Labeless Immunosensor Assay for Myelin Basic Protein based upon an AC Impedance Protocol

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Abstract.

This paper describes the development and characterisation of a labeless immunosensor for myelin basic protein (MBP) and its interrogation using an AC impedance protocol. Commercial screen-printed carbon electrodes were used as the basis for the sensor. Polyaniline was electrodeposited onto the sensors and this modified surface then utilised to immobilise a biotinylated antibody for MBP using a classical avidin-biotin approach.

Electrodes containing the antibodies were exposed to solutions of MBP and interrogated using an AC impedance protocol. The real component of the impedance of the electrodes was found to increase with increasing concentration of antigen. Control samples containing a non-specific IgG antibody were also studied and calibration curves obtained by subtraction of the responses for specific and non-specific antibody based sensors, thereby accounting for and eliminating the effects of non-specific adsorption of MBP. A logarithmic relationship between the concentration of MBP in buffer solutions and the impedimetric response was observed.

Keywords: myelin basic protein, antibody, AC impedance, immunosensor, polyaniline.

INTRODUCTION

The principle of immunoassays was first established by Yalow and Berson[1] in 1959. Their work led to the development of the widely used radioimmunoassay to examine the properties of insulin-binding antibodies in human serum, using samples obtained from subjects that had been treated with insulin.

Independently within unconnected work, the concept of a biosensor was pioneered by Clark and Lyons[2] in 1962. The original methodology involved immobilising enzymes on the surface of electrochemical sensors so as to exploit the selectivity of enzymes for analytical purposes. This idea has remained virtually unchanged since the original design, although the field has undergone continual technological developments over the last forty years.

The incorporation of antibodies into conducting polymer films was first reported[3] in 1991. Anti-human serum albumin (anti-HSA) was incorporated into a polypyrrole film, which was galvanostatically polymerised onto a platinum wire substrate. When grown in the absence of a counterion, a poor polymeric film (both in appearance and electrochemical properties) was formed, suggesting that the presence of a counterion was necessary for the polymerisation process to be successful. Amino acid analysis of the polymer using a leucine marker showed that approximately 0.1% w/v (0.2 μg) of the antibody was incorporated into the matrix. When the pyrrole anti-HSA electrode was exposed to 50 $\mu g\ ml^{-1}$ HSA for ten minutes, a new reduction peak was observed at a potential of approximately +600mV vs. Ag/AgCl. This peak increased in magnitude after a further thirty minutes in the same solution - and it was suggested this could be due to an antibody/antigen interaction with the polymer. Further work by the same group gave rise to reports of a reversible real-time immunosensor[3]. Other early work utilised a pulsed amperometric detection technique

for other analytes, including p-cresol[4], thaumatin[5] and polychlorinated biphenyls[6]. Since this early work there has been burgeoning interest in the development of electrochemical immunosensors - as detailed in several recent reviews[7-9].

Antibody-antigen interactions are by their very nature complex and the reproducible response characteristics of immunosensors requires that the affinity reaction is minimally perturbed by the fabrication procedure. We have previously shown that up to 2-3 μg antibodies for BSA and digoxin may be successfully incorporated into conducting polymer films by entrapment in a growing polypyrrole film with no detrimental effect to antibody activity[10]. Electrochemical interrogation of these films demonstrated selective interactions with the target antigens. Further work utilised an AC impedance protocol[11] as the method of interrogation for these films - and led to the development of immunosensors for digoxin and bovine serum albumin. Later work by our group studied approaches for immobilisation of antibodies onto polyaniline coated screen-printed carbon electrodes utilising a classical avidin-biotin chemistry. This enabled the construction of immunosensors for the fluoroquinolone antibody ciprofloxacin[12].

Myelin is a complex membrane which forms a sheath around axons in vertebrate species[13]. It is the most abundant membrane structure within the vertebrate nervous system, allowing the fast conduction of nerve impulses through the nerve fibres[14] due to its high resistance and low capacitance. Myelin basic protein is a cytoplasmic protein important in the process of myelination of nerves in the central nervous system since it comprises the bulk of the main line of compact myelin[13] and up to 30% of the protein content of myelin overall[14].

