Electrochemical Probing of Selective Hemoglobin Binding in Hydrogel-based Molecularly Imprinted Polymers..

Subrayal M Reddy*, Giulia Sette and Quan Phan
Chemical Sciences Division, Faculty of Health and Medical Sciences,
University of Surrey, Guildford, Surrey, GU2 7XH, UK.

* Corresponding author
Tel : 01483 686396 Fax: 01483 686401
e-mail: s.reddy@surrey.ac.uk

Abstract

An electrochemical method has been developed for the probing of hydrogel-based molecularly imprinted polymers (HydroMIPs) on the surface of a glassy carbon electrode. HydroMIPs designed for bovine haemoglobin selectivity were electrochemically characterized and their rebinding properties were monitored using cyclic voltammetry. The electrochemical reduction of bovine oxyhaemoglobin (BHb) in solution was observed to occur at -0.460 V vs (Ag/AgCl) in 150mM phosphate buffer solution (PBS). When the protein was selectively bound to the MIP, the electrochemical reduction of oxyhameoglobin could be observed at a similar peak potential of -0.480 V vs (Ag/AgCl). When analysing the non-imprinted control polymer (NIP) interfaced at the electrode, which contained no protein, the peak reduction potential corresponded to that observed for dissolved oxygen in solution (-0.65 V vs (Ag/AgCl)). MIP and NIP (in the absence of protein) were interfaced at the electrode and protein allowed to diffuse through the polymers from the bulk solution end to the electrode. It was observed that whereas NIP exhibited a protein response within 10 minutes of protein exposure, up to 45 min of exposure time was required in the case of the MIP before a protein response could be obtained. Our results suggest that due to the selective nature of the MIP, BHb arrival at the electrode via diffusion is delayed by the MIP due to attractive selective interactions with exposed cavities, but not the NIP which is devoid of selective cavities.

Keywords: Hydrogel; MIP; protein; biomimetics; electrochemistry

1. Introduction

Molecularly imprinted polymers (MIPs) are being extensively used as synthetic polymer-based receptors or as artificial antibodies, because of their ability to mimic natural systems. However, when compared to natural recognition products, they offer several advantages such as robustness, specificity, reusability and cost-effectiveness [1]. They also exhibit resistance to extreme conditions, like high temperature, acidic and basic environments, and therefore lend themselves to engineering possibilities compared with biological counterparts. Synthetic design in molecular imprinting also allows the creation of a recognition system for an analyte for which a natural receptor may not exist, making MIP materials suitable for a wide range of applications. MIPs have been used, for example, in chromatographic applications, solid phase extraction, catalysis and in MIP-based sensors for the detection of active molecules, pharmaceuticals and pollutants. Such sensors identify and quantify target molecules by converting the binding between the analyte of interest and the MIP into a readable signal [2].

To date, many publications have reported the imprinting of small molecules, such as drugs [3], sugars [4], nucleotides [5] and pesticides [6]. Currently, macromolecular imprinting is gaining momentum but is still a challenge. Protein recognition is of particular interest for biomedical and diagnostic applications. However, it presents a series of issues: when large molecules are used as templates, it is more difficult for them to penetrate the polymer network and occupy the complementary binding sites; subsequently, vacating the binding site can also be problematic; their large size and structural complexity leads to more non-specific binding sites and, consequently, to a poor recognition behaviour displayed by the MIPs; additionally, when dealing with biomacromolecules, such as proteins, the imprinting conditions need to be compatible with maintaining the quaternary structure of the protein, to ensure that their binding activities and conformations are not lost [7]. Therefore, synthesis of MIPs in organic solvents is not a viable option for proteins in general, as the two are incompatible. However, synthesis in aqueous media can be problematic as well, with the water solvent reducing the binding strength of the non-covalent interactions between template and monomer, which is crucial for the production of the imprinting effect [1]. All the above

factors and the high cost of pure proteins make effective and practical protein imprinting difficult. Different approaches are available for the imprinting of polymers with proteins, depending on the portion of protein involved in the process as template (i.e. the whole protein or part of it) [7].

Bulk imprinting is the most straightforward methodology for protein imprinting, with the advantage that the whole protein can be extracted or washed out from the matrix, whilst retaining the ability to rebind into the cavities within the MIP. Protein movement is usually facilitated by the low density of the polymer network.

Hydrogel polymer materials constitute a more suitable matrix for bulk imprinting of proteins. They are cross-linked polymers that swell upon contact with water, as a result of hydrophilic interactions between the template and the polymer [7].

In the presence of high molecular weight substances, such as proteins, a successful method to produce gels with high selectivity towards the protein of interest was found to be based on the polymerisation of a non-charged monomer, like acrylamide, and cross linking agent, such as *N,N'*-methylenebisacrylamide, that together form polyacrylamide [8]. The main functions of a cross-linking agent are to control the morphology and improve the mechanical stability of the polymer, as well as stabilising the binding sites [9].

