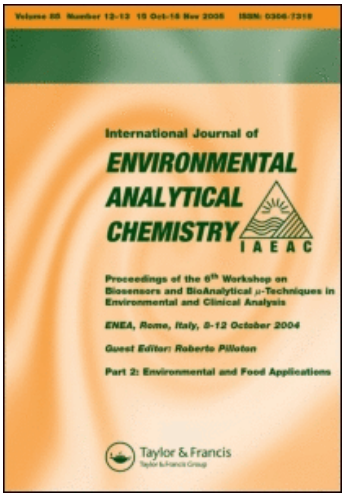

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Practical Study into Enhancing IgG Immobilisation on Screen-Printed Electrodes for Environmental Applications

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PRACTICAL STUDY INTO ENHANCING IgG IMMOBILISATION ON SCREEN-PRINTED ELECTRODES FOR ENVIRONMENTAL APPLICATIONS

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This work is focused on the influence of pH and applied potential for the immobilisation of rabbit IgG on a carbon screen-printed electrode. The orientation of IgG molecule at the surface is fundamental for activity and reproducibility of the immunosensor. As the electrode potential is increased a particular order may be brought to the immobilised antibodies, i.e. the antibodies adapt a more favourable arrangement on the surface to facilitate better binding. The response increased when changing the pH from basic to acidic medium and the reverse trend was observed for the limit of detection (LOD). When a potential was applied to the electrode, the response generally decreased and the LOD increased in the order acidic > basic > neutral pH. The LODs obtained from antibodies immobilised at acidic pH and +100 mV were better than the LODs obtained at other conditions.

Keywords: Immunosensor; Immobilisation; Screen-printed electrode; Antibody orientation; IgG; Biosensors; Alkaline phosphatase; *p*-Aminophenyl phosphate

INTRODUCTION

Screen-printing technology has been around for many years and has been instrumental in the development of biosensors. It offers an easy and inexpensive format for modification to both general and specific applications. Carbon screen-printed electrodes (SPE) are perhaps the most commonly used as the carbon ink may be modified with a range of substances including metallic particles, mediators, biocomponents, etc.

Surface modification or manipulation of solid electrodes determines their performance. Recent report showed that polishing the surface of electrodes greatly enhanced the adsorption of rabbit immunoglobulin (IgG) [1]. Indeed, polishing electrodes has been shown many times to be able to renew the activity of composite electrochemical sensors and biosensors [2–4].

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Immobilisation of biocomponents at electrode surfaces can be achieved by a variety of methods such as passive adsorption [5], covalent binding [6], physical entrapment [7] and cross-linking [8,9]. However, the surface stability of the biocomponent that is achieved by some of these techniques can lead to a decrease in the activity or damage to the protein structure. A number of inert solid supports for antibody immobilisation have been described, including glass wool [10], gold [11] and silica [12]. Numerous coupling strategies have been developed for immobilising antibodies to solid supports, usually based on activating the solid matrix and then binding pre-treated or native antibody. The most popular methods include activating a solid matrix with cyanogen bromide [13] and *N*-hydroxysuccinimide esters. These procedures rely on the random coupling of antibodies via the amine groups resulting to a random orientation. Methods have been developed to try and overcome this random orientation and focus on the site-directed immobilisation of antibodies [14–16] as shown in Fig. 1. Another method consists of hydrazide-activated supports. Coupling is achieved via oxidised carbohydrate functionalities in the antibody structure, forming covalent hydrazide bonds. Another method of immobilisation uses coupling via thiol groups at the hinge region [17].

