Enzyme-based quartz crystal biosensors for analytes of biomedical significance

By

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Abstract

The development and optimisation of the AT-cut quartz crystal sensor for hydrogen peroxide is described. The optimised hydrogen peroxide biosensor was subsequently used to determine the total cholesterol concentration in buffer and serum, plus creatinine in buffer, through a series of enzyme reactions that produced hydrogen peroxide from the analyte of interest.

The detection of hydrogen peroxide was achieved through the oxidation of the benzidines, 3,3'-diaminobenzidine (DAB), 3,3'-dimethoxybenzidine (DMOB), and 3,3',5,5'-tetramethylbenzidine (TMB), in the presence of horseradish peroxidase (HRP). The oxidised product is insoluble, but was found to remain in suspension, reducing the crystal’s ability to detect its formation. Through the inclusion of the non-ionic surfactants, Tween 80 and Triton X-100, it was found that greater adsorption of the oxidised benzidine occurred, leading to increased impedance shifts. The presence of Triton X-100 gave an improved response time e.g. with DAB the response was reduced from 21 min to 10 min.

The use of polymers with the hydrogen peroxide sensor was found to have no beneficial effect, apart from poly(vinyl chloride), which increased the impedance shift. In the presence of Triton X-100, the response was the same as that for a bare crystal, suggesting the possibility that polymers may be used to reduce crystal fouling by the oxidised benzidine.

The presence of the enzymes, glucose oxidase and β-amylase in the reaction mixture increased the impedance response by about 10 and 2-fold respectively, and reduced
the response time to 15 min from 68 min, at the concentrations used. All the proteins used had various effects upon the response, and this indicates that the method will suffer from interference (both positive and negative), due to proteins present in the biological sample.

The optimised hydrogen peroxide biosensor using Triton X-100 and DAB was used to determine total cholesterol in buffer and serum, using the additional two enzymes, cholesterol esterase (ChE) and cholesterol oxidase (ChOx). The response of the sensor to LDL (low-density lipoprotein) cholesterol gave a response time of less than 25 min. Using the optimised biosensor, a linear response for free and LDL cholesterol was obtained between 50 - 300 μM, and 25 - 400 μM, respectively. HDL (high-density lipoprotein) cholesterol was found to solubilise the oxidised DAB, and hence act as an interferent in the cholesterol determination. A linear response for cholesterol determination (at 300 μM) in serum was obtained.

The hydrogen peroxide biosensor with DAB and Triton X-100 was used to measure creatinine in buffer, through the addition of the enzymes, sarcosine oxidase (SO), creatinase (Cl), and creatininase (CA). The creatinine determination at the concentration of the enzymes used, reached an endpoint in under 3 hr.

The potential of the quartz crystal to determine low molecular solutes through a series of enzyme-based reactions, using the hydrogen peroxide detection strategy has been demonstrated.
Acknowledgements

I would like to thank my supervisors, Dr Sub Reddy and Prof. Jim Lynch for their support, guidance and encouragement, throughout the course of my PhD.

Further I wish to thank my colleagues on AY04, particularly Dave Lamb, Mu’azu Abubakar, Michelle Tickner, Nikos Karousos, Helen Davidson and Julie Paice for their friendship and support during the last three years.

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## Abbreviations

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<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>APM</td>
<td>Acoustic Plate Mode</td>
</tr>
<tr>
<td>BAW</td>
<td>Bulk Acoustic Wave</td>
</tr>
<tr>
<td>BEP</td>
<td>bis(2-ethylhexyl) hydrogen phosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CA</td>
<td>creatininase</td>
</tr>
<tr>
<td>ChE</td>
<td>cholesterol esterase</td>
</tr>
<tr>
<td>ChOx</td>
<td>cholesterol oxidase</td>
</tr>
<tr>
<td>CI</td>
<td>creatinase</td>
</tr>
<tr>
<td>CMC</td>
<td>critical micelle concentration</td>
</tr>
<tr>
<td>DAB</td>
<td>diaminobenzidine</td>
</tr>
<tr>
<td>DCB</td>
<td>dichlorobenzidine</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethylformamide</td>
</tr>
<tr>
<td>DMOB</td>
<td>dimethoxybenzidine</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked Immunosorbent assays</td>
</tr>
<tr>
<td>FETs</td>
<td>Field Effect Transistors</td>
</tr>
<tr>
<td>FPW</td>
<td>Flexural Plate Wave</td>
</tr>
<tr>
<td>fp</td>
<td>parallel frequency</td>
</tr>
<tr>
<td>fs</td>
<td>series frequency</td>
</tr>
<tr>
<td>GOx</td>
<td>Glucose oxidase</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffer saline</td>
</tr>
<tr>
<td>PolyDAB</td>
<td>poly(diaminobenzidine)</td>
</tr>
<tr>
<td>PPy</td>
<td>Polypyrrole</td>
</tr>
<tr>
<td>PVC</td>
<td>poly(vinyl chloride)</td>
</tr>
<tr>
<td>QCM</td>
<td>Quartz Crystal Microbalance</td>
</tr>
<tr>
<td>SAW</td>
<td>Surface Acoustic Wave</td>
</tr>
<tr>
<td>SO</td>
<td>sarcosine oxidase</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface Plasmon Resonance</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>TMB</td>
<td>tetramethylbenzidine</td>
</tr>
<tr>
<td>TSM</td>
<td>Thickness Shear Mode</td>
</tr>
<tr>
<td>Zp</td>
<td>parallel impedance</td>
</tr>
<tr>
<td>Zs</td>
<td>series impedance</td>
</tr>
</tbody>
</table>
Chapter 1 - Introduction
1.1 The requirements of a Biosensor

A biosensor can be defined as an analytical device, which incorporates a biological sensing element in intimate contact with a transducer for the purpose of reversible or single shot detection, of the concentration or activity of a chemical or biological species in biological samples.

