

# Development of an electrochemical flow injection immunoassay (FIIA) for the real-time monitoring of biospecific interactions

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## Abstract

Not only are sensors a revolution in analysis; they themselves are also experiencing a revolution brought about by parallel developments in sensor fabrication techniques and materials, polymer chemistry, signal processing methodologies, the increased use of biomolecular processes as a means of analyte detection, and the coupling of sensors to other techniques such as flow injection analysis. Many of these developments have been incorporated into the present study, which we are undertaking in the development of our immunosensor technology. The system described here utilises screen-printed electrodes which are low-cost, disposable devices that are simple to fabricate. Incorporated into our sensor is the electroactive polymer, polyaniline, which brings about mediatorless redox coupling between the electrode and biomolecular components attached to the polymer surface. This system also utilises enzyme-labelled antibodies as the biomolecular recognition component for the analysis of the test analyte, biotin. The system has also been integrated into a flow injection system. This has led to the monitoring of real-time antibody-antigen interactions using electrochemical methods and foreshadows the development of single-step immunosensors. (

*Keywords:* Flow injection; Immunoassay; Biosensor; Screen printed electrode; Polyaniline

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## 1. Introduction

Biosensors still offer great potential as the next revolution in analysis. They possess many characteristics that may allow them to accomplish this. However, they still fall short of many requirements to allow this to become reality. Many obstacles still lie in the way of the widespread commercialisation of biosensor sys-

tems. However, several new areas of development offer prospects for continued advancement in biosensor technology.

Even today, biosensor research concentrates nearly exclusively on simple enzyme-based biosensor systems. These may serve as good models for laboratory-based research, but are limited in scope. The range of substrates utilised by natural enzyme systems is very limited and this also limits the range of analytes that may be studied, mostly to analytes of biomedical relevance. Attempts have been made to get around this problem with enzyme-inhibition-based

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systems [1], but with these, the lack of selectivity is of great concern when it comes to the analysis of field samples. To be successful, the techniques involved must have the potential to become ubiquitous. This was one of the roots of success of the enzyme immunoassay [2]. The utilisation of antibodies in combination with enzyme-labelling systems ensured that the technique had the potential to analyse any component to which an antibody could be produced.

Electrochemical biosensors have also been held back by the need for diffusional electron transfer mediators. These are low molecular weight molecules that can diffuse rapidly between the enzyme redox site and the electrode surface. In the original Clarke-type electrode, oxygen acted as the mediator [3]. Later devices used small redox molecules such as potassium ferrocyanide and quinones to accomplish this [4,5]. Some electroactive polymers, however, do not require soluble mediators. They undergo redox cycling themselves and can couple electrons directly from the enzyme active site to the electrode surface. One such polymer is polyaniline (PANI). It also possesses several other characteristics that make it useful in biosensor design. The incorporation of dopants such as polyvinyl sulphonate (PVS) leads to enhanced morphology and conductivity at neutral pH [6]. Biomolecules can also be doped onto the surface of the polymer by electrostatic interactions with the polymer backbone [7]. In this way, diffusion of substrates and biorecognition molecules does not have to take place into and out of a polymer matrix as traditionally occurs in polymer entrapment systems [8].

Electrode fabrication technology still remains an important element of the commercial success of biosensors. For mass production, sensors must have extremely low unit costs and be capable of undergoing mass production, while still maintaining adequate levels of reproducibility. Several fabrication systems are available, most of which have been borrowed from the microelectronics industry, such as photolithography systems and thin film technologies. Another technology is screen-printing. Developed for the printing industry, this thick film system has been adapted for the electronics industries and in biosensor research. It offers many characteristics that make it a suitable system for electrode fabrication. The technology is relatively low cost, both in terms of capital and run-

ning costs. It is also an extremely flexible technology in that the time from design to manufacture is very short, and again, is low cost. This means that new designs can be rapidly changed and developed. The planar nature also means that many design ideas can be readily explored. What is still of some concern with screen printing, however, is the level of reproducibility in electrode production. This is mainly due to the nature of the inks used in production. These consist of mixtures of metal, or carbon pastes, mixed with binders and solvents. The characteristics of these inks change with time and usage [9]. The ingredients for the inks are also manufacturer's formulae and are difficult to characterise. Despite these problems, however, screen-printing is the basis for successfully commercialised enzyme electrodes [10].

Enzyme immunoassay for small haptens is usually performed using a competition immunoassay format, and thus requires several steps to perform [11]. These assay formats have been adapted for biosensor applications, but become difficult to apply on sensors manually. Flow injection systems have been developed for the automated, controlled, sequential delivery of reagents to sensors to remove operator intervention from assays, and improve reproducibility [12].