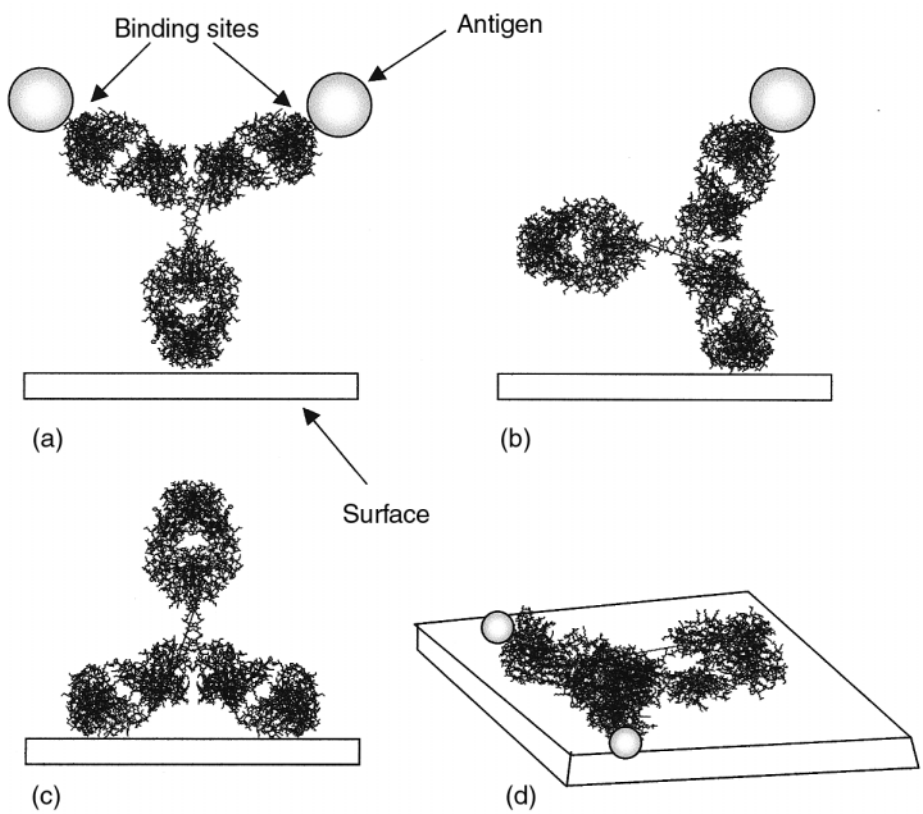

FIGURE 1 Possible orientations of antibody adopted during immobilisation indicating degree of activity: (a) fully active; (b) partially active; (c) inactive and (d) 3D representation of thermodynamically most probable orientation of the antibody on the surface.

Many different methods for immobilisation of antibodies have been described before [18–21], however, no ideal support material or method of immobilisation has emerged to provide a standard for each type of immobilisation. The quality of immunosensors primarily depends upon the selectivity and affinity of the antibodies used in the receptor unit.

In this work, a novel approach is discussed for the site orientation of IgG molecules during passive adsorption to the carbon working area of the SPE. The specific control of the primary immobilisation step is achieved by manipulation of parameters such as applied potential and pH. The analyte used in this study was 2,4,6-trichloroanisole (TCA). Previous research has identified TCA as one of the most important contaminants responsible for the musty/mouldy taste of wine [22–24].

EXPERIMENTAL

Reagents

The substrate for the electrochemical detection of alkaline phosphatase (AP EC 3.1.3.1, type VII-S, Sigma), was 4-aminophenyl phosphate (p-APP) (Universal Sensors). The DEA buffer (pH 9.5) contained 0.1 M diethanolamine, 50 mM KCl (Sigma) and 1 mM $MgCl_2$ (Sigma). The blocking buffer (pH 7.4) contained 50 mM Tris (Sigma), 1 mM $MgCl_2$ and 1% (w/v) bovine serum albumin (BSA, Sigma). Immobilisation buffers contained, for acidic pH, sodium citrate (1% NaCl) (Sigma), for neutral pH, phosphate buffered saline tablets (1% NaCl) (Sigma), and for basic pH, sodium carbonate buffer (1% NaCl) (Sigma). The analyte was TCA (Aldrich) and the polyclonal antibody raised against hapten A, pAb_{76}, was obtained from the department of biological organic chemistry, IIQAB-CSIC, Barcelona, Spain. Hapten A is a derivative of TCA. It was synthesised and conjugated to the enzyme AP by us. All other chemicals were of analytical grade or better, all solutions were prepared daily with doubly distilled water.

Apparatus

Electrochemical workstation BAS 100 B/W (Bioanalytical Systems, BAS, USA) was used to control the three-electrode set-up consisting of a carbon screen-printed strip working electrode, a Ag/AgCl (3 M NaCl, BAS) referenced electrode and a platinum wire auxiliary electrode. A multi-cell for manipulation of IgG immobilisation at SPE was designed.