For a functional biosensor, there are several fundamental requirements:\n
<table>
<thead>
<tr>
<th>Specificity</th>
<th>The ability to distinguish between various substrates, usually achieved through the biological component.</th>
</tr>
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<tbody>
<tr>
<td>Sensitivity</td>
<td>The analytes are often in micro- or nanomolar concentrations.</td>
</tr>
<tr>
<td>Accuracy</td>
<td>This needs to be less than two standard deviations of the actual concentration.</td>
</tr>
<tr>
<td>Response &amp; Recovery Time</td>
<td>Needs a quick response (ideally less than a few minutes), and a short recovery time (less than 5 min), if it is not a disposable, single use device.</td>
</tr>
<tr>
<td>Lifetime</td>
<td>The biosensor may be used once or more, this usually depends on the stability of biological material, and recoverability of the transducer.</td>
</tr>
<tr>
<td>Nature of the solution</td>
<td>The pH, temperature and ionic strength of the solution may affect the biosensor.</td>
</tr>
</tbody>
</table>
1.2 The components of a Biosensor

1.2.1 The Analyte

The chemical species can be any substance that is consumed or produced in a biochemical process, or forms a complex such as an antigen-antibody or ligand-receptor complex, that can in principle be analysed by a biosensor. The substance or analyte could include any of the following,

- Bacteria & Viruses (e.g. Salmonella, Neisseria meningitidis, smallpox),
- Drugs (e.g. Penicillins, Paracetamol, Aspirin, Cocaine),
- Body elements, e.g.,
  - Cholesterol
  - Hormones (e.g. thyroxine, insulin)
  - Carbohydrates (e.g. glucose)
  - Enzymes (e.g. creatine kinase, pseudocholinesterase)
  - Neurotransmitters (e.g. dopamine, acetylcholine)
  - Trace elements & minerals (e.g. Iron, Zinc)
  - Waste products (e.g. creatinine, urea, bilirubin)
- Environmental pollutants (e.g. Organophosphates, Mercury, polyaromatic hydrocarbons).

1.2.2 The Biological Component

The biological component of the biosensor is by nature required to be highly specific, so avoiding the problem of interference’s from other substances, which plague analytical methods. The biological sensing element may include an enzyme, antibody
or the antigen, receptor, organelle, cell or tissue (see fig. 1.1), though the cell or tissue is being used as a crude, unpurified enzyme biosensor.

Enzymes and antibodies are the most commonly used components, since they have a high specificity for a particular substrate and antigen, respectively. Other components can be used, examples include micro-organisms such as yeast and bacteria, and tissue material e.g. the banana, which was used by Sidwell & Rechnitz², and Wang & Lin⁴, to measure dopamine levels. They found that by mixing some graphite powder and liquid paraffin, with some banana, and then placing this in an electrode cup, they had built a biosensor. The banana tissue contains the enzyme, polyphenol oxidase, and this oxidises the dihydroxy form of dopamine to the quinone form. When the resulting compound is electrochemically reduced back to its original form, the result is the release of electrons producing a current, which is directly proportional to the amount of dopamine present in the sample. An amperometric sensor was developed using asparagus tissue to determine fluoride in drinking water⁵. The biological component may be in solution with the substrate, immobilised by entrapment within a membrane, or chemically attached to the transducer surface.
Fig. 1.1 Different principles of Biosensing and typical interfaces (taken from Ziegler & Gopel\(^6\)), A is the analyte, T and the arrows denote the transducer, and S is the recorded signal. (a) is a bioaffinity sensor, where the analyte (e.g. antibody, antigen) is identified by an immobilised biorecognition unit, R (e.g. antigen, antibody and DNA). (b) is a biocatalytic sensor, where the analyte is converted by immobilised enzymes (E) to products (P). (c) is a transmembrane sensor, where (i) is a transport or channel protein, (ii) is a receptor protein incorporated into a membrane (M). These structures either (i) move the analyte through the
membrane, (ii) bind the analyte and open a channel for another species, or (iii) subsequently activate a separate enzymatic cascade. (d) is a cell sensor, where immobilised living cells (C) either (i) convert or (ii) bind the analyte.

Biosensors using the whole microorganism, detect the analyte, which produces a biochemical change e.g. an increase or decrease in luminescence or fluorescence that is proportional to the concentration of the analyte present. An example is the use of *Pseudomonas fluorescens* to detect benzene, toluene, ethylbenzene and xylene. The biochemical change is a gross cellular inhibition of bioluminescence. The same bacterium has been used for the detection of naphthalene by the specific induction of bioluminescence. The luminescence does not occur naturally in this bacterium. This has been achieved by the cloning of the luminescent genes of the luminescent marine organism, *Vibrio fisheri*. *V. fisheri* cannot be used in the conditions required, so the cloned genes were moved into *P. fluorescens*, which can be found in natural soil.

Some examples of the use of enzymes, antibodies and nucleic acids with the quartz crystal are given in section 1.5.

The advantages of using enzymes as the biological component include, their high selectivity for the analyte, and their fast response time. The disadvantages include their expense, and a loss of activity over time, when immobilised. By using tissue or cells/micro-organisms for enzymatic detection of the analyte, the activity will be improved, and the expense lowered, though this will result in a loss of selectivity, and a longer response time. The major advantage of enzymatic detection is their catalytic effect, which alters a parameter within the test solution or cell. Antibodies do not have this ability, though they are more selective and sensitive. Nucleic acids are
similar to antibodies in terms of their lack of catalytic behaviour, and their high selectivity and sensitivity. The same is true of the use of receptors as the biological component.

1.3 The Transducer

There are various types of transducer, which include, amperometric, potentiometric, conductimetric, field effect transistors, optical, calorimetric, and piezoelectric. Biosensors developed using amperometric, optical and piezoelectric transducers will be discussed in depth. Some examples of biosensors developed for analytes for the other transducers are given in table 1.1 below.

<table>
<thead>
<tr>
<th>Transducer Type</th>
<th>Examples</th>
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<tbody>
<tr>
<td>Potentiometric</td>
<td>Dopamine\textsuperscript{9}</td>
</tr>
<tr>
<td></td>
<td>Hydrogen peroxide and peracetic acid\textsuperscript{10}</td>
</tr>
<tr>
<td>Conductimetric</td>
<td>Hydrogen peroxide\textsuperscript{11}</td>
</tr>
<tr>
<td></td>
<td>\textit{Salmonella and E.coli}\textsuperscript{12}</td>
</tr>
<tr>
<td>Field Effect Transistors</td>
<td>Glucose\textsuperscript{13}</td>
</tr>
<tr>
<td></td>
<td>Glucose, ascorbic and citric acid\textsuperscript{14}</td>
</tr>
<tr>
<td>Thermistors</td>
<td>Glucose\textsuperscript{15}</td>
</tr>
</tbody>
</table>

Table 1.1. Examples of biosensors with different transducers

1.3.1 Amperometric

Amperometric usually refers to the analytical technique of chronoamperometry. This technique is related to the technique of voltammetry, where an increasing or
decreasing potential is applied to the cell, until the substance of interest is completely oxidised or reduced respectively. This results in a sharp rise or decrease in the cell current to give a maximum or minimum current. The height of the peak current is directly proportional to the concentration of the electroactive material. A graph or trace of the current against potential is called a voltammogram, and the straightforward technique is known as the linear sweep voltammetry. A modification of voltammetry is cyclic voltammetry, where Ox (starting substance) is reduced, and R (reduced product of Ox) is produced, but then the potential is swept back towards the redox potential, and R is oxidised to Ox. As a result, the current increases in the negative (oxidising) direction, until an oxidation peak is reached.