The biosensor described here possesses all the elements of sensor development just described. The system utilises a screen-printed carbon paste electrode onto which a PANI/PVS film is potentiodynamically applied. Antibodies are immobilised onto the polymer surface which can then take part in immunochemical reactions with enzyme-labelled haptens; in this case, biotin. The system was used for the measurement of free biotin, but can be applied to the analysis of other analytes to which antibodies and enzyme-labelled conjugates are available. The physical characteristics of the polyaniline-based antibody sensor have been studied using electrochemical, spectrophotometric and electron microscopic techniques [13].

The electrodes were also integrated into a flow injection system. The concept of the electrically-wired immunosensor was tested [14], and real-time monitoring of antibody-antigen interactions could be performed electrochemically. The authors then go on to project how this will lead to the development of simple one step flow injection immunoassays (FIIA) in the future.

## 2. Experimental

### 2.1. Chemicals

Immunochemical reagents were purchased from Sigma-Aldrich (Poole, Dorset, UK). Anti-biotin antibody was a goat polyclonal (Sigma B3640), and horseradish peroxidase-labelled biotin (Biotin-HRP) was biotinamidocaproyl-labelled peroxidase (Sigma P2907). Horseradish peroxidase (HRP) was 200 U/mg (Sigma P 6782). Aniline was purchased from Aldrich (13,293-4), vacuum distilled and stored frozen under nitrogen. 30% hydrogen peroxide solution was purchased from Merck. Sigmafast *o*-phenylenediamine dihydrochloride substrate (OPDA, Sigma P 9187), was prepared according to manufacturer's instructions. Tween 20 (27,434-8) and polyvinylsulphonate (PVS, 27,842-4) were purchased from Aldrich Chemical.

### 2.2. Buffers and solutions

PBS is 0.1 M phosphate buffer, 0.137 M NaCl and 2.7 mM KCl, pH 6.8. This was prepared by mixing solutions containing 0.1 M  $\text{Na}_2\text{HPO}_4$ , 0.137 M NaCl and 2.7 mM KCl and 0.1 M  $\text{KH}_2\text{PO}_4$ , 0.137 M NaCl and 2.7 mM KCl to a pH of 6.8. Unless otherwise stated, all immunochemicals were prepared in PBS.

### 2.3. Instrumentation

#### 2.3.1. General electrochemical experiments

All electrochemical protocols were performed on a BAS100/W electrochemical analyser with BAS100 W software, using either cyclic voltammetry, or time-based amperometric modes.

#### 2.3.2. Screen-printed electrode fabrication

Electrodes were screen-printed onto a pre-shrunk polyester (PET) substrate (175  $\mu\text{m}$  thickness). Screen printing was performed on a manual screen printing machine. Nylon mesh screens of 90T were used. Initially, a silver conductive layer (Dupont Ag 5007E) was applied with curing at 120°C for 5 min, followed by a carbon paste (Gwent Electronic Materials C10903D14) sensor layer with curing at 80°C for 15 min. A non-conducting, UV-curable dielectric layer (Acheson Electrotag 451SS) was then placed

over the surface to define a circular electrode surface of 3 mm diameter.

### 2.4. Electrode preparation

The screen printed electrodes were cut from the PET sheet leaving excess width on either side to a width of 14 mm.

#### 2.4.1. Polymerisation of aniline on the electrode surface

Electrodes were placed in 10 ml of 0.2 M  $\text{H}_2\text{SO}_4$ , prior to the polymerisation of aniline. A platinum mesh auxiliary and a silver/silver chloride reference electrode were used. Polymerisation was performed in a glass electrochemical cell using cyclic voltammetry between -1200 and 1500 mV versus Ag/AgCl electrode at a scan rate of 100 mV/s, sensitivity of  $1 \times 10^{-3}$  A over one cycle.

A mixture of 7.8 ml 1 M HCl, 186  $\mu\text{l}$  aniline (distilled and stored under nitrogen) and 2 ml polyvinylsulphonate (PVS) were degassed under nitrogen for 10 min. Aniline was polymerised on the surface of the working electrode using ten voltammetric cycles between -500 and 1100 mV versus Ag/AgCl electrode at 100 mV/s, and sensitivity at  $1 \times 10^{-3}$  A.

#### 2.4.2. Immobilisation of anti-biotin antibody

The working electrode surface was reduced in 10 ml of PBS (degassed for 10 min under nitrogen) at -500 mV versus Ag/AgCl, sample interval of 500 ms, over 1500 s at a sensitivity of  $1 \times 10^{-4}$  A.

Antibody was previously prepared at 1 mg/ml in PBS. Very quickly after reduction was complete, PBS buffer was removed from the cell and quickly replaced with antibody solution, not under stirring or degassing. Again quickly, oxidation was performed at 700 mV versus Ag/AgCl for 1500 s.