PROCEDURES

The SPE were prepared using Electrodag® 423 SS carbon ink, Electrodag® 477 SS silver ink for conductor paths, and a Matt Vinyl White MV27 (Apollo Ltd., London, UK) for insulation layers. Screen-printer DEK 247 was used for printing. The squeegee velocity was set to 6. The electrode area was 4×4 mm, i.e. 16 mm^2. Each layer was left for 1 h to evaporate the solvent to obtain a dry path. After printing the last path, the electrodes were cured at 80°C overnight.

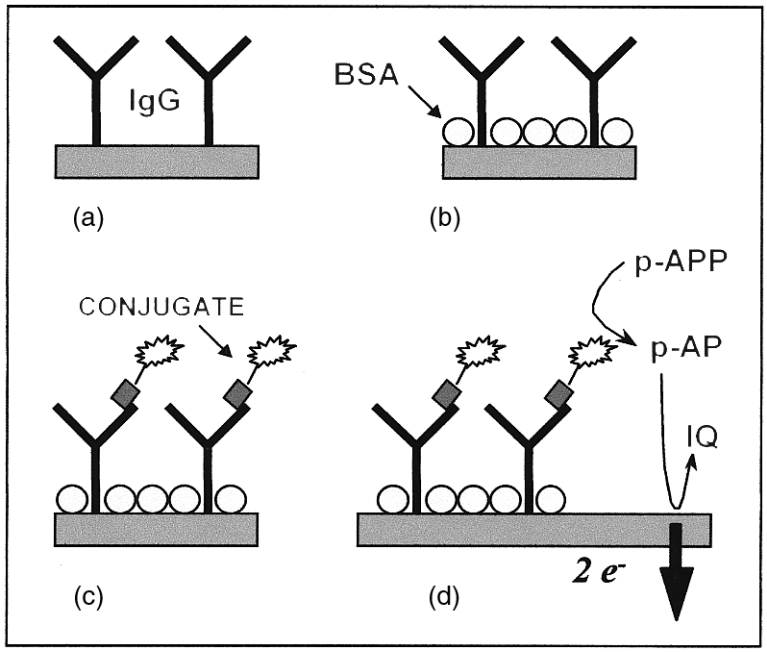

FIGURE 2 Scheme of electrode preparation illustrating steps involved: (a) adsorption of IgG; (b) blocking with BSA; (c) capture of AP conjugate; (d) amperometry of p-AP at +300 mV. IQ: imminoquinone.

A scheme illustrating the experimental design of the biosensor is shown in Fig. 2. The immunoelectrodes were prepared as follows. A drop of 5 μL of 7.5 μg/mL pAb_{76} in immobilisation buffer was spread onto the surface of the working electrode. For the direct capture experiment a serial dilution of antibody was used. 50 μL of immobilisation buffer was placed in each cell, which an electrode would occupy. The electrodes were then placed onto the multi-cell where an applied potential was introduced to the system. The electrodes were incubated for 1 h at room temperature. The unbound IgG was washed away with water. The electrode surface was then blocked by incubating in 200 μL of blocking buffer for 1 h at 37°C. The excess BSA block buffer was washed away with water. For direct capture assay, the electrodes were immersed in 150 μL of hapten A-AP conjugate (3.5 μg/mL). The antigen-antibody capture was allowed to proceed for 1 h at 37°C. For direct competitive assay the electrodes were immersed in 150 μL of a 1 : 1 mixture of hapten A-AP (3.5 μg/mL) and the TCA analyte. A serial dilution of the TCA was done in order to realise a calibration plot. The electrodes were washed for a final time with water and tested amperometrically by adding pAPP at +300 mV in a stirred DEA buffer. The preparation for "blank" electrodes was identical, except that there was no primary immobilisation step.

All amperometric experiments were performed at room temperature (22°C) in a 5 mL stirred batch cell at +300 mV containing DEA buffer. DEA solution containing 1 mM pAPP was injected for amperometry of immunoelectrodes [25,26]. Spectrophotometric detection was done using a microplate reader at 405 nm.