Amperometry is similar to cyclic voltammetry, but the potential is stepped in a square-wave fashion. The potential is increased past the would-be peak of cyclic voltammetry. Rather than the potential being measured, in this case the current is monitored over time, at a fixed potential, which results in a decay curve, this being due to the breakdown of the diffusion layer. Amperometry uses the Cottrell equation (equation 1a), where F is the faraday constant (96 487 C/mol), n is the number of electrons transferred, C_ox is the concentration of the oxidised species, i_d is the decay current i.e. when a steady state current is reached, A is the area of the electrode, and D is the diffusion coefficient.

\[ i_d = \frac{nFADC_{ox}}{\pi^{1/2}t^{1/2}} \]  \hspace{1cm} (1a)

An example of the use of the amperometric biosensor for the determination of hydrogen peroxide is the work by Razola et al.\(^{16}\). The biosensor was produced
through the immobilisation of the enzyme, horseradish peroxidase (HRP) within an
electropolymerised polypyrrole film (PPy). The result was a biosensor which was
able to determine hydrogen peroxide in the concentration range between $4.9 \times 10^{-7}$
and $6.3 \times 10^{-4}$ M, with a response time of 5 s. The biosensor was operationally stable
for 35 days. Ascorbate was studied has a possible source of interference and it was
found that it gave rise to currents 25% higher than those for $H_2O_2$ at the same
concentration.

A recent biosensor was described for the measurement of protein concentrations for
possible use in the food and clinical environments\(^{17}\). A bi-enzyme system of protease
and l-amino acid oxidase (l-AAO) was used to depolymerise the proteins into its
constituent amino acids, and then oxidise the amino acids producing hydrogen
peroxide. A linear response was observed for the concentration range 0.017 – 0.1%
(w/v), with a maximal response at 0.4% (w/v), and an assay time of 4 min. Over a 7
day test period, it was found that protease activity decreased by 40%.

More examples of amperometric biosensors for cholesterol and creatinine can be
found in chapter 3 and 4, respectively.

The major advantage of this type of sensor is the ease with which it can be
constructed. However, the major disadvantages include, the need for calibration of
the sensor both before and after the measurement, the lifetime is short, and the
oxidation of other electrochemically active species (known as interferents) which are
present in the test sample. The major interferents are ascorbate, urate and
paracetamol. This problem can be overcome through the use of polymer layers,
which are more selective for the analyte of interest and eliminate or reduce the interferences. If the interferents are not reduced, this can lead to an overestimation of the analyte concentration (a false positive signal).

1.3.2 Optical

This type of transducer includes optical fibres, visible absorption spectroscopy, chemiluminescence, light scattering techniques, and the various reflectance methods including surface plasmon resonance (SPR). Here, the concentration will be upon SPR, since it is a surface sensitive technique, which is comparable with the quartz crystal sensor. Figure 1.2 shows the method of detection for SPR.

SPR measures biological/chemical analytes of interest, by detection of binding or adsorption to the sensor surface. It is a charge-density oscillation, which propagates along the interface of the metal layer and dielectric material (the liquid or air). The metal layer is usually silver or gold, though copper and aluminium have been used. The choice of metal is important because it must exhibit free electron behaviour, and this layer must be less than one wavelength of light in thickness\(^\text{18}\). The phenomenon of SPR will only occur at a unique angle (\(\theta\)), which is determined by the refractive index (RI) of the analyte of interest, the optical properties of the prism (or grating), the type and thickness of the metal, and the wavelength of the monochromatic light\(^\text{19}\).

In order for the surface plasmon to be produced, the two vectors labelled (\(K_{ev}\) and \(K_{sp}\)) on figure 1.2, must be equal. \(K_{ev}\) is the wave vector of the evanescent field, and is given by

\[
K_{ev} = \frac{\omega_0}{c} \eta_e \sin \theta \tag{1b}
\]
where, $\omega_0$ is the frequency of incident light, $\eta_\text{p}$ the refractive index of the prism, $\theta$ the angle of incidence of the light, and $c$ the speed of light in a vacuum. $K_{sp}$ is the wave vector of the surface plasmon, and this is given by,

$$K_{sp} = \frac{\omega_0}{c} \sqrt{\frac{\varepsilon_m \eta_s^2}{\varepsilon_m + \eta_s^2}}$$  \hspace{1cm} (1c)$$

where $\varepsilon_m$ is the dielectric constant of the metal film, and $\eta_s$ is the refractive index of the dielectric medium\(^\text{18}\). So by monitoring the incidence angle at which surface plasmon occurs, the adsorption of material to the surface can followed over time.

Su and O'Shea\(^\text{20}\) have used SPR to demonstrate its ability to detect precipitation from an enzymatic reaction. They used the substrate 4-chloro-1-naphthol, with horseradish peroxidase (HRP) to determine hydrogen peroxide concentration, by correlation with the precipitation produced by the oxidation of the substrate. By adding a further enzyme, glucose oxidase, they were able to demonstrate that their SPR biosensor
could detect any analyte, which had a corresponding oxidase enzyme. The enzymes were immobilised within a thiotic acid based self-assembling monolayer, on the base of a cuvette. The enzymatically produced precipitate formed on the surface of the enzymatic layer, and this could be detected by a shift in the SPR incidence angle. SPR is a surface sensitive optical method, where surface charge-density waves are produced at the interface between two media. In this example, the cuvette and the enzyme layer/precipitate, and a change in the later (e.g. precipitation onto the surface) will produce a shift in the angle of the SPR wave. It was found that this method was able to detect $H_2O_2$ linearly up to a concentration of $7.24 \times 10^{-5}$ mM, with a limit of detection of $6.7 \times 10^{-7}$ mM. The SPR response for glucose was proportional in the concentration range, $0.7 - 8.3 \times 10^{-4}$ mM, and a response time of 150 s.

SPR has also been used to follow the biodegradation of polymer films, which were enzymatically catalysed. The polymer film, poly(ester) amide was degraded by $\alpha$-chymotrypsin; dextranase was used with a dextran hydrogel, and lipase with poly(trimethylene) succinate. It was found that a $1.0^\circ$ change in the SPR angle correlated to a 2.7 nm change in the thickness of the polymer. It was found that the rate of degradation was dependent upon the concentration of enzyme used, and if the same enzyme concentration was used with films of different thickness, the rate remained constant. The rate of degradation of the polymer was linear to the concentration of enzyme used.

Kößlinger et al. used SPR and the Quartz Crystal Microbalance (QCM) to study the non-specific/specific binding of bovine serum albumin (BSA), and the detection of HIV antigens using antibodies. The specific binding was achieved using an
immobilised anti-BSA antibody. The authors compared the results from using both techniques, and concluded that both performed the same, giving similar responses in terms of size and time, with neither having any clear advantage over the other. Their comparison of the two techniques is reproduced below (table 1.2).

