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"Species Differentiation by DNA-modified Carbon Electrodes using an AC Impedimetric Approach"

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Abstract

A simple and novel electrochemical biosensor based approach is described for differentiating between differing species of fish on the basis of DNA hybridisation events. Screen-printed carbon electrodes modified with a variety of polymers were used to immobilise commercially available DNA in a single-stranded form. AC impedimetric measurements were firstly carried out on these systems and then upon exposure to single-stranded DNA solutions. When the electrode and solution DNA were complementary, a large drop in impedance was measured; this did not occur for non-matching DNA exposures. DNA hybridisation sensors for closely related species of fish were in the first instance developed as a demonstration for this approach. Species of fish such as herrings and salmon could be differentiated by this method. This sensor format offers great promise for many DNA hybridisation applications and lends itself to mass fabrication due to the simplicity and inexpensiveness of the materials and methods used. The hybridisation results were confirmed by use of ellipsometry to measure the characteristics of similar films deposited on silicon substrates.

Introduction

Biosensor technology has developed into an ever expanding and multidisciplinary field since the Clark enzyme electrode was first reported [Clark and Lyons1962]. The determination of the human genome, the need for detection of biological pathogens and the development of genetically modified organisms have all led to a demand for simple portable DNA detection and characterisation tools. This has led to a great deal of interest in electrochemical DNA hybridisation biosensors [Gooding 2002].

Detection of and discrimination between species by studying their DNA leads directly from being able to detect and follow DNA hybridisation events. For example DNA from *Mycobacterium Tuberculosis* [Wang *et* al 1997a] can be detected using a suitable biosensor at levels as low as 3.4 nmol l^{-1} and 0.6 nmol l^{-1} for human cytomegalovirus [Azek *et al* 2000]. Usually techniques like these require use of electroactive labels or intermediates such as enzyme-substitution or transition metal complexes which bind to the DNA strands. It follows that a technique that can directly detect hybridisation offers many advantages in terms of simplicity in comparison to those that require labels.

For a successful electrochemical DNA biosensor, we firstly require a recognition layer, usually a single stranded DNA which will selectively hybridise with its counterpart. Hybridisation, if it occurs, must lead to a measurable change in the properties of the electrode layer, since during the binding of two strands of DNA, no new molecules, electrons or photons are produced.

Many workers have studied the immobilisation of DNA on electrodes surfaces [Gooding 2002]. A variety of methods for binding single stranded DNA to an electrode are available. The strong binding of thiols to gold can be used to attach modified nucleic acid strands to electrode surfaces [Levicky *et al* 1998], however, the use of thiol-substituted DNA by itself has a tendency to give poor hybridisation results due to DNA's tendency to bind flat to the gold surface. Diluting the DNA with simple alkane thiols was found to release all but the modified end of the DNA from the surface, leading to greater freedom of movement and better hybridisation of the DNA strands [Levicky *et al* 1998].

Cationic surfaces have been shown to successfully immobilise DNA by electrostatic interaction. Nicolini *et al* (1997) showed that LB films of octadecylamine deposited from subphases containing *E. coli* plasmid DNA incorporated the DNA in a single-stranded form between the layers. Soaking these systems in solutions of single-stranded DNA led to further incorporation of DNA and possible formation of the double helix. Others workers have used electrodeposition onto screen-printed carbon (Mascini *et al* 2001) pre-treatment of glass slides with polyethylene imine solution (Lang and Liu 1999) or deposition onto amino-silanised glass (Lemesklo *et al* 2001) to immobilise DNA on electrode surfaces. Hybridisation of complementary DNA was shown to occur selectively, although whether actual formation of a DNA double helix occurs is still in doubt, and it is possible that a non-helical duplex may be the preferred structure (Lemesklo *et al* 2001). Several reviews on DNA biosensors (Gooding 2002, Wang *et al* 1997b, Pividori *et al* 2000) have been published, detailing much of this previous work.

Electrically conductive polymers have been shown to be suitable substrates for the immobilisation of DNA. Oligonucleotides could be grafted to polypyrrole films and shown by quartz crystal microbalance and photocurrent spectroscopy to hybridise with their counterparts (Lasalle *et al* 2001). Simple incorporation of oligonucleotides within polypyrrole films has been shown by Wang's group to give sensors which upon hybridisation give transient current increases allowing rapid detection of the counterstrand (Wang *et al* 1999, Jiang and Wang 2001). Hybridisation of DNA immobilised in polypyrrole with its counterpart has been detected by the use of AC impedance (Cai *et al* 2003).

Previous work within our group has studied the immobilisation of species such as enzymes and/or antibodies within conducting polymer films or microelectrodes as well as their use as biosensors (Barton *et al* 2004). Antibodies for example have been successfully incorporated into polypyrrole films and this has allowed affinity based recognition for their antigens to be detected by AC impedance techniques (Grant *et al* 2003, 2004). We have attempted within this paper to describe our utilisation of similar approaches for DNA immobilisation to allow hybridisation events to be followed for analytical purposes.