A demyelinating disease is any disease of the nervous system in which the myelin sheath of neurons becomes damaged. This impairs the conduction of signals in

the affected nerves, causing impairment in sensation, movement, cognition, or other functions, depending on which nerves are involved. Multiple sclerosis (MS) which is a chronic and inflammatory disease of the central nervous system is an example of a demyelinating disease. Multiple sclerosis affects neurons within the brain and spinal cord that are involved with sensory and motor control functions. The name multiple sclerosis is derived from the multiple scars (or scleroses) that can be observed on the myelin sheaths. At the time of writing multiple sclerosis does not have a cure, although several treatments are available that may slow the appearance of new symptoms.

Multiple sclerosis is currently often diagnosed following occurrence of a number of indicative symptoms. Magnetic resonance imaging (MRI) may be used to evaluate patients who display these symptoms. MRI is capable of imaging areas where demyelination is occurring, with lesions caused by demyelination showing up as bright areas on the scan. Cerebrospinal fluid can also be utilised in the diagnosis of MS and the presence of immunoglobins can be detected in the majority of MS sufferers. It should be noted however that other conditions can also lead to the presence of these species[15].

Other demyelinating diseases include transverse myelitis, Guillain-Barré syndrome, and progressive multifocal leukoencephalopathy. There are various identified potential causes of demyelination, including autoimmune reactions[16], infectious agents[17], genetic conditions[18] and also exposure to compounds such as carbamate pesticides[19].

The presence of antibodies against myelin proteins such as MBP can be a predictor of multiple sclerosis[20]. Clinical studies of 103 patients suffering from MS demonstrated that the presence of antibodies for MBP and myelin oligodendrocyte

glycoprotein within serum could be correlated with a greatly increased level of relapse compared to patients without these antibodies. Other demyelinating conditions also lead to elevated levels of MBP in cerebrospinal fluid[21-22], for example in cases of closed head trauma[23] or acquired immunodeficiency syndrome (AIDS) dementia complex (ADC)[24]. A significant correlation has also been determined between the clinical stage of the childhood-onset cerebral form of adrenoleukodystrophy and cerebrospinal fluid myelin basic protein levels[25]. MBP levels between 4 and 8 ng l^{-1} in cerebrospinal fluid may indicate a chronic breakdown of myelin, or recovery from an acute episode. MBP levels greater than 9 ng ml^{-1} indicate that active demyelination may be occurring. Normally there should be less than 4 ng ml^{-1} of myelin basic protein in the cerebral spinal fluid.

Increased myelin basic protein levels can also occur as a result of a stroke. A stroke, also known as cerebrovascular accident (CVA), is an acute neurological injury in which blood supply to a part of the brain is interrupted. Strokes involve a sudden loss of neuronal function due to arterial or even venous disturbance in cerebral perfusion. The part of the brain with disturbed perfusion no longer receives an adequate supply of oxygen. This initiates the ischemic cascade which causes brain cells to die or be seriously damaged, impairing local brain function. An indirect result of stroke is an active breakdown of myelin or demyelination and therefore the levels of myelin basic protein in cerebral spinal fluid (CSF) can act as markers for stroke.

Commercial ELISA tests exist for myelin basic protein; Diagnostic Systems Laboratories (Texas) for example manufacture an ELISA which can measure levels of MBP in cerebrospinal fluid in four hours.

We have within this work developed a labeless immunosensor for MBP. The sensor utilises screen-printed carbon electrodes, and is modified firstly by deposition

of a conducting polymer (polyaniline) and thence biotinylating reagent. Complexion of the immobilised biotin with avidin allows the further binding of biotinylated antibodies via standard avidin-biotin interactions as described earlier[12] and as shown schematically in Figure 1. The resultant electrodes are capable of detecting the antigen within the required physiological range. Control electrodes containing non-specific IgG have also been fabricated to measure the effects of non-specific binding and permit the subtraction of these unspecific interactions from the response towards specific binding of MBP. This approach helps to increase the stability and reliability of these sensors when applied to clinical samples.