<table>
<thead>
<tr>
<th>Quantity</th>
<th>QCM</th>
<th>SPR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thickness sensitivity</td>
<td>184 Hz nm$^{-1}$</td>
<td>26.3 mdeg nm$^{-1}$</td>
</tr>
<tr>
<td>Detection limit of mAb</td>
<td>20 nM</td>
<td>23 nM</td>
</tr>
<tr>
<td>Detection limit of sera</td>
<td>1:1000</td>
<td>1:1000</td>
</tr>
<tr>
<td>Immunological sensitivity for mAb</td>
<td>0.5 nM Hz$^{-1}$</td>
<td>3 nM mdeg$^{-1}$</td>
</tr>
<tr>
<td>Penetration depth</td>
<td>126 nm</td>
<td>150 nm</td>
</tr>
<tr>
<td>Sensitive area</td>
<td>5 mm$^2$</td>
<td>5 x 10$^{-3}$ mm$^2$</td>
</tr>
<tr>
<td>Amount of molecules at the detection limit</td>
<td>10$^{-14}$ mol</td>
<td>10$^{-17}$ mol</td>
</tr>
<tr>
<td>Signal-volume noise ratio</td>
<td>2.3</td>
<td>0.45</td>
</tr>
</tbody>
</table>

Table 1.2. The overview of quantities that were used by Kößlinger et al., to compare the SPR and QCM (mAb = monoclonal antibody). The values given for the QCM are for a 20 MHz crystal.

Other work has been completed to compare these two surface sensitive techniques, and each has found that neither has the advantage over the other. The one difference noted by Spangler et al. was that they found the SPR device they were using a commercially available device called Spreeta® sensor, was more sensitive to temperature fluctuations than the QCM device. Otherwise they found no significant
differences in the portability, robustness, cost, sensitivity, detection limits, or ease of operation between the two techniques.

The devices used by Vikinge et al.\textsuperscript{24} are both commercially available. The SPR experiments were performed on a BIAlite\textsuperscript{®} instrument produced by Biacore AB (Sweden). This device has been used as an immunosensor\textsuperscript{25}, and to measure cellular\textsuperscript{26} and molecular interactions\textsuperscript{27; 28}. A review of Biacore and some of its applications is Fivash et al\textsuperscript{29}.

The QCM device used is produced by Q-Sense AB (Göteborg, Sweden). This device measures the series resonant frequency ($f_s$, described in section 2.1) and the dissipation factor ($D_s$). The dissipation factor is a ratio of the energy dissipated per oscillation and the total energy stored in the system. The device allows the determination of $f_s$ and $D_s$ at several harmonics of the quartz crystal, i.e. the fundamental frequency and several overtones. The influence of the viscosity, elasticity, thickness and density properties of the material, being measured on the crystal response can be better understood through the use of mathematical modelling. This device has been used as an immunosensor\textsuperscript{30}, cellular adsorption sensor\textsuperscript{30;32}, and to measure surfactant adsorption\textsuperscript{33}, and molecular adsorption\textsuperscript{24;34;35}.

Some of the advantages of the SPR biosensor include the lack of reference electrode, and it does not suffer from electrical interference caused by electrochemically active substances such as ascorbate and urate. The disadvantages include the need for the signal to be produced within a few hundred nanometres of the sensing surface. Another disadvantage identified by Spangler et al.\textsuperscript{23} is the SPR’s sensitivity to
temperature fluctuations, which may in part be due to the laser, which is used to measure reflectivity angle changes heating the sample medium.

1.3.3 Piezoelectric Devices

The piezoelectric effect occurs when pressure is applied to the crystal, so that the crystal lattice is deformed, resulting in a dipole moment in the molecules of the crystal. This type of transducer usually involves the use of a crystal, which has a natural resonant frequency of oscillation, which can be modulated. In addition to crystals, there are other substances, which demonstrate a piezoelectric effect, and these include ceramic materials, e.g. barium titanate, some organic polymers e.g. poly(vinylidene fluoride) (PVDF). The piezoelectric quartz (silicon dioxide) crystal (PQC) is the most commonly used piezoelectric device, the reason being its electrical, mechanical and chemical properties are well suited for use as a mass detector.

There are several different types of piezoelectric device, the main four being thickness-shear mode (TSM), surface acoustic wave (SAW), flexural plate wave (FPW), and acoustic plate mode (APM). Figure 1.3 illustrates the different techniques.
Figure 1.3 The structures of TSM, SAW, FPW, and APM (taken from Grate et al.\textsuperscript{36}). This diagram shows the various views of the different piezoelectric set-ups, and the direction of the propagated wave.

The SAW device uses two sets of interdigitated electrodes at either end of the quartz crystal, and the wave created is a surface-localised, shear-horizontal-polarised acoustic wave (known as Rayleigh waves).\textsuperscript{37} These waves are commonly confined within a waveguide layer to prevent wave diffraction into the bulk, and these are called Love Wave devices (see figure 1.4). This device is commonly prepared from ST-cut quartz crystals (cut at 42° angle from the z-axis), which have a fundamental frequency of between 30 and 300 MHz.\textsuperscript{36} The thickness of the SAW device is usually greater than 10 times its wavelength. An example of a SAW wave device is that used by Gizeli \textit{et al.}\textsuperscript{38}, where they used three devices, two were quartz operating at 108 and 155 MHz, and the other was a lithium tantalate (LT) device operating at 104 MHz. These were used with a waveguide prepared from Novolac, of various thicknesses to determine the effect upon IgG binding to surface-immobilised protein A. The SAW device has most commonly been used for immunosensor development.\textsuperscript{37, 39}
Figure 1.4 The SAW device used by (and taken from) Saha et al., showing the interdigitated electrodes, and the polymer waveguide.

The FPW device produces a wave (called a Lamb wave) which travels through the device from one set of interdigitated electrodes to the other. This device is usually less than 5 μm in thickness (substantially less than λ for this device), and supported by a surrounding silicon layer. The FPW device used by Nguyen et al. is shown in figure 1.5, where it was used as a micropump, integrated into a thermal flow through sensor.

Figure 1.5 The FPW pump device with a flow through sensor used by Nguyen et al.
In appearance the acoustic plate mode (APM) sensor looks similar to the SAW device, with a set of interdigitated electrodes at either end of the device, but it is substantially thinner at 3 to 10 $\lambda$. The other difference is the propagation of the wave, not only a Rayleigh wave across the surface between the two interdigitated electrodes, but also a shear wave, which is reflected between the plate surfaces as it progresses between the two interdigitated electrodes. This device has been used to determine mercury contamination within groundwater\textsuperscript{41}, and the pollution of water by engine oil\textsuperscript{42}, the experimental setup of the later is shown in figure 1.6.