Haemoglobin is a vital protein and mutations in genes that encode for the protein's subunits result in hereditary diseases, such as sickle cell anaemia, thalassaemia and haemoglobinopathies. These are diagnosed only after complicated and time-consuming procedures have been carried out. MIPs could offer a new method for the screening, diagnosis and monitoring of haemoglobin disorders, that is rapid, sensitive and selective [1]. Haemoglobin molecules consist of four polypeptide chains: two identical α chains of 141 amino acid residues each and two identical β chains of 146 residues. The α and β chains have different amino acid sequences but fold in similar three-dimensional structures. Each chain comprises one haem group (a porphyrin group with an atom of iron at its centre). A single polypeptide chain combined with a single haem group is a subunit of haemoglobin. In the complete molecule, these subunits form a pair of αβ dimers ($\alpha_1\beta_1$ and $\alpha_2\beta_2$) that associate together to obtain a tetramer. In horse, man and

many other mammals haemoglobin has a molecular weight of 64500 g/mol [10, 11]. Its diameter is 5.5 nm and its isoelectric point (pI) is 7.1 [1].
Oxygen is transported by haemoglobin to the tissues that most need it. This is possible because rapidly metabolising tissues generate large amounts of hydrogen ions and carbon dioxide and, at the same time, the affinity of haemoglobin for oxygen is pH dependent. In fact, above pH 6.0, increasing pH value results in an increased oxygen affinity of the protein. From values of pH of 7.4, the oxygen affinity of haemoglobin deceases as pH decreases, allowing the protein to release oxygen more readily in response to higher levels of protons and carbon dioxide [10].

Cyclic voltammetry offers a viable, non invasive method for the characterisation of highly hydrated soft materials, such as gels. In fact, it can allow us to measure the diffusivity of molecular probes through the material [12]. In this paper, we demonstrate the electrochemical characterization of hydrogel-based molecularly imprinted polymers for oxyhaemoglobin based on the electrochemical activity of the template molecule.

2. Experimental

Acrylamide, methylenebisacrylamide, ammonium persulphate, tetramethylethyldiamine, sodium dodecyl sulphate (SDS) and bovine haemoglobin (BHb) were all purchased from Sigma (Poole, UK). Phosphate buffered saline (PBS) tablets were purchased from Oxoid (Basingstoke, UK), whilst acetic acid (AcOH) was purchased from Fisher Scientific (Loughborough, UK).

2.1 MIP and Non-Imprinted Polymer (NIP) Gel Preparation

Fifty-four milligrams of acrylamide and 6 mg of methylenebisacrylamide were dissolved each in 0.5 ml of de-ionised water, in separate containers, and vortexed to aid their dissolution. The two solutions were then added to 12 mg of BHb, vortexing after each addition. Volumes of 10 µl of 10% (w/v) ammonium persulphate, the initiator, and of 20 µl of 5% (v/v) tetramethylethyldiamine, the catalyst of the reaction, were added and the solution was vortexed at each addition. The solution was then deoxygenated by purging with nitrogen for 5 min and left to polymerise overnight at room temperature, in order to

obtain a material with the consistency of a gel. A non-imprinted polymer (NIP), used for control purposes, was made in an identical manner but in the absence of the BHb template.

2.2 Characterisation of Gels

Once polymerised, the gels were passed through a 75 μm sieve (Endecotts Ltd., London, UK). The granulated gels were each washed with 2 ml volumes of de-ionised water, centrifuged and the supernatant fractions were collected for analysis. This process was repeated for four further times and was followed by the elution of the template, using five 2 ml volumes of 10% (w/v):10% (v/v) SDS:AcOH. The combination of SDS and AcOH has a significant effect on the template removal. SDS is known to form micelles at concentrations above 8mM. Since the overall solution's pH is 2.8, which is much lower than the pI of Hb, SDS interacts with a positively charged protein. This results in the protein being attached and wrapped around the negatively charged surface of the SDS micelles. In this way, the template protein could be therefore removed from the polymer. It is likely that the micelles cannot physically penetrate into the imprinted cavities, however the close contact between the micelle and the charged protein in the cavity still allow for favourable electrostatic interactions that will cause the protein to unravel. The acetic acid, as well as lowering the pH of the solution, also disrupts intermolecular hydrogen bonds within the protein molecule. This helps further in the denaturation of the protein.

After the elution washes, the gels were washed with four 2 ml volumes of de-ionised water in order to remove any SDS:AcOH left in the polymer and the supernatants were discarded. The wash and elution steps were carried out using the Centaur II centrifuge (Fisher Scientific, Loughborough, UK) at 3000 rpm for 5 min.