EXPERIMENTAL SECTION

Sodium dihydrogen orthophosphate, disodium hydrogen orthophosphate, sodium chloride, hydrochloric acid, were obtained from BDH (Poole, Dorset, UK). Potassium chloride was obtained from Fisher Scientific UK Ltd, Loughborough, UK. Aniline, MBP (from mouse, M2941), myelin basic protein antibody (monoclonal anti-MBP from rat, M9434), polyclonal IgG (from mouse, I5381), the biotinylation kit (part no. BK101), biotin 3-sulfo-N-hydroxysuccinimide, neutravidin, bovine serum albumin (BSA), potassium ferrocyanide and potassium ferricyanide were obtained from Sigma-Aldrich, Gillingham, Dorset, UK. All water used was obtained from a Purelab UHQ Deioniser (Elga, High Wycombe, UK). Commercial screen-printed carbon electrodes (Figure 2) containing carbon working and counter electrodes and an Ag/AgCl reference electrode were obtained from Microarray Ltd, Manchester, UK. The surface area of the working electrode was 0.2178 cm^2.

Phosphate Buffered Saline (PBS), pH 7.4 stock solution was prepared containing 0.14 mol l^{-1} NaH_2PO_4, 0.52 mol l^{-1} Na_2HPO_4 and 0.0051 mol l^{-1} NaCl.

Aniline buffer, pH 1-2 was prepared containing 0.5 mol l^{-1} KCl, 0.3 mol l^{-1} HCl and 0.2 mol l^{-1} aniline.

For antibody biotinylation, the procedure outlined in the BK101 kit was followed (see manufacturers instructions for details). Biotinylated antibodies were kept frozen in aliquots of 200 µl at a concentration of 1 mg ml^{-1} until required.

Cyclic voltammetry (Sycopel Potentiometer, Sycopel Scientific, Tyne & Wear, UK) was utilised to deposit polyaniline films on the carbon electrodes. Screen-printed carbon electrodes were placed in aniline buffer and cycled from -200 mV to +800 mV vs. Ag/AgCl for approximately 20 cycles, (the number of cycles could be varied to ensure the same quantity of polyaniline was deposited on each electrode). Deposition was terminated at +800 mV to ensure the polyaniline remained in its conducting form. Following deposition the polymer coated electrodes were rinsed in water.

30 µl of biotin-sulfo-NHS (10 mg ml^{-1} in water) was placed on the polymer coated working electrode surface for 24 hours. The sensors were rinsed with copious water and 30 µl of avidin (10 µg ml^{-1} in water) placed on the working electrode for 1 hour - followed by further rinsing in water. Then 30 µl biotinylated antibody (1 mg ml^{-1} in water) was added followed by rinsing. Finally, non-specific interactions were blocked by BSA (10^{-6} M in PBS); the sensors are ready to use at this point, however, if opted, can be stored in PBS at 4°C up to 24 hours.

AC impedance measurements were performed using an ACM Auto AC DSP frequency response analyser. Antigen solutions for AC impedance were prepared by diluting the required concentration of antigen in 30ml of PBS, pH 7.4. A range of concentrations were utilised; since levels should be less than 4 ng ml^{-1} for a healthy

individual, we set our minimum level at 1 ng ml^{-1} with an upper limit of 1 μg ml^{-1}, so as to cover the clinical range.

The sensors were first interrogated without antigen addition. Following this, each sensor was exposed to the required antigen concentration for 30 minutes, rinsed well with deionised water and then subjected to impedance interrogation. Potassium ferrocyanide (10 mM) and potassium ferricyanide (10 mM) in PBS buffer were utilised as a redox couple for impedimetric measurements. Three electrodes were used for each measurement. A frequency range from 10 kHz to 1 Hz was measured, with a peak amplitude of 5 mV and a DC offset of +400 mV against Ag/AgCl.