Carbon screen printed electrodes were fabricated and attempts made to immobilise DNA on their surfaces using either electrostatic adsorption approaches on polyethylenimine (Nicolini *et al* 1997), or immobilisation via incorporation into electrodeposited polyaniline or polydiaminobenzene (Barton *et al* 2004). These electrodes were then placed into solutions of single stranded complementary DNA and their AC impedance monitored with time. Control experiments were performed using buffer or non-complementary DNA.

Experimental Section.

Herring DNA and salmon DNA were purchased from the Sigma Chemical Company (Poole, Dorset, UK). Aniline hydrochloride, disodium hydrogen orthophosphate monohydrate, sodium dihydrogen orthophosphate 12-hydrate, sodium chloride, and diaminobenzene dihydrochloride (all 'AnalaR' grade), were purchased from BDH (Poole, Dorset, UK). All chemicals were used without further purification.

All water used was purified with a ELGA Purelab UHQ purifier. The pH7 buffer was made by dissolving $NaH_2PO_4H_2O$ (0.55 g), $Na_2HPO_412H_2O$ (2.11 g) and NaCl (7.73 g) in water and making up to 1 litre.

Screen printed electrodes (Fig. 1a) were designed with carbon working and counterelectrodes and a Ag/AgCl reference electrode. The electrodes were fabricated using a DEK 248 screen printer. Conductive inks (carbon type 422SS and Ag/AgCl type 6088SS) were supplied by Acheson Industries Europe (Reading) and printed onto Melinex plastic sheets.

For the polyethylene imine coated films, the working electrode was dipped into a 1% solution of polyethylenimine for 5 minutes, withdrawn and allowed to dry. The electrode was then placed in single stranded herring or salmon DNA solution (0.2 mg/ml in water denatured by boiling for 5 minutes) for 5 minutes and then rinsed. For the ellipsometric measurements the substrates were silicon wafers with a native oxide layer, cut to size. They were then immersed in 1% polyethylenimine solution for 5 minutes, removed and dried, then placed in ssDNA solution for 5 minutes, rinsed and dried.

For electrodeposition of DNA containing polyaniline films, a technique based on one previously reported (Barton *et al* 2004) was used. Aniline hydrochloride (20 mg) and herring DNA (6 mg) were mixed in water (30 ml) and stirred overnight to allow good dissolution. This solution was then heated to 95^{o}C for 5 minutes to denature the DNA into a single stranded form. The solution was rapidly cooled to room temp using crushed ice. 0.17 ml of conc. HCl was added to ensure polyaniline deposited in the conductive form (Cooper and Hall 1992). A screen-printed electrode was then placed

into the solution and voltammetrically scanned for 20 cycles (-800 to +800 mV vs Ag/AgCl, 50 mV s^{-1}), so as to deposit polyaniline/DNA as shown by the cyclic voltammogram.

A similar method was used for electrodeposition of DNA containing polydiaminobenzene films (Barton *et al* 2004). O-diaminobenzene dihydrochloride (27 mg) and herring DNA (6 mg) were mixed in pH 7 buffer (30 ml) and stirred overnight to allow good dissolution. This solution was then heated to 95^{o}C for 5 minutes to denature the DNA into a single stranded form and again rapidly cooled to room temp in crushed ice. A screen-printed electrode was then placed into the solution and scanned voltammetrically for 20 cycles (0 to +800 mV vs Ag/AgCl, 50 mV s^{-1}), causing deposition of polydiaminobenzene/DNA as shown by the cyclic voltammogram.

Batches of these electrodes were fabricated and interrogated via AC impedimetric approaches. Electrodes were immersed in fresh solutions of single stranded DNA solutions (0.2 mg/ml herring, salmon or calf thymus in pH 7 buffer) and the impedance measured with time over a range of frequencies (1Hz-10kHz). Initial investigations were carried out using heated solutions (i.e. straight after boiling) but the change in temperature had major effects and appeared to mask the effects of any hybridisation. For this reason solutions were rapidly cooled to room temperature in ice before interrogation of the films was attempted.

The ellipsometric measurements were performed using M-2000V^{TM} spectroscopic ellipsometer, J.A.Woollam Co., Inc. The spectra and ellipsometric parameters Ψ and Δ in the range of 370 -1000 nm were measured using the DARCE^{TM} (diode array rotating compensator ellipsometry) method. Since the parameter Ψ, related to the amplitude ratio of *p* - and *s* - components of polarised light ($\Psi=tn(A_p/A_s)$) is not very sensitive to the changes in thin film thickness, the spectra of the other parameter $\Delta = \varphi_p - \varphi_s$, which is a phase shift between *p* - and *s* - components, is presented here.

Ellipsometric measurements were performed at an angle of incidence of 68^{o} in the special cell (1) , shown schematically in Fig. 1(b). The cell containing two transparent

windows (2) was sealed by the silicon sample (3) through the rubber O-ring (4); the injection of solutions into the cell was carried out through inlet/outlet tubes (5).

Measurements were carried out on herring or salmon ssDNA coated silicon wafers. The initial measurement was carried out with the cell filled with pH 7 buffer. Then a freshly boiled and cooled 0.2% DNA solution was injected into the cell and left for three hours. Attempts were made to measure ellipsometry during the exposure but consistent results could not be obtained due to refractive index changes within the solution. Therefore after three hours, the cell was flushed with fresh buffer and ellipsometry measured. In this way consistent results could be obtained.