RESULTS AND DISCUSSION

SPE Capture Assay

The effect of manipulating both pH and potential during immobilisation of pAb_{76} for the direct capture assay with hapten A-AP conjugate was investigated. The results of this experiment are depicted in Fig. 3. The capture immobilisation studies show that the best response was achieved at acidic pH and that as the potential was increased the slope increased. This trend in increased sensitivity was observed for acidic, neutral and

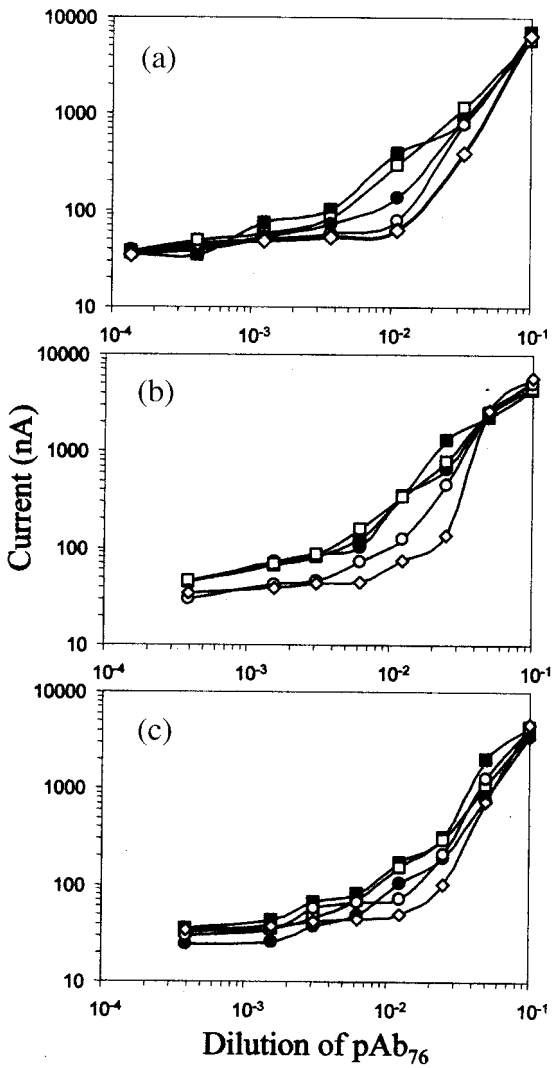

FIGURE 3 Comparison of effect of applied potential: (■) open; (□) +100 mV; (●) +200 mV; (○) +300 mV and (◇) +400 mV, on the immobilisation of pAb_{76} raised against hapten A in capture assay with hapten A-AP (3.5 μg/mL) in (a) acidic, pH 4, (b) neutral, pH 7 and (c) basic, pH 9.

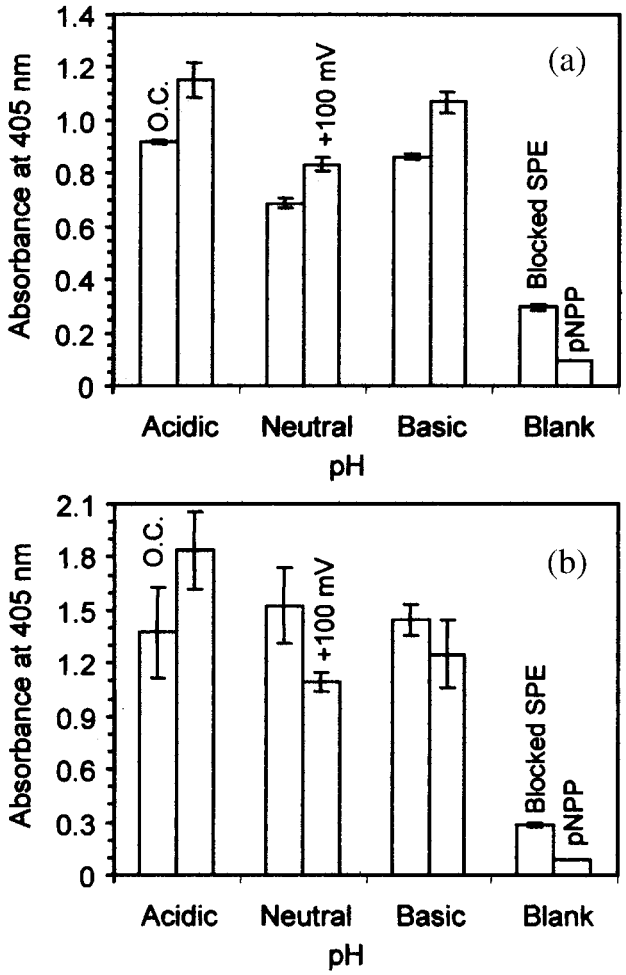

FIGURE 4 Capture comparison of immobilised pAb_{76} on the carbon surface of SPE with (a) goat anti-rabbit IgG labelled with AP and (b) hapten A-AP illustrating effect of pH and potential on affinity using spectrophotometric detection.

basic pH ranges. As there was no apparent effect of pH on this trend, it can be assumed that this order is due to the applied potential. Therefore, as the applied potential increases, more control and order may be brought to the system.