During this oxidation, the antibody becomes electrostatically attached to the polymer surface. Antibody was carefully recovered from the cell and re-stored for later use.

### 2.5. Colourimetric enzyme immunoassay

A colourimetric enzyme immunoassay was performed on the surface of the screen-printed

electrodes to verify antibody–antigen interaction. Anti-biotin-immobilised screen-printed electrodes were incubated for 1 h with 10  $\mu$ l of either 1 mg/ml biotin-HRP, HRP or PBS. The electrodes were washed with a few drops of PBS/0.5% (v/v) Tween 20 and 10  $\mu$ l of OPDA substrate added, and incubated for 30 min. The substrate was then transferred to the wells of a microtitre plate (Nunc) and 50  $\mu$ l of 2 M H<sub>2</sub>SO<sub>4</sub> added, and the absorbance read at 492 nm.

## 2.6. Electroanalytical procedure

Electrodes that had been immobilised with anti-biotin antibody were subjected to the appropriate immunochemical incubations as described in the sections below. To perform this, the electrode was disconnected from the electrochemical apparatus and allowed to lie in a horizontal position and the appropriate immunochemicals added. Following this, the electrode was rinsed gently with PBS/0.5% (v/v) Tween 20. The electrode was reattached to the electrochemical analyser and placed in 10 ml PBS. Amperometric experiments were performed at  $-100$  mV versus Ag/AgCl, with a sample interval of 500 ms and sensitivity of  $1 \times 10^{-4}$  A.

Following the reaching of the electrode steady state 1  $\mu$ l additions of 1 M hydrogen peroxide substrate were made to the system as appropriate and the increase in catalytic current monitored.

## 2.7. Immunoassay development

### 2.7.1. Optimisation of biotin-HRP concentration

Anti-biotin antibody-immobilised electrodes were incubated with 10  $\mu$ l of biotin-HRP in concentrations ranging from 0 to 2 mg/ml for 1 h, followed by the basic amperometric procedure in Section 2.6.

### 2.7.2. Pre-incubation of antibody-modified sensor with biotin

Anti-biotin-immobilised electrodes were pre-incubated with 10  $\mu$ l of 1 mg/ml biotin in PBS for periods between 5 and 30 min. This was followed by the addition of 10  $\mu$ l 0.05  $\mu$ g/ml biotin-HRP for 30 min. The basic amperometric procedure was then performed according to Section 2.6.

### 2.7.3. Competition electrochemical immunoassay

Biotin at various concentrations from 0 to 1 mg/ml was applied to the surface of the working electrode for 30 min at room temperature in a volume of 10  $\mu$ l. This was followed by the addition of 10  $\mu$ l biotin-HRP at 0.05 mg/ml for a further 30 min. This was again followed by the basic amperometric procedure described in Section 2.6.

## 2.8. Design of batch and flow injection electrochemical cells

For further analytical procedures, the system was refined by designing a batch cell, with integrated reference and auxiliary electrodes and a reduced solution volume of approximately 2 ml (Fig. 1a). Stirring was provided by an electric motor mounted above the cell. Polymerisation of aniline was conducted in a glass electrochemical cell according to Section 2.4.1, before transfer to the batch cell. Reduction and oxidation of the polymer in the presence of 1 ml of 1 mg/ml anti-biotin antibody was performed in the cell according to Section 2.4.2. The electrode was then either used *in situ* or transferred to a flow injection device (Fig. 1b), also with integrated platinum wire and silver/silver chloride wire electrodes incorporated. The flow rate was typically 10  $\mu$ l/s.

## 3. Results and discussion

### 3.1. Electrode preparation

Fig. 2 presents the cyclic voltammogram of PANI/PVS on the surface of the screen printed electrode. This shows that aniline has become polymerised and is also conductive and electroactive. Discernible are the two oxidation (a) and (b) and the two reduction (c) and (d) peaks of the polymer. CVs from screen-printed electrodes are typically comparable with those previously obtained for glassy carbon electrodes [15] (data not shown).

Oxidation of the polymer in the presence of macromolecules such as an antibody results in their electrostatic attachment to the polymer backbone. This has been shown to occur directly with the catalytic response brought about by enzyme immobilisation