The reload step was performed by adding 2 ml of a 3 mg/ml solution of BHb, per ml of gel, and by allowing the protein to associate with the imprinted gel for 10 min. The resulting solution was centrifuged at 3000 rpm for 5 min and the supernatant discarded. The wash (four 2 ml volumes of de-ionised water) and elution (five 2 ml volumes of 10% (w/v):10% (v/v) SDS:AcOH) steps where then repeated [1]. The same procedure was applied to a different batch of NIP and MIP gels using a 150mM solution of phosphate

buffered saline (PBS), instead of de-ionised water. MIPs and NIPs of different loading state were prepared as shown in Table 1 and used in subsequent electrochemical characterization studies.

2.3 Electrochemical Characterisation of MIP and NIP Hydrogels

The electrolyte buffer, PBS was analysed at different pH values: 3.1, 5.0, 6.9, 7.3, and 8.5. The pH of PBS is normally around 7.3. It was adjusted by addition of 1M HCl, to obtain lower values of pH, or by addition of 100 mM NaOH, for higher pH values. Solutions of PBS at various pH values were used to make up BHb solutions at a concentration of 10 mg/ml, which were then analysed on an Autolab II potentiostat/galvanostat system (Utrecht, Netherlands).

The method was optimised to give the following conditions: bare glassy carbon (GC) working electrode, Ag/AgCl reference electrode (stored in 3M KCl when not in use), platinum counter electrode, 100 mV s^{-1} scan rate, and the applied potential swept between 0 and -0.9 V vs (Ag/AgCl). The equilibration time was 5 min. The equilibration time is necessary to equilibrate the modified electrode with the solution in the cell. Modifications to the working electrode were not needed. All electrochemistry experiments were conducted under ambient air conditions, as oxygen presence was necessary to characterise the oxy form of the haemoglobin.

Electrochemical characterisation of NIP and MIP hydrogels at various protein loading states (see Table 1) were also carried out in PBS solution at the normal buffer pH of 7.4. The glassy carbon electrode was modified by separately layering 20 mg of each granulated gel on the electrode. The thin film of gel was held in place at the electrode with a nylon netting and dialysis membrane (The Scientific Instrument Centre Ltd., Winchester, UK). The dialysis membrane cut off was, in this instance, 35 kDa, meaning that only molecules with a molecular weight lower than this can pass through the pores of the membrane. Therefore, only the conductive ions present in the buffer solution were able to reach the gel and the electrode, whilst the Hb could not diffuse through the membrane and be lost to the bulk solution. This was particularly advantageous for the characterisation of the MIP and NIP polymers. Water tight clamping of the netting and membrane onto the glassy carbon electrode shaft was achieved by using a rubber “o-ring”.

2.5.3 Rebinding Study

Eluted NIP and MIP gels (i.e. NIP3, NIP5, MIP 3 and MIP 5 in Table 1) were also analysed on the CV system, in order to study the rebinding of BHb in the cavities of the MIPs. The glassy carbon electrode was modified by placing a thin layer of granulate MIP or NIP gel (20 mg) directly onto the electrode. The gel was again held in place at the electrode with a nylon netting but this time covered over with a 0.8 μm pore size polycarbonate membrane (Osmonic Inc., Minnetonka, USA) with an additional nylon netting placed on top and the construction was held at the glassy carbon electrode with the aid of a rubber 'O' ring. The polycarbonate membrane was chosen because its pores are small enough to retain the gel (75 μm particle size) and, at the same time, big enough to allow BHb in solution to diffuse through.

The modified electrode was firstly placed in a solution of PBS, at its normal pH of 7.3, and analysed after a 20 min period of equilibration. Subsequently, a 10 mg/ml solution of BHb in PBS was placed in the cell. Cyclic voltammograms were obtained at 5 minute intervals for 40 min, and the solution stirred in between measurements.

3. Results & Discussion

Haemoglobin is expected to produce an electrochemical signal because of the four Fe containing haem active centres per protein molecule. However, the extended three-dimensional structure results in the inaccessibility of the electroactive iron centres. It can therefore be difficult for Hb to undergo heterogeneous electron transfer. As a result, no detectable current appears at conventional electrodes ([13, 14]). However, denaturation of Hb results in conformational changes allowing the haem groups to be electrochemically reduced at glassy carbon electrodes [15].

Since denaturation of proteins can be caused by both acidity and alkalinity, it was therefore important to determine the effects of the pH on the signal produced by the redox reaction of BHb at the electrode. The data could be used to corroborate the hypothesis that the template molecule undergoes an electrochemically detectable conformational change (similar to that induced by pH change) during the process of binding selectively within the imprinted sites.