RESULTS AND DISCUSSION

Deposition of polyaniline:

The voltammograms for the deposition of polyaniline onto the screen printed carbon electrodes[12] are depicted in figure 3 and imply a steady in situ formation of polymer at the electrode surface. As the number of scans increases, voltammetric peaks are seen between +350-400 mV vs Ag/AgCl, corresponding to the oxidation and reduction of surface bound polyaniline. The increase in current from scan 10 to 20 is due to the increase in polyaniline thickness and coverage of the electrode[11].

Impedance profiles of the electrodes:

A series of Nyquist curves were obtained for the sensors after exposure to various levels of MBP in PBS (Figure 4a). As can be seen, there is a steady decrease in the impedance of the electrodes with increasing antigen concentration. The relative impedances of the system for various antigen concentrations, can be obtained by dividing the impedance for each frequency when a given concentration of antigen has

been applied - by the impedance (at the same frequency) for the sensor exposed to zero antigen concentration; these are shown in Figure 4b. This figure clearly shows that the impedance of the electrodes steadily increases with the concentration of MBP. Myelin basic protein is a mixture of epitopes with molecular weights of 14-21 kDa[14]. However the commercial antibody recognises the peptide strand between amino acids 36 to 50 which is common to all the epitopes. The increases in impedance are most probably due to binding of the protein to the surface, since the protein molecules are likely to be insulating in nature. As can be seen, much larger increases in impedance are observed at the lower frequencies, which again indicates deposition of insulating material. Therefore it was decided that changes in impedance at 1 Hz would be used as a measurement of antigen binding.

The impedance spectra consists of two components, the real (Z') component where the impedance in phase with the AC potential waveform is measured and the imaginary (Z") where the impedance is 180° out of phase. It is important to differentiate between the individual components of the total impedance of the cell. Previous work by our group showed that while both the imaginary and real components increase, the increase in the real component dominated the total increase in the impedance[11,12]. Although in this case changes in both real and imaginary components are visible and again the real component is the major component of the total impedance, it was also found (perhaps more importantly) that the real component offers far greater reproducibility in comparison to the imaginary contribution[11,12].

Figure 5a shows the percentage increase in Z' across a range of antigen concentrations. As can be seen, there is a steady increase in impedance as antigen concentration increases towards a concentration of about 100 ng ml^{-1}. Levels higher than 100 ng ml^{-1} do not appear to lead to even higher impedances, but rather tend

towards a plateau, figure 5a. This possibly indicates saturation of the specific binding sites. Any further changes in impedance beyond this level are likely to be due to non-specific interactions.

Non-specific interactions have the potential to interfere with immunosensor performance, leading to erroneously elevated results. This could be addressed by utilisation of a second sensor containing either no antibodies - or alternatively by exploitation of a non-specific antibody. For this reason an identical set of immunosensors were fabricated utilising a non-specific IgG antibody in place of the specific MBP antibody. The non-specific antibody was a commercial polyclonal IgG antibody, from the same species (mouse) as the specific MBP antibody. This was biotinylated using an identical protocol and then incorporated into the sensor using identical conditions, buffers, concentrations etc as utilised for the specific antibody electrodes. Whereas specific binding of MBP will only occur when the anti-MBP antibody is present, electrodes fabricated using a non-specific antibody should undergo the same non-specific binding events as those fabricated using specific antibodies. It therefore follows that utilisation of both types of electrodes followed by simple subtraction of non-specific electrode response from those of the specific electrodes should allow the non-specific responses to be cancelled out.

Results for the non-specific IgG electrodes were obtained in exactly the same manner as for the specific electrodes and the calibration plot for these measurements are shown within figure 5b. As can clearly be seen, there is a much lower response for the non-specific antibody, showing that although there are non-specific interactions, between the concentration ranges of 1-100 ng ml^{-1}, they comprise a minor component of the detected response.