Results and Discussion

Polyethylenimine immobilised DNA

AC impedance measurements across a range of frequencies (1-10,000 Hz) were performed on polyethylenimine immobilised single stranded herring DNA films immersed in the DNA solution. Due to the many decades over which the scale of the plots extend, the raw data fails to show any visible changes on these scalings. However when the change in impedance is plotted with respect to time, differences immediately becomes apparent. The data for each frequency was therefore processed by dividing the initial impedance of the polyethylene/herring ssDNA modified electrode immediately following immersion into the DNA solution, by the impedance measured at given time intervals. A typical plot of this type is shown in Fig 2(a). The mean change in impedance over the frequency spectrum was recorded and plotted against time in Fig 2(b).

Since the introduction of complementary DNA was found to give rise to changes in impedance which could be measured, AC impedance data of single stranded herring DNA polyethylenimine immobilised films upon non-complementary and complementary DNA exposure were determined for comparison. Fig. 2(b) shows impedimetric changes following exposure to herring (complementary), salmon (non-complementary) and exposure to buffer as a control.

It can be seen that a large drop in impedance occurs when we have herring DNA in solution, i.e. following exposure to complementary DNA, strongly indicating that the change in the AC impedimetric measurements is indeed due to the hybridisation of complementary DNA strands. A similar drop in impedance upon hybridisation has been noted by other authors for DNA adsorbed on carbon nanotube modified electrodes (Cai *et al* 2003) and is thought to be due to the higher conductivity of double stranded DNA with respect to single stranded DNA (Boon and Barton 2002). The change in impedance is also seen to decrease with continued complementary DNA exposure as further hybridisation continues. Neither the buffer solution or the solution containing salmon DNA display this behaviour.

It can also be seen that the changes in impedance are more pronounced at lower frequencies upon exposure to complementary DNA, suggesting that the DNA hybridisation primarily leads to a lowering of the capacitance of the interrogated film.

Similar results (not shown for brevity) were obtained when salmon DNA was immobilised on PEI, i.e. the electrodes showed a noticeable drop in impedance when exposed to complementary DNA but not upon exposure to either non-complementary DNA or buffer.

Polyaniline immobilised DNA.

The voltammograms for the deposition of polyaniline/DNA are depicted in Fig. 3(a). and imply a steady *in situ* formation of polymer at the electrode surface. As the number of scans increases peaks appear between +350-400 mV vs Ag/AgCl corrsponding to the oxidation and reduction of surface bound polyaniline. The increase in current from scan 10 to 20 is possibly due to the increase in polyaniline thickness and coverage of the electrode. As before, polyaniline/herring DNA electrodes were exposed to a solution of single stranded herring DNA in phosphate buffer and the impedance monitored with time; similar salmon DNA or just buffer solutions were both used as controls. The results of this study are shown in Fig. 3(b).

In a similar manner as before it can be seen that a large drop in impedance occurs upon exposure to herring DNA in solution, i.e. exposure to complementary DNA, indicating once again that hybridisation of complementary DNA strands is occurring. Neither the buffer solution nor the solution containing salmon DNA display this behaviour. Once again the changes in impedance are more pronounced at lower frequencies upon exposure to complementary DNA (not shown for brevity), suggesting a lowering of the capacitance of the interrogated film upon exposure and complementary DNA hybridisation. The overall effect also appears to be greater for polyaniline immobilised DNA than for PEI immobilised DNA.

It can be seen that for both immobilisation schemes, presence of non-complementary DNA causes a small upward drift in impedance. It is noteworthy that studies on polypyrrole immobilised nucleotides by previous workers, (Wang *et al* 1999), clearly showed that some interaction still occurs between non-complementary DNAs but

leads to current changes in the opposing direction to those between complementary DNAs.

Polydiaminobenzene Films

Single stranded herring DNA containing polydiaminobenzene were in a similar manner formed on the surface of electrodes. Voltammograms clearly show a cumulative formation of an insulating layer of polymer on the electrode surface, Fig. 4(a).

Again a drop in impedance is observed when exposed to herring ssDNA, Fig. 4(b). A drop in impedance for salmon ssDNA may also be observed and it is possible that this indicates some unspecific binding to the surface, making this electrode less-selective than either polyaniline or polyethylenimine modified electrodes. The drop in impedance was, however, larger for the samples exposed to herring ssDNA. Again the changes in impedance appeared to be mainly capacitive in nature for the complementary DNA and also for the non-complementary DNA changes in impedance observed. No drop in impedance occurred for samples immersed in simple phosphate buffer.

Effect of DNA Concentration with time

Electrodes coated with polyethylenimine immobilised single-stranded herring DNA as before were used for studying the effects of DNA concentration on the observed impedance with time. Electrodes were placed in varying concentrations of complementary DNA as before and the impedance monitored over periods of time exceeding 3 hours. Two observations can be made. As can be seen from Fig. 5, increasing the concentration of DNA has a marked effect on the impedance change, however it is not a simple linear relationship. Higher concentrations lead to an initial higher rate of hybridisation, probably due to a simple diffusional gradient but continue to lower the impedance throughout the time frame of this study. It would therefore appear that both DNA concentration and extended time periods affect the observed impedance and by inference the degree of DNA hybridisation. We can therefore conclude the DNA concentration imparts diffusional control of DNA mass transport to the electrode surface, but that DNA hybridisation still acts as a rate

limiting step. The electrode response to DNA hybridisation is, in conclusion, therefore under mixed mass transport and kinetic control.