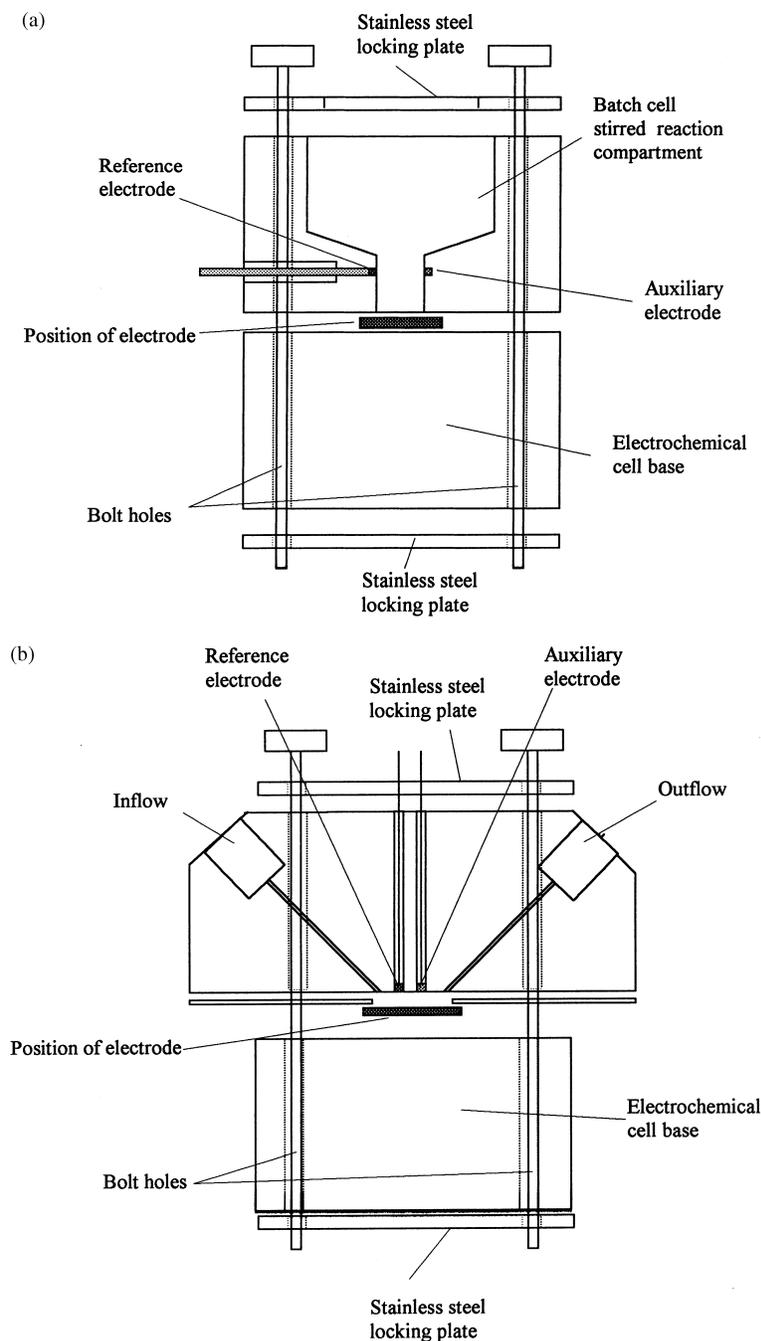


Fig. 1. (a) The perspex batch cell comprised an upper reaction vessel and a lower base, between which was clamped the screen printed electrode. Platinum wire auxiliary and silver/silver chloride wire reference electrodes were incorporated into the reaction vessel with external connections as shown. (b) The flow injection cell was composed of perspex into which had been drilled holes for inflow and outflow. Platinum and silver/silver chloride electrodes were placed at the base of the cell to come into close association with the screen-printed working electrode. The flow chamber was formed by the use of a PET spacer (260  $\mu\text{m}$ ). The screen-printed electrode was clamped into place between the upper and lower sections as shown.