3.1 pH Dependency of Haemoglobin Electrochemical Signal

The cyclic voltammetry results obtained from the analysis of 10 mg/ml BHb solutions in PBS, at pH 7.3, are compared with PBS alone and are shown in Fig 1a. The signal for PBS alone at different pH values (between 3.5 and 8.5) was found to be consistent at around -0.65 V. This cathodic peak is due to electrochemical reduction of oxygen dissolved in PBS. As expected, when PBS was deoxygenated by bubbling pure nitrogen into the cell, the peak for oxygen reduction disappeared.

Addition of BHb caused a decrease in peak reduction potential, proving that a signal for the protein in solution can be obtained (Fig 1b). A cathodic peak current, but no anodic peak, was observed, in agreement with the results reported by X. Chen et al. (1998). The signal is a consequence of the direct electrochemical reduction of bonded oxygen in oxyhaemoglobin [16]. The cathodic peak potentials were found to be around -0.410 V vs (Ag/AgCl), which is close to values previously reported for the electrochemical response of Hb ([17, 18]). The positive increase in peak potential is because the haemoglobin bound oxygen is more susceptible to electrochemical reduction compared with free oxygen in solution due to an electrocatalytic process. The peak reduction potential for

haemoglobin bound oxygen changed with different pH values: in general, the lower the pH, the less negative the potential for reduction. In fact, the BHb solution at pH 3.1 had the lowest reduction potential (-0.374 V vs (Ag/AgCl)). However, the signal for the BHb solution at pH 5.0 was similar to that produced by the solution at pH 6.9, with reduction peaks detected at -0.417 and -0.427 V vs (Ag/AgCl) respectively. This could be due to changes in the conformation of the protein under different conditions of pH allowing more or less access to the electrode of the oxygen bound to the protein. Also, it was found that the cyclic voltammogram of BHb in a solution at pH 7.3 was similar to that of BHb in a solution at pH 6.9, with the two signals almost completely overlapping (at around -0.44V vs (Ag/AgCl)) with protein at pH 7.3 exhibiting a slight increase in peak current value compared with pH 6.9. At pH 8.5, BHb has a similar reduction potential (-0.449 V vs (Ag/AgCl)). However, it was noted that the current output was larger than at any other pH value. Although a larger signal was obtained at pH 8.5, PBS at pH 7.3 was selected as the supporting electrolyte in subsequent experiments, given that Hb sensors would be normally used under physiological conditions. A similar overall shift in bioelectrocatalytic wave for oxygen reduction with pH was also observed by Gao et al. [19] who showed that the peak reduction potential (vs SCE) shifted from -500mV to -300mV (ie -455mV to -255mV vs Ag/AgCl) when changing the pH from 9 to 5 respectively. In our study of a 4 unit reduction in pH, we observed a comparable shift from -480mV to -350mV (vs Ag/AgCl). Note, that in the former paper, a tetrafluroborate salt of hyaluronic acid film was used to immobilize the haemoglobin at the electrode, whereas our studies relied on probing available protein at the electrode/protein solution interface. This difference could explain the difference observed in peak reduction potential changes with protonation of the protein at lower pH.

3.2 HydroNIP and HydroMIP Electrochemical Characterisation

Figs 2a shows the cyclic voltammograms obtained from the NIPs at different stages of the washing and reloading procedure (see Table 1) and compares them with the responses from the respective MIPs (Fig 2b), which were used as control samples.

The peak seen in NIP 1 coincides with the reduction peak for free oxygen in solution (at around -0.6V vs (Ag/AgCl)) whereas MIP 1 shows a cathodic peak at -0.481V vs (Ag/AgCl) corresponding to reduction of haem-based oxygen from within the MIP-bound protein.

NIP2 and MIP 2 (after water washing only of NIP 1 and MIP 1 respectively) were found to give rise to very similar responses to NIP 1 and MIP 1 respectively. The NIP 2 and NIP 1 similarity would be expected since there is no protein to be removed from NIP 1. The difference between MIP 2 and MIP 1 is that the water washes are only designed to remove the excess BHb in MIP 1 leaving behind the selectively bound protein within the cavities of the gel. Upon SDS and acetic acid washing of MIP 2, the electrochemical behaviour of the protein removed MIP (MIP 3) was found to be remarkably different from the ones observed for MIP 1 and MIP 2, most notably lacking the typical peak that indicates the presence of BHb at around -0.48V vs (Ag/AgCl) but instead exhibiting the cathodic peak for the reduction of free oxygen, much like NIP 3.