The results for a capture comparison between goat anti-rabbit IgG labelled with AP and hapten A-AP for pAb_{76} immobilised at acidic, neutral and basic pH and at open and applied potential are shown in Fig. 4. These results represent the total amount of pAb_{76} that are actively orientated toward capture for either labelled antibody or hapten. Figure 4(a) shows that at either acidic or basic pH the response is similar, while at neutral pH the response is not as good. As a potential is applied to the immobilisation step there is an increase in response across the pH range. Figure 4(b) illustrates results for a more specific capture assay. The polyclonal antibodies have been raised against this hapten and there is little difference between the responses obtained across the pH range at open potential (O.P). Also, as a potential is applied there is

TABLE I Limit of detection (LOD), IC_{50} and linear range values obtained for competitive assay between free TCA and hapten A-AP with pAb_{76} immobilised under influence of pH and applied potential

pH	*Applied potential* mV	*LOD* ppt	IC_{50} ppb	*Linear range* ppb
4	0	500	59	2.7–180
	100	8	67	0.09–130
7	0	64	27	0.6–110
	100	4900	28	10–64
9	0	44	47	0.2–43
	100	19000	50	28–83

only an increase in response at the acidic pH, while there is a decrease in response at both the neutral and basic pH. The absorbance is also high and the development time is short, in the order of 10–15 min.

SPE Competitive Assay

The influence of pH and applied potential on immobilised pAb_{76} for the competitive assay between free TCA and hapten A-AP was studied. A comparison between antibodies immobilised at different pH ranges and between an open circuit and applied potential during the adsorption step of the IgG molecules was investigated. The results are shown in Table I. The general response trend mirrors that obtained from the capture assay, acidic > basic > neutral pH range. The limit of detection (LOD) varies significantly when comparing both pH and potential parameters. The observed trend is that as the pH is increased from acidic to basic the LOD decreases. There is an initial dramatic decrease in the LOD (by at least three orders of magnitude) as the pH is increased from acidic to neutral and it only decreases slightly further as the pH reaches the basic range.

As a potential is applied to the electrode during the immobilisation stage, the reverse trend is observed for the LOD in comparison to the open circuit system. The LOD increases in the order acidic < neutral < basic pH range for pAb_{76} immobilised at +100 mV. There is also an initial significant difference in the LOD as the pH is increased. Perhaps the most interesting result obtained from this study is the fact that the LOD achieved for the competitive assay, where the pAb_{76} were immobilised at acidic pH at + 100 mV, was better than the LOD obtained from the competitive assay where the pAb_{76} were immobilised at basic pH and at no potential applied.

CONCLUSIONS

Manipulation of antibody immobilisation by control of orientation at the surface of the SPE enhances the response from the antibody–antigen interaction. As the potential is increased a certain order is brought to the immobilised antibodies. This can be seen from the increased slope that is observed during the capture assay Fig. 3. This order is maintained irrespective of the buffer pH used for immobilisation and is only dependent on the applied potential.

During immobilisation, two forces (electrostatic and adsorptive) are involved in the orientation of the antibody at the carbon surface of the SPE. The antibody net charge is