Once the non-specific responses have been subtracted (figure 5a - figure 5b), a corrected plot (figure 5c) shows the calibration curve for the corrected sensor response. Between a concentration range of 1-100 ng ml^{-1}, there is a near linear correlation of the corrected impedance change with the log_{10} of concentration (R^2=0.976). Between the lower range of 1-15 ng ml^{-1}, the correlation of the impedance change with the log_{10} of concentration is further improved (R^2=0.990). The limit of detection of this system was found to be approximately 1 ng ml^{-1}, based a multiple of x3 the standard deviation of the baseline samples - and combined with the good linear response, indicates that we have developed a sensor with good performance within the required range of detection for myelin basic protein.

As yet we have not attempted to either reverse the antibody-antigen binding process - or remove the antibody and replace it with fresh protein. In view of the low potential unit cost of the electrodes it would be simpler just to use sensors as single use devices. However for this to be feasible there must both be good sensor to sensor and batch to batch reproducibility. We have found that repeated runs on the same electrode give reproducible results with RSD's<2%. We have also determined the electrode-to-electrode reproducibility of responses to have RSD's<5% variability in a series of trials.

CONCLUSIONS

We have demonstrated the construction of an immunosensor for the myelin basic protein using a combination of screen-printed electrodes coated with conducting polyaniline and an immobilised polyclonal antibody. Interrogation of the electrodes by AC impedance demonstrated the detection of the antigen. Linear correlation of the impedance change with the log_{10} of concentration (R^2=0.977) was observed between

concentrations of 1-100 ng ml^{-1}. A similar logarithmic relationship was observed in our earlier work with ciprofloxacin immunosensors[12].

Further work utilising samples of cerebrospinal fluid with various levels of MBP will be undertaken, and it is expected that the use of a dual antibody (specific and non-specific) electrode system should allow subtraction of any non-specific binding events to allow the detection of MBP levels in "real" samples. These studies will be reported in a future publication.

One major drawback associated with immunosensors is the strength of the antibody/antigen binding which can render the interaction essentially irreversible. This would lead to the sensor rapidly becoming saturated upon multiple usage, a process which would be very difficult to reverse by simple washing. The use of inexpensive screen printed electrodes as a template for these sensors will allow for the production of simple - and more importantly inexpensive single-use immunosensors, thereby eliminating the need for washing and re-use of sensors.

Commercialisation of this research is proceeding through a new spin-out company ELISHA Systems Ltd., who are in the process of developing a range of labeless immunosensors for a number of different antigens.

ACKNOWLEDGEMENTS

This work, including funding for GT, GZG and FD, has been supported by the European Community Framework VI NMP2-CT-2003-505485, “ELISHA” contract.

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LIST OF FIGURES.

Figure 1. Schematic of antibody modified electrodes showing the assembly of avidin and biotinylated electrodes and binding of biotinylated antibody.

Figure 2. Screen-printed carbon electrodes used within this work.

Figure 3. Deposition of conducting polyaniline films by cyclic voltammetry between -0.2 to +0.8V at 50 mv s^{-1}; current transients are shown for the 1st, 10th and 20th cycles.

Figure 4. (a) Bode and (b) Nyquist plots of a typical specific anti-MBP modified electrode exposed to various concentrations of antigen (MBP); for brevity not all concentrations are shown, but just for 0, 1, 2, 4, 8, 10 and 100 ng ml^{-1}.

Figure 5. Calibration curves showing the increase in the real component of impedance at 1Hz for: (a) specific anti-MBP modified electrodes exposed to varying concentrations of antigen (MBP) (b) IgG modified electrodes exposed to antigen under identical conditions (c) corrected calibration curves where the non-specific response has been subtracted form the specific response (curve a – curve b). All data points are means for the responses of three electrodes; error bars give a measure of the reproducibility of the system.