S-M Chen and C-C Tseng [20] have characterised the bioelectrocatalytic properties of haemoglobin by the probing the direct electrochemistry of didodecyldimethylammonium bromide (DDAB) film modified electrodes. It is known that enhanced electron transfer rates from haemoglobin can be achieved in the presence of such polyion surfactants compared with a bare electrode. The latter authors demonstrated that direct electrochemical reduction potential for O_2 reduction is -600mV (vs Ag/AgCl) and can be enhanced in the presence of a DDAB film on the electrode with the potential increasing to -450 mV vs Ag/AgCl. In the presence of Hb in the DDAB film, the bioelectrocatalytic reduction of oxygen occurs at -100mV vs Ag/AgCl. In our study with polyacrylamide gel particles containing Hb, the bioelectrocatalytic reduction of oxygen occurs at -480 mV vs Ag/AgCl which is more comparable with that reported by H. Lu et al. [21] for thin film polyacrylamide films containing Hb (-275 mV vs Ag/AgCl (calculated)). Our data is therefore comparable with other reported work.

3.3 Protein Rebinding in MIPs Characterised using Cyclic Voltammetry

NIP3 and MIP3 correspond to NIP and MIP after pre-washing with SDS/AcOH followed by water washing and pre-equilibrating with PBS solution. Therefore, neither the NIP nor MIP contain any protein at this stage. Fig. 3 shows the time dependent analysis of BHb re-uptake from bulk solution by NIP 3 (Fig. 3a) and MIP 3 (Fig. 3b).

With NIP3, after 15 minutes of contact with a concentrated solution of the protein, a shift in the peak reduction potential of the gels is observed. The signal increases overtime until a steady state is obtained. Confirmation of the fact that the peak results from the reduction of BHb comes from the comparison of the potential at which the peak for BHb in the pH study is found. The reduction potential is similar when compared to the pH study (respectively, -0.470 V and -0.438 V vs (Ag/AgCl)), and can be explained by considering that the BHb is not in solution, but is immobilized and interfaced directly at the electrode surface because of the gel matrix. As expected with MIP3 (Fig. 3b), the reduction peak for BHb was absent.

Fig 4 shows the time dependent voltammetric analysis of BHb reuptake by NIP 5 (Fig. 4a) and MIP 5 (Fig. 4b), corresponding to NIP and MIP washed and conditioned with SDS and AcOH only (i.e. no PBS conditioning). For the NIP 5 gel, 10 minutes of protein exposure was sufficient to produce a change in the signal from the NIP washed and equilibrated with PBS. The potential of the developing reduction peak was found to be at around -0.480 V.

The time dependent analysis of MIP 5 showed a different trend. The reduction peak for BHb was not observed for the imprinted gel until a small change in signal appeared at 40 minutes, followed by a marked increase at 45-50 minutes.

The reduction potentials for the peaks obtained from the analysis of NIP 3 and 5 were very similar, respectively -0.470 and -0.480 V vs (Ag/AgCl). This was expected since both gels underwent a similar treatment (wash with PBS, elution with AcOH:SDS, equilibration with PBS).

The smaller peak current for MIP2 compared with MIP1 is likely due to the removal of non-selectively bound BHb in MIP2, after the water washing stage. The fact that the majority of the signal remains after water washing suggests that the majority of the protein is still selectively bound within the gel and the protein is accessible to the electrode. This would be possible if the cavities allowed the partial exposure of protein allowing it to be probed by the electrode. Upon SDS and acetic acid washing, the electrode signal for oxyhemoglobin disappears and is replaced with the reduction peak for oxygen in solution. The elution washing with AcOH and SDS has caused the BHb to be denatured and removed from the MIP cavities, leaving a protein-void polymer gel with electrochemical characteristics similar to those of the NIP.

The electrochemical signal to hemoglobin rebinding within SDS/AcOH/water washed MIP and NIP (MIP3 and NIP 3 respectively) after pre-conditioning each with PBS were also investigated. With NIP3, whereas initially there is only the oxygen in solution signal, with increased time of exposure to protein solution, the oxygen signal is displaced by the hemoglobin signal. This suggests that Hb has diffused to the electrode from the outer bulk solution, through the polycarbonate membrane and has met with little resistance from the NIP particles interfaced at the electrode surface. In contrast, MIP3 shows no signal for haemoglobin even after 40 minutes exposure, which suggests that the MIP 3 (comprising protein-selective cavities) is offering more resistance to the diffusion of protein to the electrode, probably due to selective binding of the diffusing protein into the MIP cavities. The same would have been expected for MIP 5, but the latter actually behaves more like NIP 3 and NIP 5. The difference between MIP 3 and MIP 5 is that whereas the former was conditioned with PBS, the latter was placed on the electrode directly after SDS/AcOH washing and without PBS conditioning. MIP 5 would appear to allow easier access of protein to the electrode than MIP 3. This could be attributed to a MIP-Hb interaction with residual SDS/AcOH during uptake of the protein within the cavities of the polymer. This would subsequently result in a conformational change in the protein. The experiment shows that approximately 30-35 minutes needs to lapse before a significant signal for BHb is observed. This time delay is needed in order to allow