DNA Hybridisation; Electrochemical Interrogation.

From our results it appears that DNA hybridisation at a surface leads to an overall drop in impedance. DNA is known to complex to a positively charged polymer surface and hybridisation would be expected to increase the quantity of DNA at the surface. A drop in impedance could be linked to a facilitation in the electron transfer process between the electrodes and the external environment. It should not in this context be forgotten that the DNA is immobilised on the carbon surface via a positively charged polymer. While insulating proteins typically show an electron tunnelling coefficient of $1.4Å^{-1}$ (Arnaut and Formosinho 1996), the conductivity of DNA can vary from insulating to wire-like (as recently reviewed, Boon and Barton 2002), greatly depending on the presence of mismatches and on base-pair stacking. This indicates that charge transfers of this nature may be very sensitive to the DNA structure and could be facilitated following hybridisation. Therefore we have the possibility of the DNA aiding transfer of electrons through the positive polymer layer and possibly affording a drop in impedance. We have within previous studies shown facilitation of electron transfer between a gold surface and ferricyanide ion using an electrode modifying self-assembled organic layer (Collyer *et al* 2003).

As yet we have not attempted to reverse the hybridisation, heating the electrodes or electrostatic polarisation may reverse hybridisation and regenerate the sensor. Care would have to be taken however that we simply did not remove all the DNA from the electrode and in view of the low potential cost of the electrodes it would be simpler just to use each sensor once.

DNA Hybridisation; Interrogation via Ellipsometry.

Adsorption of herring and salmon ssDNA was studied independently with optical ellipsometry. Typical results of these measurements are shown in Fig. 6 as spectra of an ellipsometric parameter $\Delta(\lambda)$. First, the samples with freshly deposited ssDNA were measured, then a ssDNA solution was injected and after 3 hours flushed out with fresh buffer. The sample was then re-measured, the measurements were always

carried out with the cell filled with a standard phosphate buffer solution. Ellipsometry is known to be a sensitive analytical tool for thin film study. The measured ellipsometric parameters Ψ and Δ, representing the amplitude ratio and phase shift between *s*- and *p*- components of polarised light, respectively, are related to the thickness and refractive index of thin films on the surface of light reflective substrate, e.g. silicon.

Fig. 6(a) shows a substantial vertical shift of Δ(λ) spectra, when a sample which had a previously deposited layer of PEI/ ssDNA was exposed to a solution of complementary ssDNA, while exposure of non-complementary ssDNA produces minimal spectral changes. A large downward shift of the spectrum in Fig. 6(a), corresponds to an increase in the adsorbed layer thickness due to hybridisation, of the single stranded DNA possibly with formation of a double helix.

However single strands of salmon and herring DNA do not hybridise with each other, and thus do not cause substantial changes of Δ(λ) spectra. Only a minimal spectral shift in the opposite direction (upwards) in Fig. 6(b) is observed; this could be interpreted as a slight increase in the refractive index of the layer or a small loss of material to solution.

The obtained results prove independently the binding of single strand DNA molecules from the solution to the complementary molecules adsorbed electrostatically onto PEI layer. Binding occurs when we have a herring-herring or salmon-salmon match but not when the DNAs are mismatched.

Conclusions

Polyethylenimine, polyaniline and polydiaminobenzene modified electrodes containing single-stranded DNA have been shown to undergo hybridisation upon exposure to complementary single-stranded DNA. These hybridisation events have been interrogated by an AC impedimetric approach with the results showing that complementary DNA hybridisation gives rise to a lowering of the capacitive properties of the electrode/polymer film in solution. The clearest differential occurs for both polyethylenimine and polyaniline electrodes, polydiaminobenzene showing some unselective binding. Both approaches have their advantages, however the use of polyaniline allows finer control and monitoring of the deposition process. Large multi-electrode sheets could be screen-printed and electrodeposited which would still allow mass production and the resultant lowering of costs.

Ellipsometric measurements have confirmed that species selective binding occurs at silicon surfaces, and in the first case we have demonstrated species differentiation between two species of fish, namely herring and salmon.

We have described a simple electrochemical approach for detection of DNA hybridisation at an electrode surface. The sensor developed is of a simple format and is inexpensive to manufacture and therefore holds promise for use as a disposable sensor strip. It shows high species selectivity and reasonable response times. Future work will be focussed towards allowing faster response times and greater specificity by the use of shorter DNA fragments of specific sequence immobilised within a polymer matrix.

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Legends to Figures

Fig. 1. Schematics of (a) the screen-printed carbon electrode (b) the ellipsometry cell.