![Figure 1.6 The experimental arrangement used by Kostial et al.\textsuperscript{42}.](image)

In the TSM device, the wave is propagated perpendicular into the bulk of the solution/air away from the surface. This device is commonly prepared using an AT-cut quartz crystal, the frequency of which is dependent upon the thickness of the crystal, as is seen in equation 1d, below (from Uttenthaler et al.\textsuperscript{43}).
where, \( f_0 \) and \( f_n \) are the resonant frequency of the fundamental \((n=1)\), and the \( n^{\text{th}} \) overtone \((n=3, 5, \ldots)\), and \( v \) is the velocity of sound (i.e. \( v = 3340 \text{ m/s} \) in an AT-cut quartz crystal), and \( d \) is the thickness of the TSM device (usually 0.5\( \lambda \)). The latter is related to the wavelength of the device \((\lambda = 2d/n)\).

There are a number of different cuts for quartz crystals, the angle at which each cut is taken, leads different vibrational modes. Two cuts known as the AT- and BT-cut vibrate exclusively in the TSM. The AT-cut crystal is the most popular cut for biosensor technology. The AT-cut crystal is one which has been cut at an angle of +35°15' from the z-axis. This type of crystal has a stable resonant frequency over a range of temperatures, and this is due to the temperature coefficient being nearly zero. Other cuts include X-, Y-, and SC-cut. Throughout this report, the AT-cut crystal will be discussed.

The modulation of the frequency of the quartz crystal is dependent upon the physical properties of the crystal, for example, electrode area, and cut, but also the medium interfacing with the crystal. The resonant frequency is proportional to the overall mass of the crystal, and this is demonstrated in the Sauerbrey equation\(^{44} \). The Sauerbrey equation provides a relationship between a change in frequency (\( \Delta f \)) (in Hz) and the corresponding change in surface mass (\( \Delta m \)) (in grams/cm\(^2\)), and this is shown below,

\[
\Delta f = -2f_0^2 \frac{\Delta m}{A(p_\text{d}h_0)^{1/2}} \quad (1e)
\]
Where, $A$ refers to the sensing area of the crystal ($cm^2$), and $f_0^2$ is the fundamental resonant frequency (squared) in Hz, $\rho_q$ is the density of the quartz crystal, and $\mu_q$ is the shear modulus of the quartz crystal. This equation provides the relationship between frequency and mass for measurements in a dry environment, if the crystal is being used at the fundamental frequency. The frequency depends upon the mass of the crystal and any modifications made to the surface of the crystal, e.g. electrodes, polymer membranes. These modified devices can measure the adsorption of an analyte on the surface, since this produces a corresponding change in the resonant frequency. The transducer can be used to measure at the picogram level.

The quartz crystal has recently been used more and more in a liquid environment, and under these conditions the relationship between the frequency and mass is no longer straightforward. It was found that the frequency of oscillation is dependent on other parameters, such as viscosity ($\eta$), density ($\rho$), and the conductivity of the solution. Their equation for quartz crystals in a liquid environment is shown below.

\[
\Delta f = -f_0^2n \sqrt{\eta_L \rho_L} \left( \frac{\mu_q \rho_q}{\rho_q} \right)^{1/2}
\]  

(1f)

Where $\Delta f$ is the change in frequency, $f_0$ is the fundamental frequency of the crystal, $n$ is the number of sides of the crystal, which are coated, $\eta_L$ is the viscosity of the solution, $\rho_L$ is the density of the solution, $\rho_q$ is the density of the quartz crystal, $\mu_q$ is the shear modulus of the quartz crystal. Equation 1f assumes the quartz being treated as a lossless elastic solid, and the liquid as a pure viscous fluid. The equation did take into account the density and viscosity of the liquid, but did not address the influence...
of interfacial factors, such as the roughness of the quartz, the thickness of the quartz, and any deposited mass on the electrode surface. Kanazawa and Gordon\textsuperscript{45} assumed that the transverse displacement of the quartz surface (actually the electrode surface) is identical to that of the adjacent liquid layer, called the no-slip boundary condition.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure1.png}
\caption{One-dimensional model of the crystal/liquid composite resonator. $\rho_L$, $\eta_L$, and $l_L$ are the density, viscosity and thickness of the liquid layer, respectively, $l_p$ is the penetration depth of the shear wave into the liquid (taken from Benes \textit{et al.}\textsuperscript{46}). The penetration depth is proportional to the density and viscosity of the liquid, $\delta = (2\eta/\rho\omega)^{1/2}$, where $\delta$ is the penetration or decay depth, $\eta$ is the viscosity of the liquid, $\omega$ is the angular velocity of the shear wave ($2\pi f$), and $\rho$ is the density of the liquid\textsuperscript{47}.

\textbf{Figure 1.7} One-dimensional model of the crystal/liquid composite resonator. $\rho_L$, $\eta_L$, and $l_L$ are the density, viscosity and thickness of the liquid layer, respectively, $l_p$ is the penetration depth of the shear wave into the liquid (taken from Benes \textit{et al.}\textsuperscript{46}). The penetration depth is proportional to the density and viscosity of the liquid, $\delta = (2\eta/\rho\omega)^{1/2}$, where $\delta$ is the penetration or decay depth, $\eta$ is the viscosity of the liquid, $\omega$ is the angular velocity of the shear wave ($2\pi f$), and $\rho$ is the density of the liquid\textsuperscript{47}.

Martin \textit{et al.}\textsuperscript{48}, considered the Quartz Crystal Microbalance (QCM), with a simultaneous mass and liquid loading. They determined that the viscous coupling of
the liquid medium to the oscillating surface of the device results in both a decrease in the resonant frequency and a dampening of the resonance. Therefore since both mass and liquid loading affect the resonant frequency, the measurement of the resonant frequency alone cannot distinguish between changes in the surface mass from those in the solution properties. They showed that by measuring the electrical characteristics over a range of frequencies near resonance, a differentiation could be made between these loading mechanisms. It was also commented upon how the electrical characteristics of the QCM, are influenced by the coupling of the mechanical displacement and electrical potential in the quartz, which leads to mechanical interactions between the QCM and the contacting media. This is of particular significance near the QCM resonance, the point where the amplitude of the crystal’s oscillation is at its greatest.

Thompson et al.\textsuperscript{49} found that the viscosity near the surface of the QCM could differ from that of the bulk fluid, due to hydrophilic or hydrophobic forces. By changing the nature of the surface, the frequency shift observed when the crystal was exposed to water increased with a hydrophobic surface.

Rajakovic et al.\textsuperscript{50} reported that the interfacial viscosity decreases with a hydrophobic surface, and that some slip at the surface occurs. These effects will reduce the coupling and hence the equivalent resistance below that predicted by the present model based on the bulk viscosity, and the no slip assumption.