diffusion of the protein through the polymer, given that the protein would experience attractive/favourable interactions with template selective cavities throughout the MIP layer which is countered by the denaturing effect of residual SDS/AcOH within the film en route to the electrode. The subsequent delayed arrival of partially denatured protein to the vicinity of the electrode can be electrochemically detected. This delay is not evident in NIP 5 as the NIP is not selective for the protein. Consequently, the protein would have a less tortuous path to reach the electrode.

Useful information can be obtained through the comparison of the rebinding study performed on MIP 5 with the voltammogram produced by the bare glassy carbon electrode immersed in a 10 mg/ml solution of BHb in PBS at pH 5.0 (Fig. 2a). The conformation of the protein at pH 5.0 is different from the one at pH 7.3. The reduction potential of BHb is the same for both the pH 5 protein only study and the MIP 5 protein re-uptake study after 50 minutes (-0.417 V vs (Ag/AgCl)); this further supports that there is some protein conformational change occurring in MIP 5 similar to the protein in solution at pH 5.0.

4. Conclusions

The direct electron transfer of haemoglobin at a hydrogel-based MIP modified electrode was achieved without the need for an artificial electron mediator. Moreover, we have been able to use cyclic voltammetry to characterize selective and non-selective protein binding within hydrogel-based molecularly imprinted polymers and NIPs respectively on the surface of a glassy carbon electrode. The method proposed may be used for further studying the direct electrochemistry of redox proteins and the development of synthetic receptor-based electrochemical biosensors and bio-medical devices.

Tables and Figures

Table 1 : Summary of the sample states used for electrochemical analysis

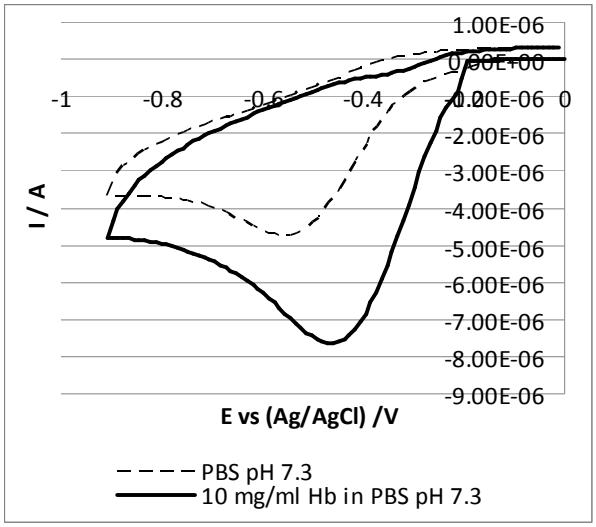
Figure 1 Cyclic voltammograms of (a) PBS only and bovine haemoglobin (10mg/ml) in PBS at pH 7.3 and (b) haemoglobin in PBS at pH values between 3-8.

Figure 2 Cyclic voltammograms of (a) NIP and (b) MIP (NIP1 and MIP1 – freshly prepared and granulated gels; NIP2 and MIP2: gels after PBS washing; NIP3 and MIP3: gels after SDS/AcOH treatment followed by PBS washing.)

Figure 3 Cyclic voltammograms of (a) NIP3 and (b) MIP3 at time intervals between 0 and 40 minutes. (NIP or MIP was retained at the electrode under a 0.8 micron polycarbonate membrane and the electrode immersed in 10mg/ml haemoglobin solution in PBS for a period of 40 minutes.)

Figure 4 Cyclic voltammograms of (a) NIP5 and (b) MIP5 at 5 min time intervals between 0 and 40 minutes. (NIP or MIP was retained at the electrode under a 0.8 micron polycarbonate membrane and the electrode immersed in 10mg/ml haemoglobin solution in PBS for a period of 50 minutes.)