Fig. 2. (a). Plot of relative impedances at different frequencies for a polyethylenimine/herring ssDNA modified electrode scanned whilst immersed in 0.2% herring ssDNA/buffer solution for differing periods of time compared to the same electrode immediately after immersion in the solution, (♦) 4 min, (■) 8 min, (▲) 21 min, (X) 36 min, (∗)57 min, (●)77 min, (+) 100 min, (△)141 min (□) 188 min. (b) Plot of mean relative impedances over the range of frequencies for an polyethylenimine/herring ssDNA modified electrode scanned after exposure to pH 7 buffer (■), 0.2% complementary DNA (♦), 0.2% non-complementary DNA (▲).

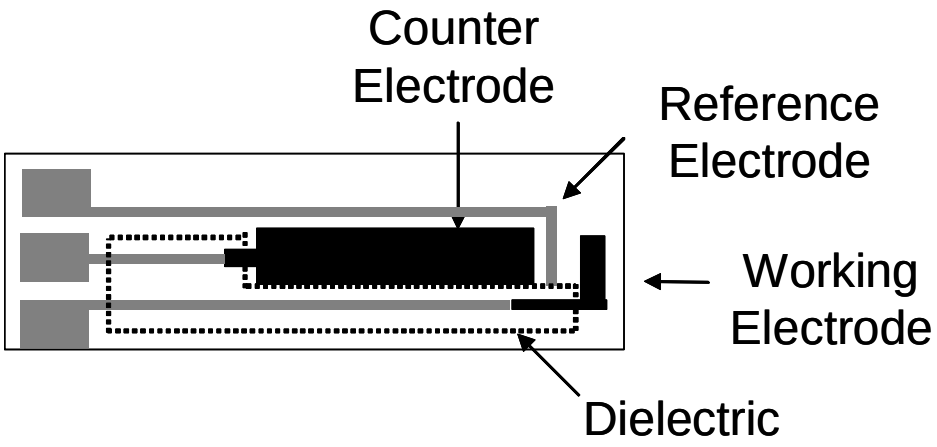
Fig. 3. (a). Cyclic voltammograms showing the deposition of material from a aniline/herring ssDNA solution (1^{st}, 10^{th} and 20^{th} sweeps only for clarity). (b). Plot of mean relative impedances over the range of frequencies for an polyaniline/herring ssDNA modified electrode scanned after exposure to pH 7 buffer (■), 0.2% complementary DNA (♦), 0.2% non-complementary DNA (▲).

Fig. 4. (a). Cyclic voltammograms showing the deposition of material from a diaminobenzene/herring ssDNA solution (1^{st}, 10^{th} and 20^{th} sweeps only for clarity). (b). Plot of mean relative impedances over the range of frequencies for an polydiaminobenzene/herring ssDNA modified electrode scanned after exposure to pH 7 buffer (■), 0.2% complementary DNA (♦), 0.2% non-complementary DNA (▲).

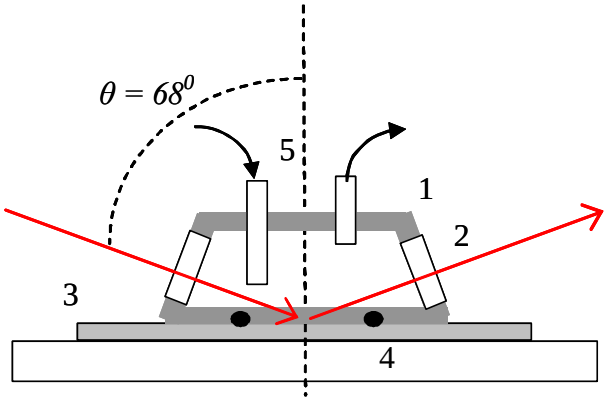
Fig. 5. Plot of mean relative impedances over the range of frequencies for an polyethylenimine/herring ssDNA modified electrode scanned after exposure to pH 7 buffer (X), 0.01% complementary DNA (■), 0.1% complementary DNA (♦), 0.2% complementary DNA (▲), 1% complementary DNA (●)

Fig. 6. Ellipsometry study of single stranded salmon DNA immobilised on Si/PEI: (exposure to (a) 0.2 % complementary single stranded DNA; (b) 0.2% non-complementary single stranded DNA).

Figure 1.


Counter
Electrode
Reference
Electrode
Working
Electrode
Dielectric

a.


θ = 68⁰
5
1
2
3
4

b.

Figure 2.