Fig 1

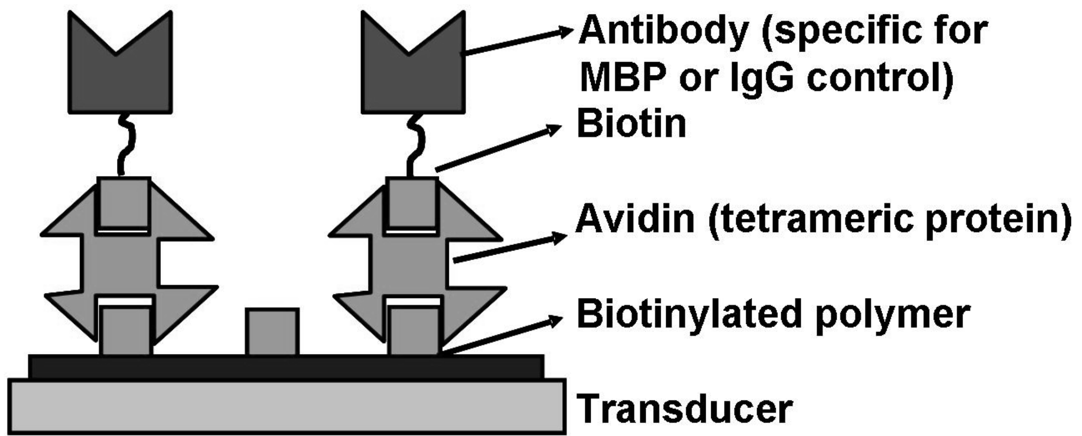


Fig 2

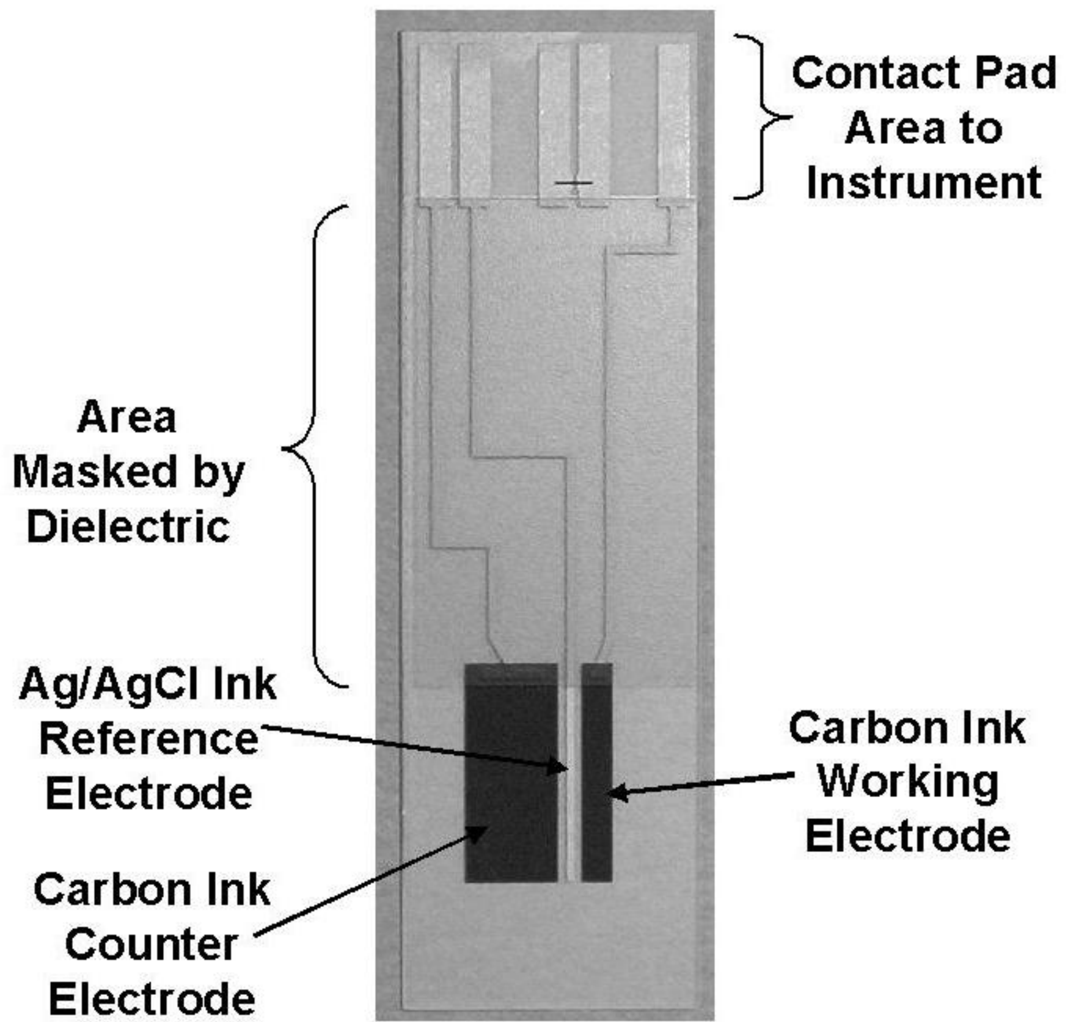