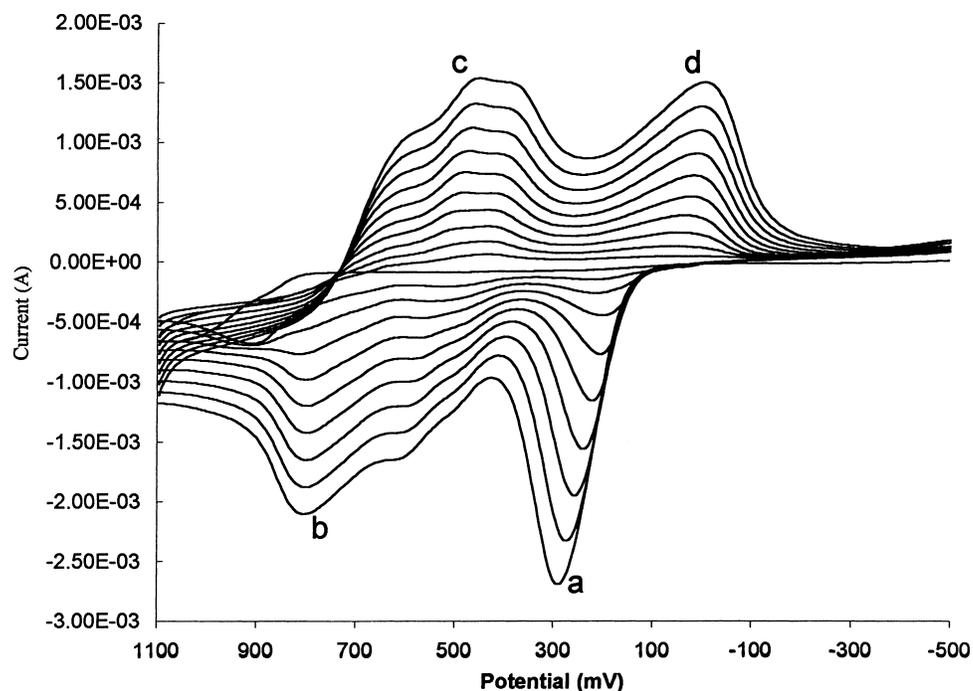


Fig. 2. Cyclic voltammogram of polyaniline on the surface of the screen-printed electrode. Peaks a and b are oxidation peaks and c and d reduction peaks showing the redox changes from aniline to polyemeraldine cation radical and from polyemeraldine cation radical to polyemeraldine.

[15,7]. In this case, however, attachment was demonstrated indirectly by the interaction of the antibody with its peroxidase-labelled hapten, biotin. Colourimetric immunoassay was performed on the electrodes and this demonstrated an elevated binding over controls of peroxidase and buffer alone, with values of 0.154, 0.099 and 0.066 absorbance units at 492 nm for biotin-HRP, HRP and buffer, respectively. Specific binding was also demonstrated electrochemically (Fig. 3). This also demonstrated that no significant background binding interactions resulted from the protein HRP.

### 3.2. Immunoassay development

Due to the nature of haptens, the most frequently employed immunoassay technique for their analysis is the competition immunoassay [16]. For the analysis of biotin, the parameters for a functioning assay strategy were elucidated empirically.

Initially, the biotin-HRP concentration and its effect on the assay was investigated (Fig. 4). It can be seen that above 2 mg/ml, the conjugate was reaching saturation. However, due to limitations on reagents, a concentration of 0.05 mg/ml biotin-HRP was chosen as the competitor concentrations for future analyses.

Initial attempts to incubate biotin and biotin-HRP on the electrode surface did not lead to effective competition, even though these reagents perform adequately when used in enzyme immunoassay (data not shown). This phenomenon has been noted previously for certain antibodies when applied to biosensor applications (unpublished observations). This may be a characteristic of different rate constants of the antibody for the hapten and the enzyme conjugate. In enzyme immunoassay, only the overall equilibrium constant is assessed as the system is allowed to reach a quasi-equilibrated state [16]. In biosensor applications, however, the association rate constants may dominate as the important factor in the assay, and this may be significantly lower for the hapten than for the

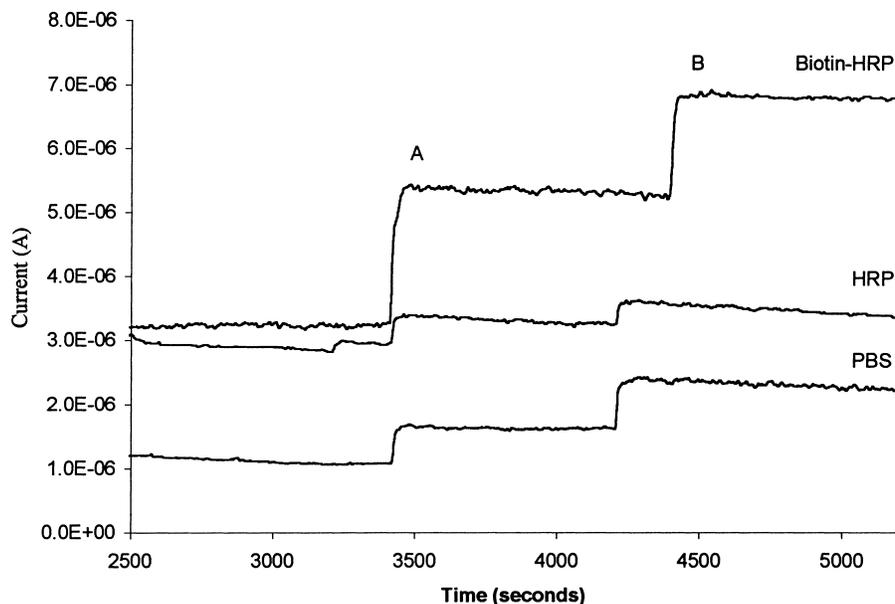


Fig. 3. Amperogram illustrating the specific interaction of biotin-HRP with anti-biotin antibody on the surface of the screen-printed electrode following addition of (A)  $1\ \mu\text{l}$  and (B)  $2\ \mu\text{l}$  of  $1\ \text{M}$  hydrogen peroxide substrate. The HRP control produced signals that were comparable with incubation with PBS alone, demonstrating that the signals generated were due to the uncatalysed decomposition of hydrogen peroxide.