Fig 1a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10 mg/ml Hb in PBS pH 7.3

Fig. 1b

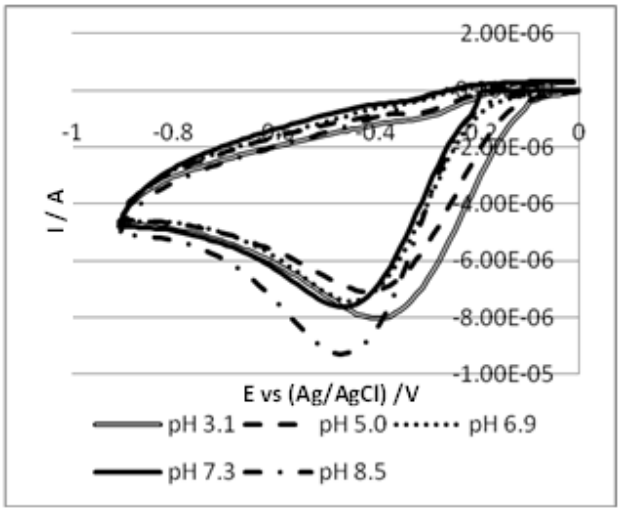
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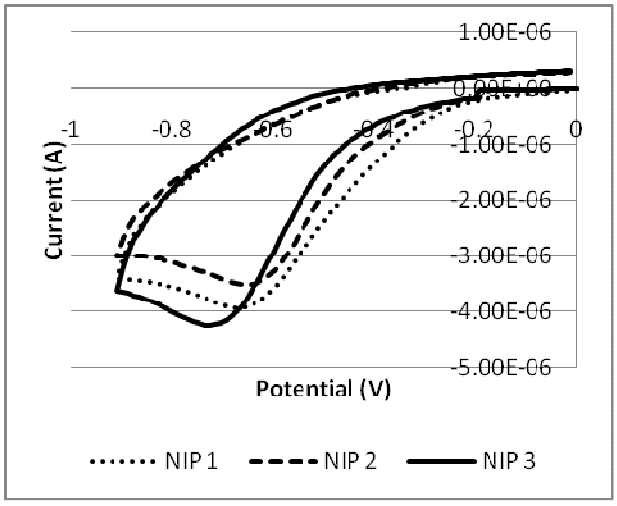
Fig 2a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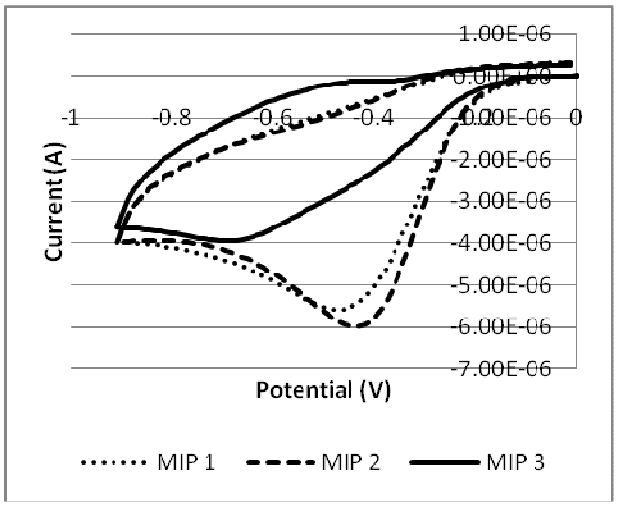
Current (A)
Potential (V)
NIP 1
NIP 2
NIP 3

Fig 2b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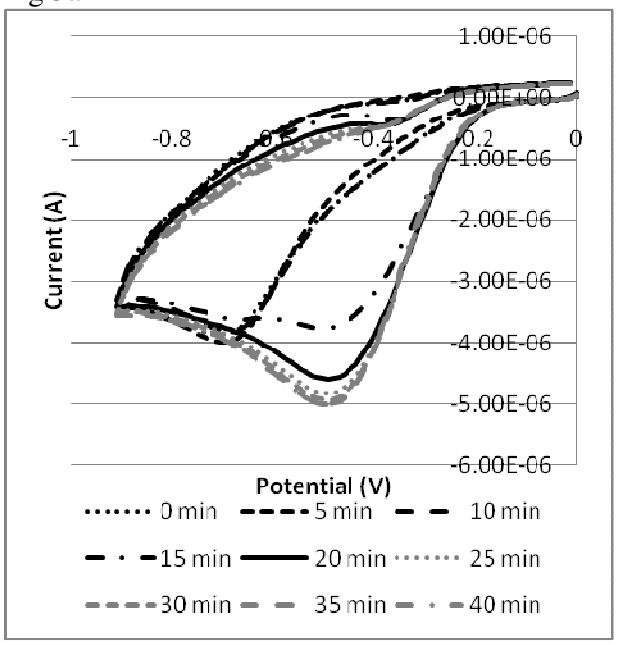
Current (A)
Potential (V)
MIP 1
MIP 2
MIP 3