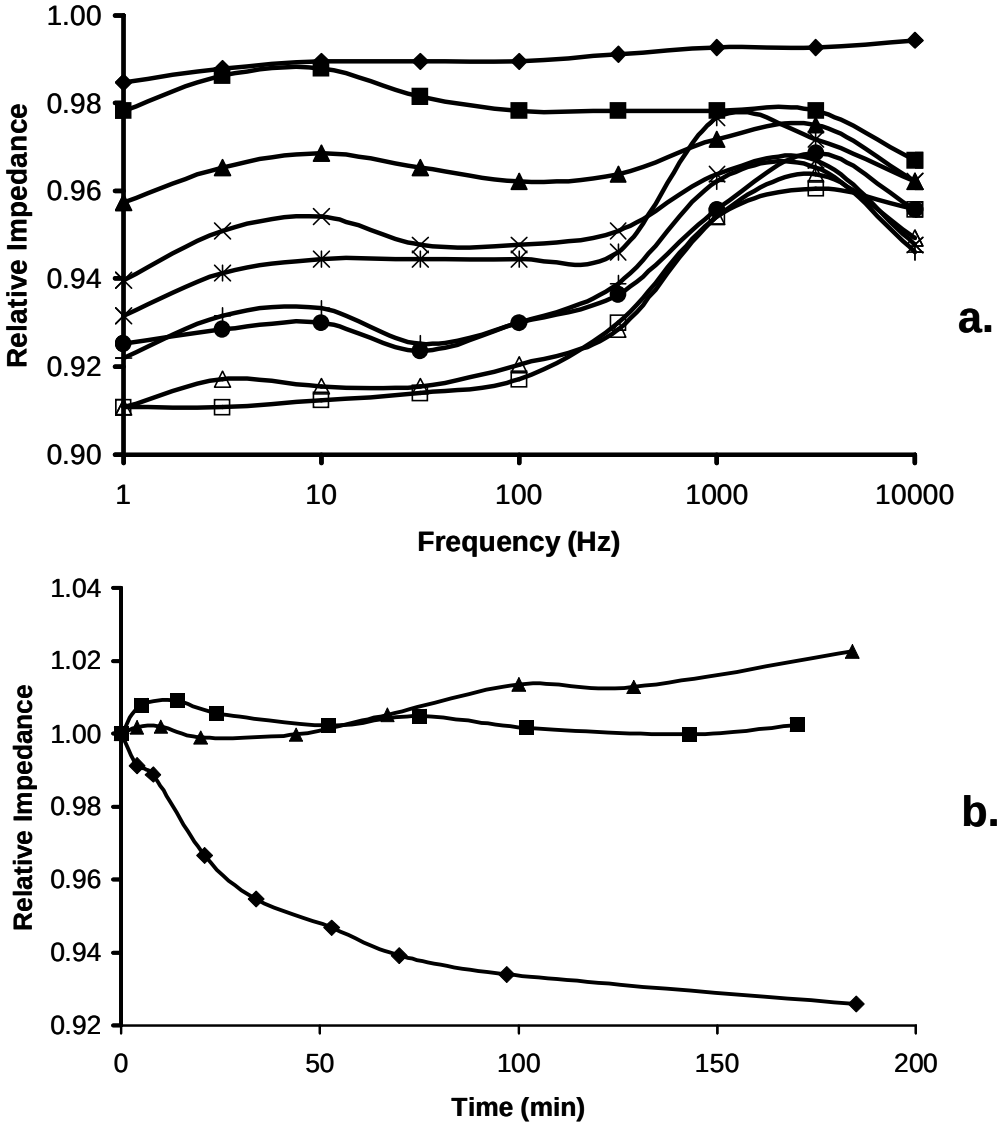

1.00
0.98
0.96
0.94
0.92
0.90
Relative Impedance
1
10
100
1000
10000
Frequency (Hz)
a.
1.04
1.02
1.00
0.98
0.96
0.94
0.92
Relative Impedance
0
50
100
150
200
Time (min)
b.

