It would be much appreciated if you could kindly donate only a small amount of blood samples (20 ml) in order to help my research in diabetes monitor and control. Please contact me at a.biela.s06@cranfield.ac.uk or at 01525863771 if you are interested and willing to help.

The blood samples will be collected by a medically qualified person to ensure the lowest risk. Many thanks in advance.

Best regards,
Anna

Investigational e-mail, version 1, June2007

Anna Biela
28.09.2007

Cranfield Health Ethics Committee,

I will explore my project: Biosensor / Biomimetic sensor for diabetes (HbA1c nanosensor), Reference No 08/07 alongside the project: Mechanistic Studies on Ultrafast Eletrochemical Nanobiosensors on Diabetes, Reference No 04/07 by using the same type of sample – 10 samples (of 10ml each).

Yours faithfully,

Anna Biela

Appendix 3: Value of HbA1c determined by different methods in ClinCheck controls

ClinChek® - Control
Diabetes Control, Level I, II
Diabeteskontrolle, Level I, II

Order No./Best.-Nr.: 11092
Lot No./Ch.-B.: 818
Exp. Date/Verfallsdatum: 05/2010

Constituent / Bestandteil	Method of Analysis/ Analysenmethode	Unit/ Einheit	Mean Value/ Sollwert	Control Range/ Kontrollbereich
Hemoglobin A1c / Hämoglobin A1c	Bayer / Siemens (immunologic) / Bayer / Siemens (immunologisch)			
	Level I	%	5.7	5.2 - 6.2
	Level II	%	10.0	9.2 - 10.8
	Beckman (immunologic) / Beckman (immunologisch)			
	Level I	%	5.1	4.6 - 5.6
	Level II	%	9.2	8.4 - 10.0
	BioRad (HPLC)			
	Level I	%	5.5	5.0 - 6.0
	Level II	%	8.9	8.1 - 9.7
	Dade Behring / Siemens (immunologic) / Dade Behring / Siemens (immunologisch)			
	Level I	%	5.5	5.0 - 6.0
	Level II	%	9.1	8.3 - 9.9
	Menarini (HPLC)			
	Level I	%	4.9	4.4 - 5.4
	Level II	%	9.4	8.6 - 10.2
	Olympus (immunologic) / Olympus (immunologisch)			
	Level I	%	5.3	4.8 - 5.8
	Level II	%	10.1	9.3 - 10.9
	RECIPE (HPLC)			
	Level I	%	4.8	4.3 - 5.3
	Level II	%	8.7	7.9 - 9.5
	Roche (immunologic) / Roche (immunologisch)			
	Level I	%	5.3	4.8 - 5.8
	Level II	%	9.6	8.8 - 10.4
	Tosoh (HPLC)			
	Level I	%	5.1	4.6 - 5.6
	Level II	%	9.6	8.8 - 10.4
	IFCC-Reference-Method* / IFCC-Referenzmethode*			
	Level I	mmol/mol	31.1	30.9 - 31.3
	Level II	mmol/mol	76.9	74.7 - 79.1

*: These values (IFCC-calibrated) were obtained in the reference laboratory of Instand e.V., Düsseldorf. The other values (DCCT-calibrated) were derived from an external quality assessment in July 2008.

Diese Werte (IFCC-kalibriert) wurden im Referenzlabor von Instand e.V. in Düsseldorf ermittelt. Die anderen Werte (DCCT-kalibriert) wurden aus einem Ringversuch im Juli 2008 abgeleitet.

Caution / Achtung:
In Germany Hb A1c is subject to the guideline of the German Medical Council for quality assurance. For assessment of the internal quality control, please also refer to table B 1a of the guideline.

Hb A1c unterliegt in Deutschland der Richtlinie der Bundesärztekammer zur Qualitätssicherung laboratoriumsmedizinischer Untersuchungen. Zur Bewertung der internen Qualitätskontrolle beachten Sie bitte auch Tabelle B 1a der Richtlinie.

RECIPE
CHEMICALS + INSTRUMENTS GmbH Labortechnik
Dessauerstr. 3 D-80992 Munich / Germany
Tel.: +49 / 89 / 54 70 81 - 0 Fax: +49 / 89 / 54 70 81 - 11
Internet: www.recipe.de e-mail: info@recipe.de

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