The no-slip boundary condition fails to consider the sensor-liquid interface boundary conditions, such as surface roughness, interfacial viscosity and free energy.
Schumacher et al. proposed that anomalously large shifts can be attributed to liquid trapped in microscopic surface cavities. However, when Martin et al. studied the effect of surface roughness, they concluded that there was no slip nor liquid ordering at the solid-liquid interface, because the trapped liquid due to the surface roughness can account for their experimental results. Yang and Thompson have demonstrated that the response of the Bulk Acoustic Wave (BAW) sensor in liquids is controlled by the molecular boundary conditions that exist at the sensor-liquid interface.

Ferrante et al., introduced the interfacial slip parameter, this accounted for the slip between the surface of a sensor vibrating in the transverse shear mode, and a liquid which is in contact with the surface of the sensor. By measuring the impedance of the sensor with hydrophilic and hydrophobic coatings in water-glycerol solutions, they were able to use non-linear regression analysis of the experimental values of impedance to determine the interfacial slip parameter.

In figure 1.8, a typical impedance (Z) vs. frequency (f) trace is shown, identifying the $Z_s$ (series resonance impedance) and $Z_p$ (parallel resonance impedance). The $Z_s$ and $Z_p$ are the two points at which the phase angle is zero, corresponding to the maximum and minimum impedance measured during the frequency-impedance sweep, the corresponding frequencies are known as $f_s$ (series resonance frequency), and $f_p$ (parallel resonance frequency). With dampening the $Z_s$ will increase, and the $Z_p$ will decrease, so the distance between these two points will decrease, while the plot becomes more flattened, as the frequency values do the opposite to their corresponding impedances.
Fig. 1.8 A typical impedance (Z) vs. frequency characteristic for a 10 MHz AT-cut quartz crystal immersed in aqueous phosphate buffer.

When used in a liquid loaded environment, the series resonance is determined by the mechanical properties of the liquid (i.e. density, viscosity and mass), while the parallel resonance is determined by both mechanical and electrical (i.e. conductivity and dielectric constant of the liquid)\textsuperscript{55}, and this is why the series resonance is the better parameter for monitoring changes in the liquid loading at the crystal surface. Throughout this work, the series impedance was used to quantify the crystal response during the experiments.
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The operation of the quartz crystal has been described in terms of corresponding electrical properties, this is known as the Butterworth van-Dyke model (see figure 1.9).

\[ Z_0 \]
\[ C_q \]
\[ L_q \]
\[ R_q \]

Figure 1.9 The Butterworth van-Dyke model equivalent circuits for (a) the parallel impedance \((Z_p)\), and (b) the series impedance \((Z_s)\). The capacitance, \(C_0\) represents the static capacitance, which arises from the positioning of an electrode either side of the quartz crystal.

The \(C_q\) is the mechanical elasticity of the quartz, \(L_q\) the inductance due to the initial mass of the quartz crystal and overlayer (e.g. liquid upon crystal surface), and \(R_q\) is the resistance arising from the energy losses arising from viscous effects of the liquid layer, internal friction, and damping induced by the crystal holder. The circuit for \(Z_s\) is composed of the various mechanical components of the quartz crystal, whereas \(Z_p\) has an additional component \(Z_0\), which corresponds to the electrical properties of the quartz crystal and the overlayer\(^5\).

The impedance is a measure in part of the mechanical impedance, and this is composed of another two components. These are known as the real part, i.e. the mechanical energy losses, and the imaginary part, i.e. the mechanical energy storage.
at the surface. Hartmann et al. have related the imaginary impedance ($Z_{im}$) to the change in the series frequency, as shown below.

\[ \Delta f_s = -f_s \frac{Z_{im}}{\pi \sqrt{\rho_q \eta_q}} \]

This equation is similar to the equation described by Kanazawa & Gordon (see equation 1f), and from the two, it would seem that the imaginary impedance is related to the density and viscosity of the liquid. This demonstrates that the changes in frequency are related to changes in the impedance, and so either parameter can be used for detection of mass adsorption events and changes in viscoelasticity at the crystal surface.

One advantage of this transducer is the lack of interference caused by electrochemically active substances such as urate and ascorbate, which is a problem for amperometric and voltammetric transducers. Another advantage is the low cost of the construction of the quartz crystal biosensor. The major disadvantage is the requirement for the signal-producing event to occur within a few hundred nanometres of the transducer surface for successful detection. Therefore fouling of the crystal surface could lead to a reduction in its sensitivity, which limits the lifetime of the crystal. This can be overcome by the use of polymer layers, but then problems of detecting the signal can be encountered, due to the reduced detection region of the crystal.
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1.5 Quartz Crystal Biosensors

The quartz crystal has been used to measure various analytes, which are of medical, environmental, and food biotechnological importance. In the medical area, this has generally with a view to replacing the present analytical techniques with methods, which are more accurate and easier and/or cheaper to use. This includes detection of various diagnostic analytes such as creatinine, which is indicative of kidney problems, and detection of pathogens such as African swine Fever\(^58\). The QCM has been used to measure environmentally significant compounds such as pollutants, and toxic gases. In the food industry, it may be utilised to measure the fermentation process or food spoilage. The area of interest in this review is the QCM as a medical biosensor, and therefore this is the area of discussion.

1.5.1 Enzyme-based Biosensors

Patolsky \textit{et al.}\(^59\), used a modified quartz crystal biosensor for glucose detection. They immobilised the two enzymes, glucose oxidase and horseradish peroxidase through the use of glutaraldehyde, and active ester groups to the electrode of the quartz crystal.

The quartz crystal used in this biosensor was an AT-cut, and a fundamental frequency of 9 MHz. The coverage of the enzymes was analysed through the resulting frequency changes of the crystal. The quantitative analyses were made from the resulting frequency changes as the insoluble precipitate was deposited onto the crystal surface.
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Benzo-4-chlorocyclohexadienone is the product of the oxidation of 4-chloro-1-naphthol by horseradish peroxidase (equation 1.1). The degree of precipitation of the insoluble product was directly proportional to the concentration of hydrogen peroxide in solution, or produced enzymatically. The quartz crystal biosensor was used to follow the accumulation of the precipitate at the crystal's electrode surface, and through the use of the Sauerbrey equation the mass of precipitate could be calculated from the change in frequency. The authors found that they had successfully constructed a biosensor, which could be used to measure any analyte through the use of the relevant oxidase enzyme.

Karousos et al.\textsuperscript{60} have recently described a quartz crystal biosensor for the determination of organophosphate and carbamate based pesticides. Through the use of an enzyme cascade (acetylcholine-esterase, and choline oxidase), which resulted in the production of hydrogen peroxide from the initial substrate of acetylcholine. The production of hydrogen peroxide was monitored through the use of the benzidine, 3,3'-diaminobenzidine, which in the presence of horseradish peroxidase produced an insoluble precipitate on the top electrode of the quartz crystal. The precipitate led to a frequency/impedance shift proportional to the degree of precipitation. The pesticides
chosen are known to inhibit the activity of the first enzyme in the enzyme cascade, acetylcholine-esterase, and the amount of inhibition is proportional to the concentration of the pesticide. So the amount of precipitation was proportional to the degree of the hydrogen peroxide produced, which was dependent upon the activity of the initial enzyme, and therefore the concentration of pesticide present could be determined. Using this system, the authors were able to measure pesticides at concentrations of 1 ppm (parts per million) in PBS, with a response time of 25 min.