Fig 3a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25 min
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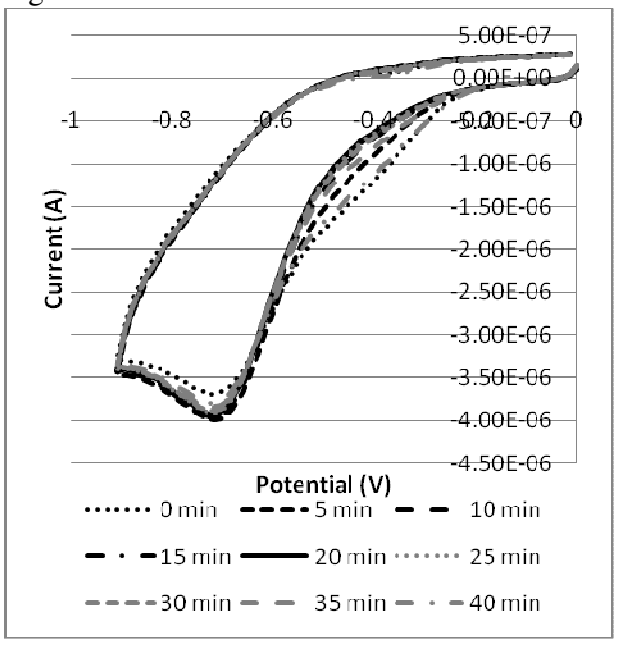
40 min

Fig 3b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-3.50E-06
-4.00E-06
-4.50E-06
Current (A)
Potential (V)
0 min
5 min
10 min
15 min
20 min
25 min
30 min
35 min
40 min

Fig 4a

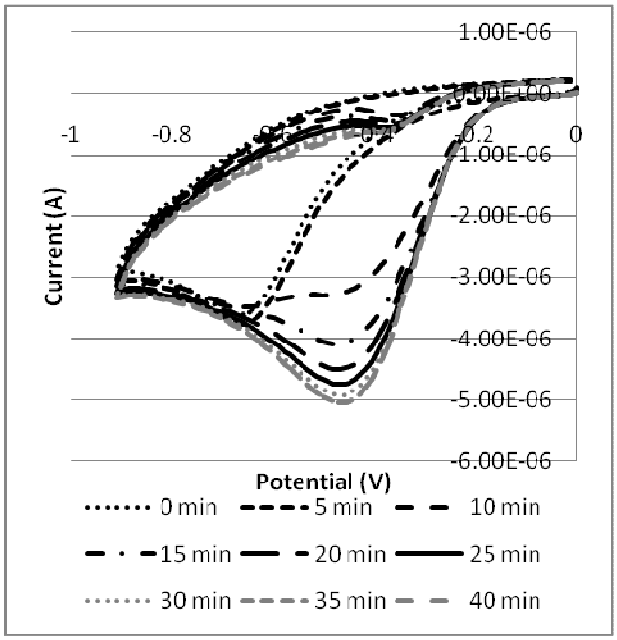
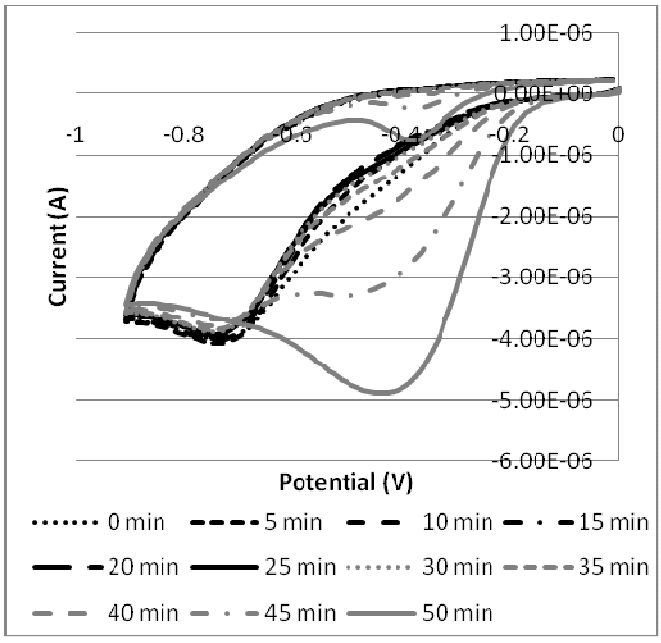

1.00E-06
0.00E+00
-1
-0.8
-0.6
-0.4
-0.2
0
-1.00E-06
-2.00E-06
-3.00E-06
-4.00E-06
-5.00E-06
-6.00E-06
Current (A)
Potential (V)
0 min
5 min
10 min
15 min
20 min
25 min
30 min
35 min
40 min

Fig 4b


1.00E-06
0.00E+00
-1
-0.8
-0.6
-0.4
-0.2
0
-1.00E-06
-2.00E-06
-3.00E-06
-4.00E-06
-5.00E-06
-6.00E-06
Current (A)
Potential (V)
0 min
5 min
10 min
15 min
20 min
25 min
30 min
35 min
40 min
45 min
50 min

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