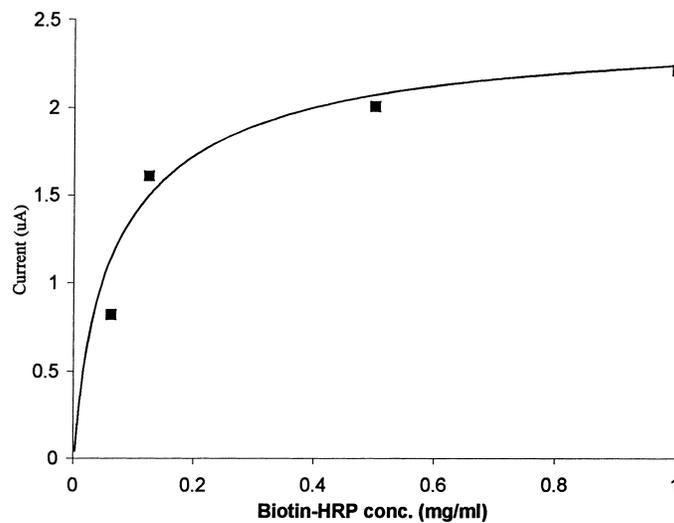


Fig. 4. Effect of the concentration of biotin-HRP incubated on the surface of the electrode on catalytic signal generated upon the addition of hydrogen peroxide substrate. Catalytic response was shown to be dependent on the concentration of biotin-HRP incubated on the electrode surface. Saturation was reached above  $2\ \text{mg/ml}$  biotin-HRP.

hapten-enzyme conjugate, which may not result in effective competition over the reduced incubation periods used. This hypothesis was tested and free biotin allowed to pre-incubate on the electrode surface prior

to the introduction of the competitor, biotin-HRP. It was found that increasing the pre-incubation time of biotin on the electrode did indeed reduce the amount of biotin-HRP attaching to the surface, thus reducing

the signal. It was deduced that this phenomenon was a characteristic of the antibody/antigen system being used and was not a flaw in the biosensor methodology being applied. Later experiments thus incorporated the pre-incubation step.

### 3.3. Competition enzyme immunoassay for biotin

A competition enzyme immunoassay for free biotin on the surface of the screen-printed electrode was established. As is typical of antibody antigen interactions in a competition immunoassay format, the signal produced was inversely proportional to the free analyte concentration. Plotted semi-logarithmically, this produced the characteristic sigmoidal plot (Fig. 5). The assay's highest sensitivity was in the region of 0.01–0.02 mg/ml, with an extended linear region from 0.03 to 1 mg/ml.

### 3.4. Integration with flow injection

Initial experiments with flow injection were to establish that both the batch system and flow injection system were comparable with one another. Fig. 6 shows a comparison of the signals produced as a result of batch and flow injection experiments performed on the same electrode. These showed good correlation for equivalent concentrations of substrate, demonstrating that stirring and flow rates were not limiting the diffusion of substrate to the electrode surface. A control using a 1 mg/ml HRP solution incubated on the electrode surface showed that there were only negligible background signals.

The reproducibility of the electrode response was also investigated. The duplicate amperograms in Fig. 7 showed good precision. Triplicate responses yielded a standard error ( $n=3$ ) below 6.4% at the 8 mM hydrogen peroxide concentration level.

### 3.5. Electrically wired antibody-based sensors and the monitoring of real-time biological interactions

The concept of the 'electrically wired' sensor stems from the principle that only redox reactions coupled to the electrode surface will bring about significant changes in catalytic current [14]. Reactions that occur in the bulk solution away from the elec-

trode surface take electrons from the surrounding bulk electrolyte and not from the sensor surface. This idea was tested on this sensor in a flow injection set up. The electrode had been prepared as previously described, with anti-biotin, the concept being that if HRP and hydrogen peroxide were passed across the electrode surface together, they would not generate a large degree of catalytic current, even though substrate turnover was taking place because no specific interaction between antibody and antigen would have occurred. This was tested in the following experiment where 0.5 mg/ml HRP and 8 mM hydrogen peroxide were allowed to flow over the electrode surface. This yielded a signal of  $1.237 \pm 0.137 \mu\text{A}$ . However, when a solution of biotin-HRP and hydrogen peroxide were passed over the surface, a large increase in catalytic current was produced (Fig. 8). The gradual increase in catalytic current is typical of the association phase of antibody-antigen interactions seen in other real-time interaction monitoring such as BIAcore<sup>TM</sup> and IA<sub>sys</sub><sup>TM</sup> [17,18]. Fig. 8 also shows that this phenomenon was concentration-dependent, with slower binding rates for lower concentrations of enzyme-labelled hapten. The catalytic signal was fully restored when the conjugate/substrate mixture was replaced with hydrogen peroxide alone.