Figure 3

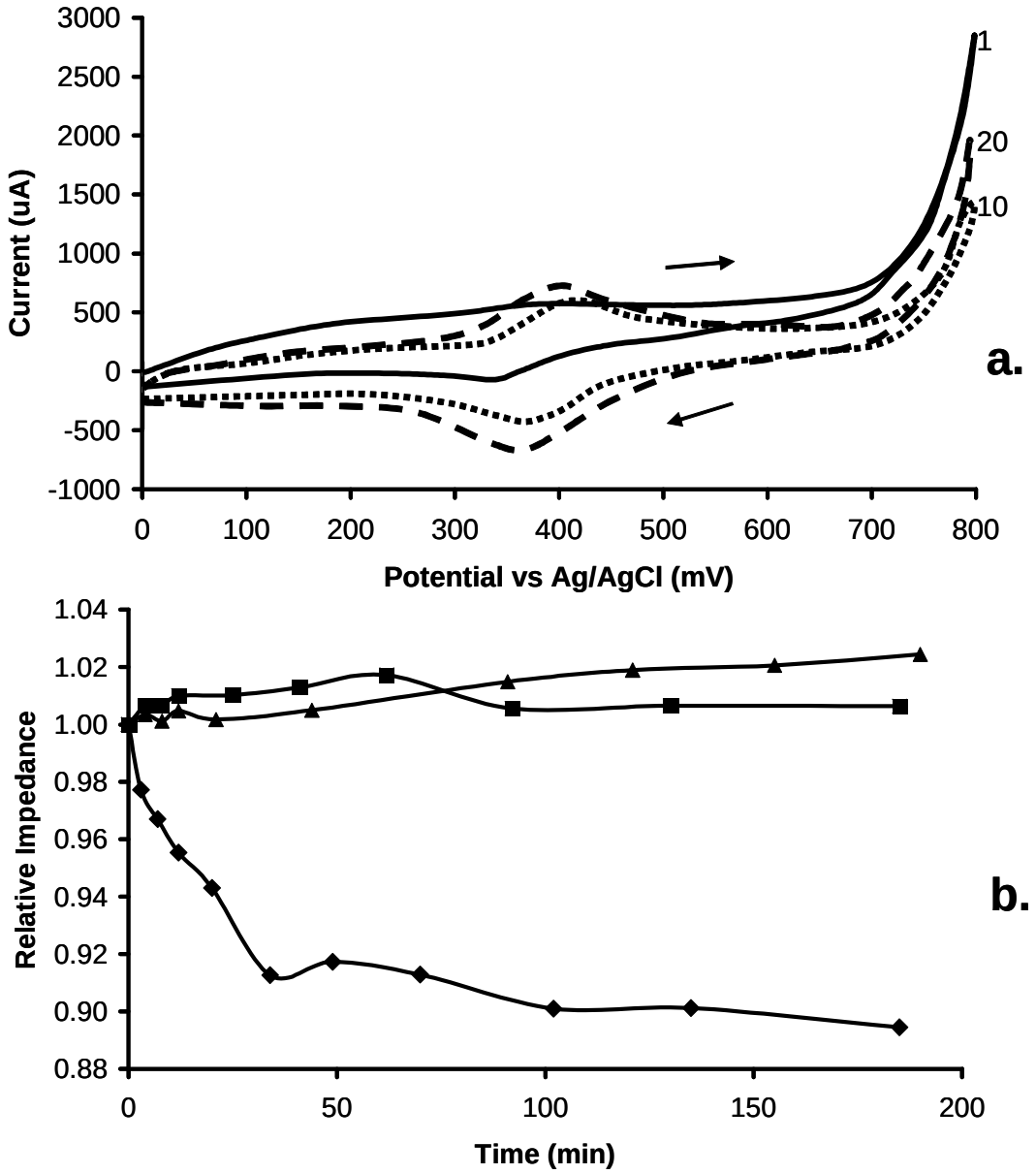

3000
2500
2000
1500
1000
500
0
-500
-1000
Current (uA)
0
100
200
300
400
500
600
700
800
Potential vs Ag/AgCl (mV)
1
20
10
a.
1.04
1.02
1.00
0.98
0.96
0.94
0.92
0.90
0.88
Relative Impedance
0
50
100
150
200
Time (min)
b.

Figure 4.