### 1.5.2 Quartz Crystal Immunosensors

One use of the quartz crystal is the measurement of cocaine levels. This was achieved by using an antibody to one of the two major metabolites of cocaine, benzoylecgonine. The gold electrodes of the quartz crystal were precoated with one of the following protein A, protein G or glutaraldehyde, to compare the various immobilisation procedures. The antibody was immobilised on the surface of the precoated quartz crystal. It should be noted that protein A is a polypeptide isolated from *Staphylococcus aureus* that binds to the Fc region of antibodies without interacting at the antigen binding site, and protein G performs the same function but protein G is more efficient in terms of binding. The resulting modified quartz crystals were incubated in a cocaine standard solution for 30 minutes, and then the frequency was measured. To determine the modified crystal's selectivity for cocaine, they were tested using several drug compounds that are widely used, these included testosterone, progesterone and barbital. This study concluded that the protein G immobilisation was the more effective method, and that the addition of other analytes had little or no effect upon the response.
Ebersole and Ward\textsuperscript{62} used an antigen-antibody complex to measure adenosine-phosphosulfate (APS) reductase and human chorionic gonadotrophin (hCG). The anti-APS reductase antibody was immobilised upon the surface of the QCM, to which was bound the APS reductase, and this in turn was bound by an anti-APS reductase-alkaline phosphatase conjugate. This formed a bound sandwich complex, which when exposed to 5-bromo-4-chloro-3-indolyl phosphate (BCIP), produces the oxidised dimer of BCIP that is subsequently deposited on the QCM surface, resulting in a frequency change which is proportional to the concentration of analyte (APS) present.

Human chorionic gonadotrophin was measured by using the sandwich complex method, with a horseradish peroxidase conjugate. The sandwich was immobilised on a separate nylon membrane positioned in close proximity to a polyvinylferrocene (PV-Fc) film on the QCM.

Through the use of sulphosuccinimidyl 6-[3-(2-pyridyldithio)propionamido] hexanoate (sulfo-LC-SPDP) thiolation, Park \textit{et al.}\textsuperscript{63}, were able to immobilise an anti-\textit{Salmonella} antibody onto the quartz crystal. The sensor was used at 35\textdegree C and pH 7.2, has these allowed optimal detection of the bacteria. The \textit{Salmonella} samples were prepared in 0.08 M phosphate buffer, which was found to give maximum sensitivity. The sensor was able to determine the concentration of \textit{Salmonella} in the range 3.2 x 10\textsuperscript{6} - 4.8 x 10\textsuperscript{8} CFU per ml, giving a good correlation with the frequency shifts. The system could be used for more than one measurement, through the use of 1.2mM NaOH, which regenerated the sensor.
Recently, Uttenthaler et al.\textsuperscript{43} have used the quartz crystal for the detection of M13-phage. The M13-phage was chosen because it has been well characterised and antibodies against it are commercially available, plus the weight of the phage is similar to pathogenic viruses. They used a range of quartz crystal of different fundamental frequencies from a standard 19 MHz, to a number of high fundamental frequency crystals (39, 56, 70, and 100 MHz). The capture antibodies were immobilised onto the crystal surface through the use of the crosslinker, dithiobis-succinimidyl-prpionate (DSP), and these crystals were used to capture the phage in a flow through arrangement. It was found that the lowest detection limit of $10^9$ pfu/ml (pfu = plague forming units), gave a frequency response which was 200 times larger using the 56 MHz crystal than the standard 19 MHz crystal.

### 1.5.3 Quartz Crystal DNA sensors

Su et al.\textsuperscript{54} produced a quartz crystal biosensor capable of detecting platinum-based drugs, by measuring the attachment of nucleic acid (calf thymus DNA) to the palladium electrodes. Compounds that have the ability to bind to nucleic acids have been used as chemotherapeutic agents. An example is the drug cisplatin (cis-Diaminodichloroplatinum (II)), which is used for its antitumour activity in ovarian, bladder, and testicular cancer. It was found that the detection limit for the drugs, cis- and transplatin was approximately $10^-7$ M. The changes in $f$, were attributed to the kinetic process of the binding of the hydrolysis products of the drugs to the nucleic acids. In producing this biosensor, the authors are able to perform direct monitoring of DNA-platin drug interactions.
A DNA quartz crystal biosensor was used to determine the level of bacterial toxicity in environmental samples. A quartz crystal biosensor was coated with steptavidin, to which were immobilised biotinylated 23-mer complementary probe for a gene involved in the production of an extracellular toxin by *Aeromonas hydrophila*. This bacterium can be found in fresh water, and has been associated with infections, such as septicaemia, meningitis and wound infections. The crystal surface was modified with thiol/dextran to enable the covalent binding of steptavidin. The different *Aeromonas* strains were isolated from samples of water, vegetable and human specimens, and the DNA amplified using PCR. It was established that there was no non-specific binding to the probe, through the use of a non-complementary oligonucleotide. The crystal surface with the immobilised probe could be regenerated upon the addition of 1 mM HCl. When the biosensor was used with real samples, it was able to distinguish between those bacterial strains containing the target gene and those that did not.

1.6 Aims & Objectives of the Project

The final objective of this project is the demonstration of the possibility of the acoustic quartz crystal being used for the measurement of biomedically significant analytes. The aim of this project is to produce a rapid, selective and sensitive biosensor for the determination of cholesterol and creatinine in biological samples. The determination of the analytes will be achieved enzymatically through the use of oxidase enzymes, which produce hydrogen peroxide, and this will react with a benzidine in the presence of horseradish peroxidase. This reaction will result in the formation of a precipitate, which will adhere to the crystal surface causing a change in
the parameters measured, the magnitude of which will be proportional to the concentration of the initial analyte.

The first objective is the optimisation of the hydrogen peroxide determination. This will be accomplished through the use of a number of benzidines to identify the best in terms of response size and time. The choice of benzidine will probably be determined by its solubility limit, and hence the concentration range over which it can be used. Further a number of polymers and surfactants will be used to alter the surface chemistry of the top electrode, to enhance the degree of adsorption, and overcome the problem of suspension formation, which cannot be detected by the quartz crystal. The polymers may be used for the immobilisation of the enzymes resulting in a biosensor with the potential for multiple use.