This phenomenon is extremely important for the future development of antibody-based electrochemical sensors. Until now, immunoassay procedures have been transferred to biosensors without any significant change in the experimental methodology, save for a different underlying substrate and detection method. Typically, the tedious numbers of assay steps which include various incubations, washings, etc., were carried over in these methodologies, leaving anywhere from six to 13 immunoassay steps to perform [19–21]. Such cumbersome systems have not been employed as useful analytical methodologies. The phenomenon of electrical wiring, however, removes the necessity for a physical and temporal separation step between bound and unbound conjugate material in a competition immunoassay. Since both species can be present in the same physical environment, but only the bound species is responsible for the generation of a catalytic signal, no separation step is required. This will lead ultimately to the development of single-step assays in which both labelled and unlabelled analyte are present with enzyme substrate in the same compartment. As

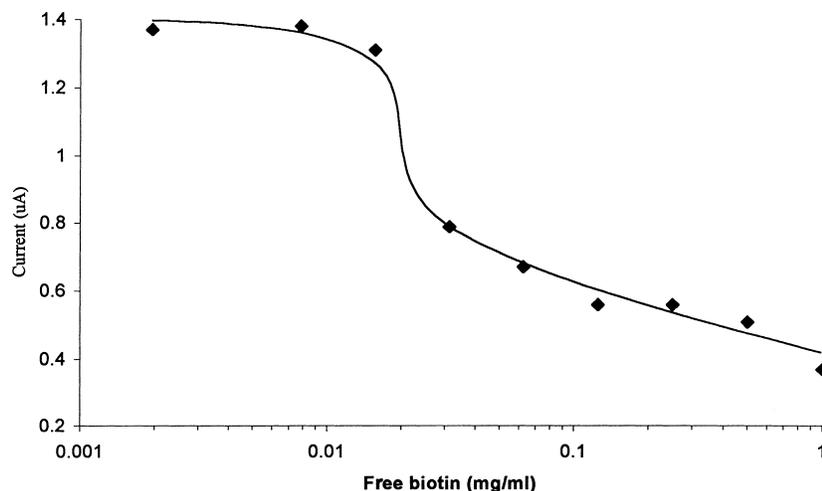


Fig. 5. Semi-logarithmic plot of the competition electrochemical immunoassay for biotin. This results in the typical sigmoidal curve, characteristic of antibody–antigen interactions, with the most sensitive range lying between 0.01 and 0.02 mg/ml biotin. A linear range was also present between 0.03 and 1 mg/ml biotin.

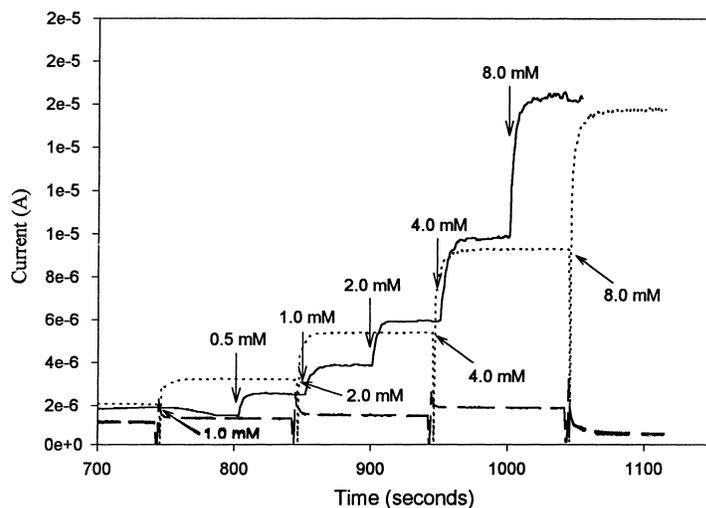


Fig. 6. Comparison of the response from batch cell and flow injection electrochemical systems. When incubated with 1 mg/ml biotin-HRP, the anti-biotin-immobilised sensor yielded comparable signals when incubated with 0.5, 1, 2, 4 and 8 mM hydrogen peroxide in either the batch cell (solid line), or the flow cell (dotted line). Incubation of the anti-biotin immobilised sensor with 1 mg/ml HRP (dashed line) produced only marginal increases in catalytic current equivalent to the background generated by the uncatalysed decomposition of hydrogen peroxide, indicating that non-specific binding of HRP to the antibody or polymer was not occurring.

competition takes place at the electrode surface, and binding takes place, a catalytic signal inversely proportional to the analyte concentration is generated without the need for the removal of any of the reagents from the surface.

### 3.6. Single step electrochemical immunoassays

The antibody used in this system, however, does not lend itself to the development of such single step assays for the reasons already outlined in this paper.