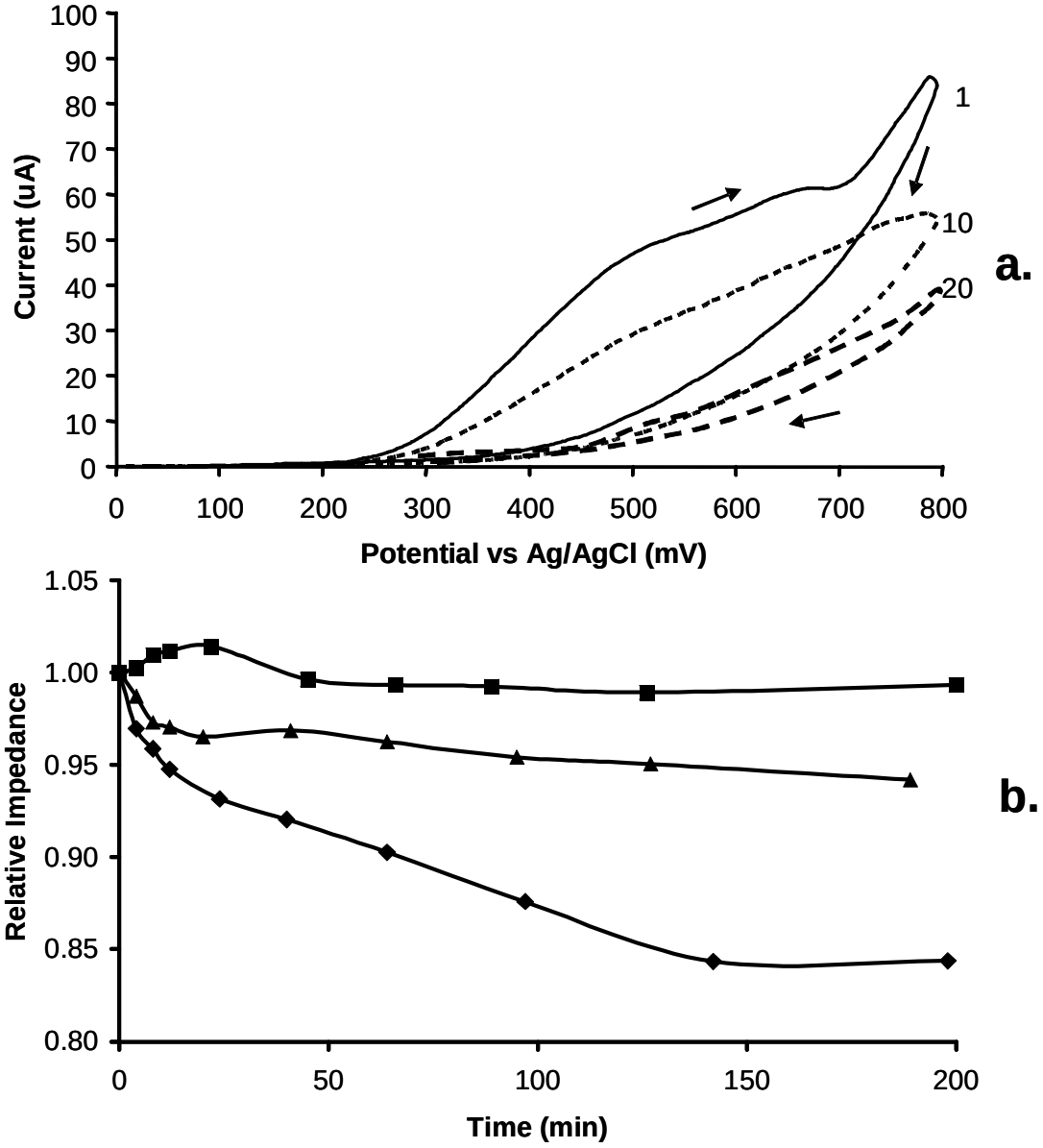
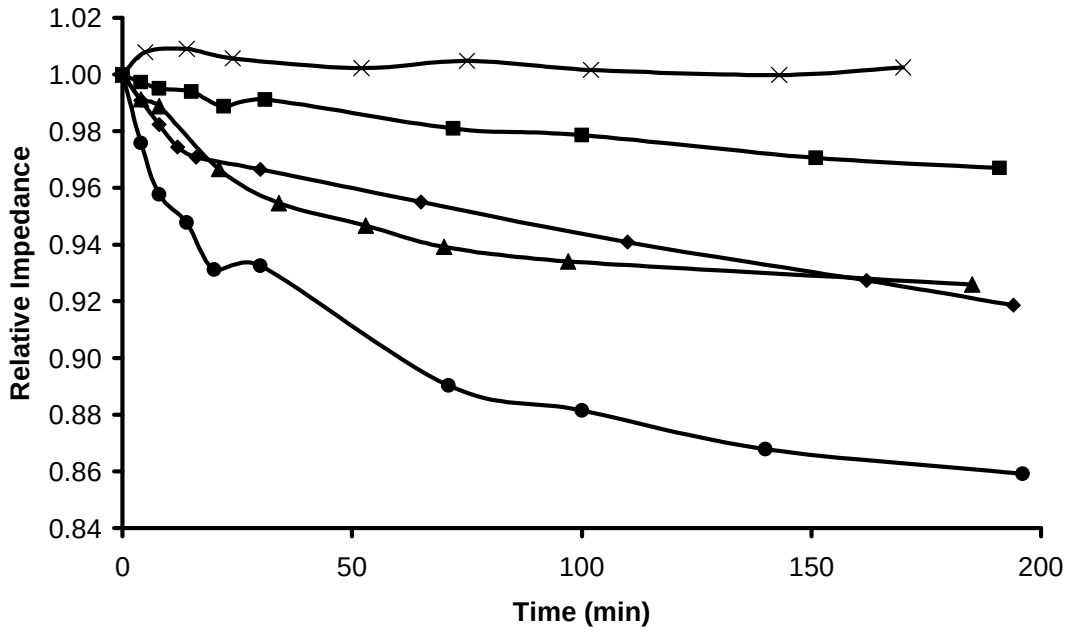

Current (uA)
Potential vs Ag/AgCl (mV)
1
10
20
a.
Relative Impedance
Time (min)
b.

Figure 5.


1.02
1.00
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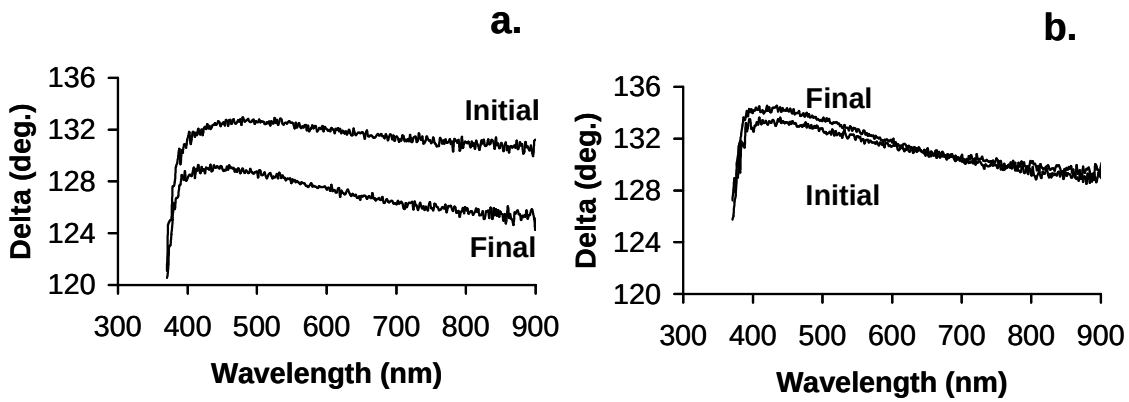
0.86
0.84
Relative Impedance
0
50
100
150
200
Time (min)

Figure 6.


a.
b.
Delta (deg.)
136
132
128
124
120
300
400
500
600
700
800
900
Wavelength (nm)
Initial
Final
Final
Initial