Fig 3

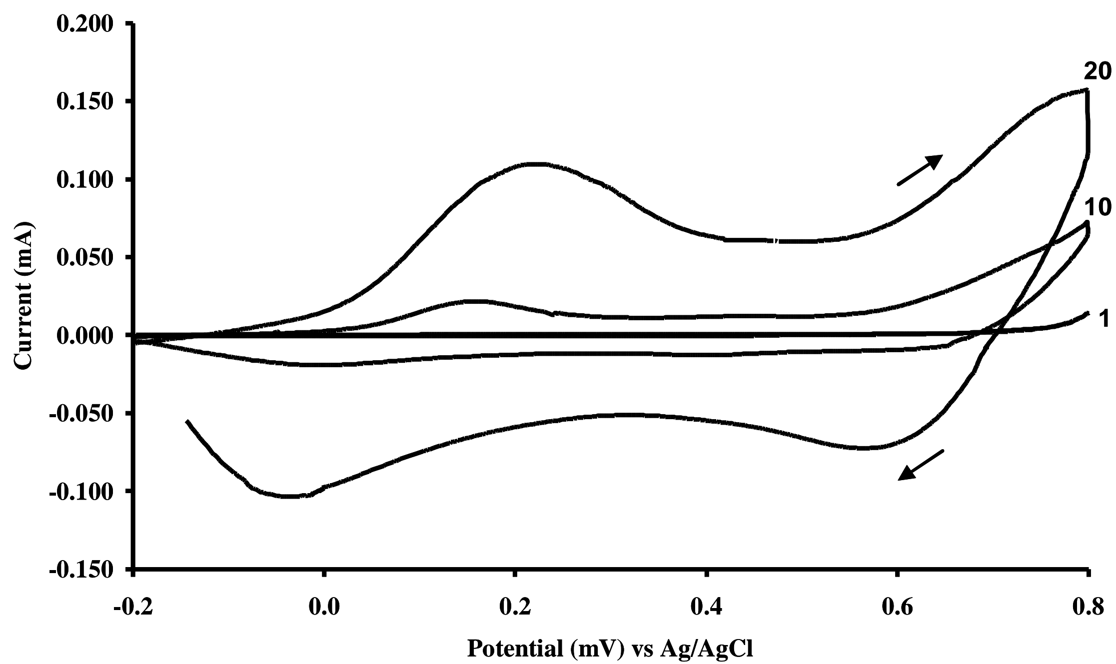
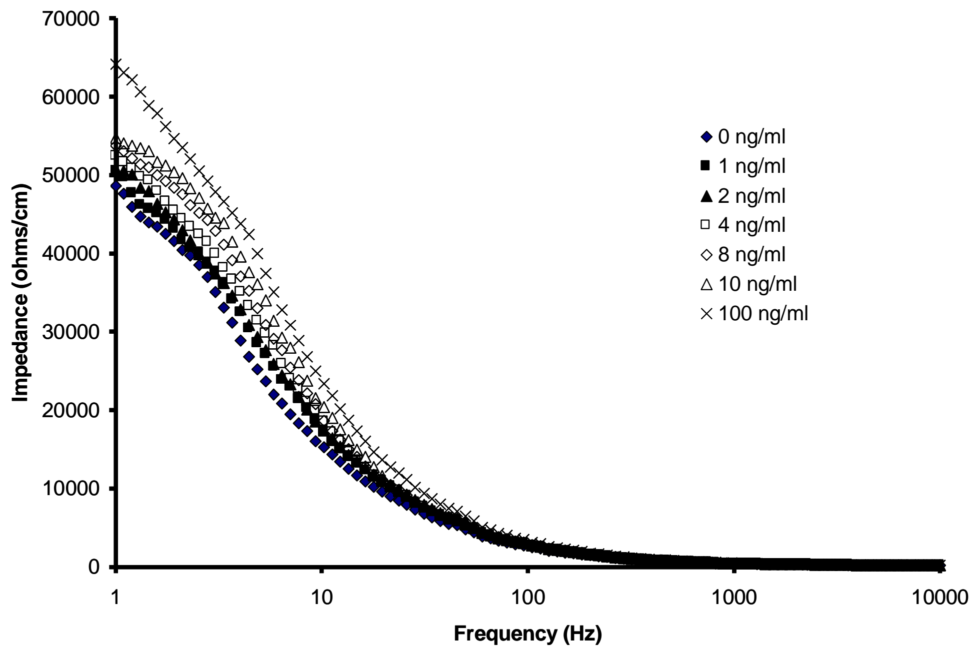