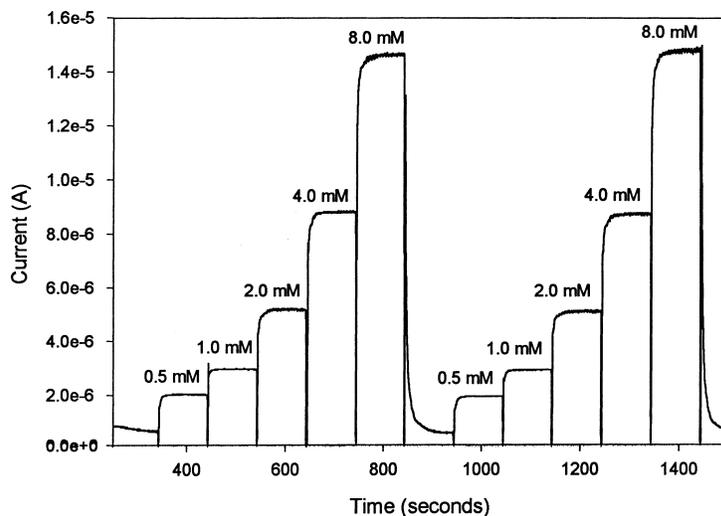


Fig. 7. Amperogram showing the reproducibility of catalytic signals of the anti-biotin-immobilised sensor, following incubation with 1 mg/ml biotin-HRP. Repeated injection of 0.5, 1, 2, 4 and 8 mM hydrogen peroxide restored comparable catalytic signals.

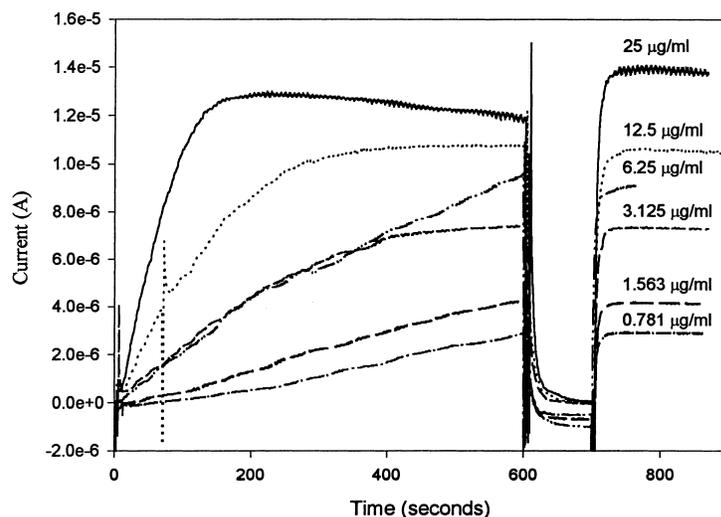


Fig. 8. The real time electrochemical analysis of the biospecific interaction of biotin-HRP with anti-biotin antibody immobilised onto a polymer-modified screen-printed electrode. Concentrations of biotin-HRP along with 8 mM hydrogen peroxide were allowed to flow over the surface of the electrode for 600 s. PBS buffer was then flowed over for 100 s, before being replaced with PBS containing 8 mM hydrogen peroxide. It can be seen that the rate of interaction of biotin-HRP with the surface is concentration-dependent. The effect is confirmed as a permanent one by the replacement of buffer with hydrogen peroxide, whereupon, the catalytic current is restored, showing that a genuine biospecific interaction event has taken place.

Some experimentation was performed using this antibody, to demonstrate that a competition system is feasible utilising this scenario. The mixing of a large molar excess of free biotin (1 mg/ml) with the

biotin-HRP (5 µg/ml), however, did not significantly reduce the rate of interaction of biotin-HRP with the surface or when free biotin was cycled over the surface for a period, before introduction of the conjugate

(data not shown). With the correct choice of antibody and antigen, however, we believe that single step flow injection immunoassays are possible with this system.

#### 4. Conclusions

The electroactive polymer, polyaniline has been shown to be well adapted for the development of an antibody-based biosensor. Coupled to screen-printed electrodes, the system was comparable to traditional glassy carbon electrode devices, and was suitable for the development of a competition electrochemical immunoassay for the test analyte biotin.

The screen-printed immunosensor was adapted to a flow injection system and this was used to demonstrate the principle of the electrically-wired immunosensor. The system was shown to be capable of monitoring real-time antibody–antigen interactions in a comparable manner to surface plasmon resonance-based systems, but with greatly reduced equipment complexity. The illustration that physical and temporal separation of bound and unbound species in a competition immunoassay format is unnecessary opens the way to greatly simplified flow-injection immunoassay strategies, with the prospect of single-step FIIA very close at hand.

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