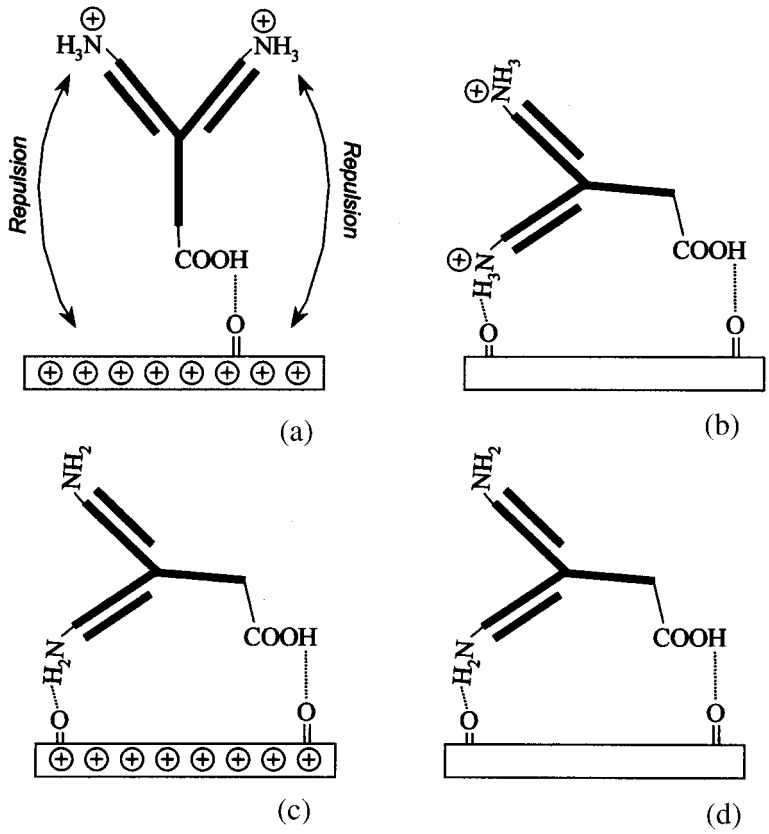

FIGURE 5 Scheme illustrating dipole–dipole interactions occurring between antibody and surface of SPE during immobilisation at: (a) acidic pH and +100 mV; (b) acidic pH and no potential applied; (c) neutral and +100 mV; and (d) neutral and no potential applied.

determined by the pH value of the solution that drives the free amino and carboxylic groups through the pK_A equilibria. pH changes cause formation of local charges at the Ab molecule, which will determine how the Ab will orientate at the charged electrode surface. Carbon electrode contains carboxyl groups that may, when deprotonised, repulse negatively charged or attract positively charged molecules, or orientate dipoles. Hydrogen bonds may also play an important role. An applied potential at the electrode will be added (or subtracted) to the surface charge. These phenomena may help to repulse the capture site of the Ab away from the electrode surface and further free it for antigen binding (Fig. 5(a)). Other combinations of pH and potential will lead to situations shown in Fig. 5 which will be most likely assembled in 3D as shown in Fig. 1(d). In this case, as the surface is very close to both binding sites, large antigens may be discriminated sterically, while small ones will bind easily. A low potential of +100 mV was chosen, because higher positive or negative potential will oxidise or reduce the Ab itself, leading to random results.

This theory may explain the experimental data shown in Fig. 4(b). Electrostatic forces are much stronger than other forces driving adsorption. These dipole–dipole and ion–dipole interactions are responsible for both attraction and repulsion of areas within the antibody molecule for the surface. However, these forces do not

always compliment the capture of the antigen, as can be seen from Fig. 4(b) where initially at acidic pH there is an increase in activity as a potential is applied, but as the pH is raised to neutral and basic the antibody activity toward the hapten decreases. This is the reverse trend as seen for the larger antibody molecule and may have a steric effect on the competitive assay where smaller molecules can diffuse more easily to the surface (Fig. 1d).

The effect of pH on sensitivity for immobilised antibodies during the competitive assay was in the order acidic > basic > neutral. As a potential was applied this trend was also observed, however, the response was generally lower. The LOD with no applied potential during immobilisation increased in the order basic < neutral < acidic, and the reverse trend was observed when a potential was applied. In fact the LOD obtained from antibodies immobilised at acidic pH at +100 mV was better than the LOD obtained from antibodies immobilised at basic pH and no applied potential. Comparison of the IC_{50} values showed a good correlation between data obtained with an applied potential and an open circuit. One may see a pivotal effect, with the IC_{50} value (Table I) as a focal point and the LOD decreasing or increasing depending on pH.

During the immobilisation stage the need to focus on the surface interactions at the carbon electrode–electrolyte interface is vital. In effect it is these two parameters, which through simultaneous manipulation can either enhance or reduce sensitivity and detection limit. A balance between pH and applied potential must be established in order to achieve the optimum conditions during immobilisation. In this case the best result was obtained by immobilising at acidic pH 4.0 and with an applied potential of +100 mV.

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