Fig 4.



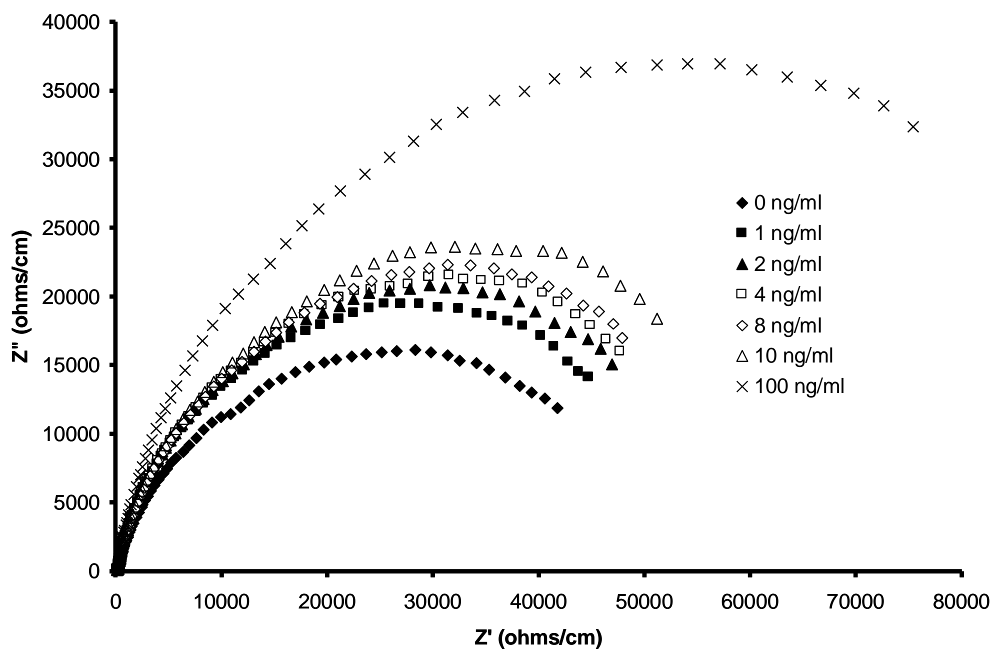


Fig 5