The second objective is the employment of the optimised hydrogen peroxide biosensor for the determination of cholesterol and creatinine in buffer. It is intended that hydrogen peroxide will be produced from these analytes, through the addition of a number of enzymes, namely cholesterol oxidase and cholesterol esterase for total cholesterol determination, and sarcosine oxidase, creatinase, and creatininase for creatinine measurement. The conditions of analyte detection will be optimised by identifying the appropriate concentration of the enzymes to achieve this. These biosensors will then be used for analyte determination in biological samples.

The third objective will involve the identification of possible interferents of the quartz crystal method of analyte determination used. Potential interferents to be
experimented with include the plasma proteins, ascorbic acid, paracetamol and uric acid.
Chapter 2 - Optimisation of Hydrogen Peroxide

Determination using the Quartz Crystal Acoustic Wave Sensor

Surfactant Section published in
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City University, London
Chapter 2- Hydrogen Peroxide Optimisation

2.1 Introduction

2.1.1 Background

The idea of using the quartz crystal biosensor with an enzymatic precipitation reaction was first described by Ward et al.\textsuperscript{62}, whose work was discussed in the literature review (section 1.5.2). The strategy described here was first reported by the publication of results by Reddy et al.\textsuperscript{66}, where they had used an AT-cut thickness-shear-mode (TSM) quartz crystal to detect glucose. The detection of the glucose was accomplished via two enzyme reactions (equations 2.1 and 2.2), resulting in the oxidation of 3,3'-dimethoxybenzidine (DMOB). The enzymes used were glucose oxidase (GOx) and horseradish peroxidase (HRP).

\begin{align*}
\text{Glucose} & \xrightarrow{\text{GOx}} \text{Gluconic acid} & \text{Hydrogen Peroxide} \\
& + \text{O}_2 & + \text{H}_2\text{O}_2
\end{align*}

(2.1)
Chapter 2- Hydrogen Peroxide Optimisation

The peroxidase results in the oxidation and dimerisation of the DMOB\textsuperscript{67}, the product of which, bis(3,3'-dimethoxy-4-amino)azobiphenyl, is sparingly soluble and therefore precipitates onto the surface of the quartz crystal, causing a frequency and impedance shift, which is monitored using an impedance analyser. The authors\textsuperscript{66} used the $Z_p$ impedance to monitor the deposition of the precipitate because they found the $f_p$ to be "dissipative", for this same reason throughout this report the series impedance is used to measure the degree of precipitation.

It was concluded that the resulting biosensor lacked sensitivity at a glucose or hydrogen peroxide concentration of less than 60\mu M. The explanation for this phenomenon was, that the product, bis(3,3'-dimethoxy-4-amino)azobiphenyl is slightly soluble, and consequently insufficient amounts were produced to induce a response from the crystal. It was noted that with increasing concentration of glucose or hydrogen peroxide, saturation of the crystal was achieved. The insoluble bis(3,3'-dimethoxy-4-amino)azobiphenyl was binding to the gold surface through hydrogen
bonds via the 1° amine group of the oxidised bis(3,3'-dimethoxy-4-
amino)azobiphenyl. The concentration of glucose, which could be detected, was
limited by the concentration of DMOB present, which was determined by its
solubility (160 μM). In conclusion, it was noted that for medically relevant glucose
assays (2-30 mM in blood) dilution would be necessary. It was commented that
solute gating membranes could be employed to protect the quartz crystal from protein
and cellular interferences.

Alfonta et al.⁵⁸ developed a quartz crystal biosensor that could be used for the
detection of acetylcholine (ACh). They assembled a three enzyme layer on a gold
electrode (for use with cyclic voltammetry and Faradaic impedance spectroscopy), or
on a gold electrode on the quartz crystal. The three enzymes were acetylcholine
esterase (which produces choline from acetylcholine), choline oxidase (which
oxidises choline giving H₂O₂), and HRP (which oxidises tetramethylbenzidine, giving
an insoluble product similar to that of DMOB).

The quartz crystal used was a 9MHz, AT-cut. For this work the precipitation was
produced through the use of 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB),
which the authors sonicated and heated to 45°C, and then cooled to 25°C before use
in any experiments. TMB has a structure similar to DMOB as can be seen in equation
2.2. The authors concluded that they had successfully displayed that the
multienzyme-layered assembly can be organised on an electrode support, leading to a
biocatalytic cascade that leads to the precipitation of an insoluble product on the
electrode support, and used this method to detect and measure acetylcholine levels.
The authors did not investigate the possibility of using the enzymes in solution, to
establish whether the immobilisation of enzymes to the surface may be restricting the access of the oxidised TMB to the surface. The enzyme layer could be acting as a diffusion-limiting barrier, which could have an effect upon the dynamic range of the biosensor.

2.1.2 The Benzidines

In the original work done by Reddy et al.\textsuperscript{66}, they used DMOB, to detect glucose, Alfonta et al.\textsuperscript{68}, used TMB to detect acetylcholine. To further this work, this chapter discusses the use of two additional benzidines as well as DMOB and TMB, to detect the intermediate product of an oxidase reaction, hydrogen peroxide. The benzidines chosen were 3,3'-diaminobenzidine (DAB) and 3,3'-dichlorobenzidine (DCB). All the benzidines have the basic structure shown in figure 2.1, and their main differences are highlighted in table 2.1. DAB and TMB are regularly used in histochemical staining of tissue samples\textsuperscript{69-71}. TMB has been used as a substrate for enzyme-linked Immunosorbent assays (ELISA)\textsuperscript{72,73}.

The purpose of using different benzidines is to determine whether any particular one offers a significant advantage in terms of,

(a.) having a higher solubility in the reduced form and a high insolubility in the oxidised form
(b.) an increased rate of reaction as measured by the high rate of decrease in the impedance
(c.) a more significant change in impedance at 300 μM of H\textsubscript{2}O\textsubscript{2}
(d.) the toxicity of the benzidine, i.e. whether the non-carcinogenic compounds DAB (in the dihydrochloride form), and TMB out-perform DMOB and DCB (the two carcinogenic benzidines).

![Fig. 2.1 The general structure of the benzidines used in this study.](image)

<table>
<thead>
<tr>
<th>Name</th>
<th>Side Groups</th>
<th>MW</th>
<th>Solubility (mM)</th>
<th>Absorption $\lambda_{\text{max}}$ of product (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMOB</td>
<td>OCH$_3$</td>
<td>244</td>
<td>0.16</td>
<td>455-475$^{74}$</td>
</tr>
<tr>
<td>DAB</td>
<td>NH$_2$</td>
<td>214</td>
<td>*Soluble (&gt; 5)</td>
<td>465$^{75}$</td>
</tr>
<tr>
<td>TMB</td>
<td>CH$_3$</td>
<td>240</td>
<td>0.42</td>
<td>465$^{76}$</td>
</tr>
<tr>
<td>DCB</td>
<td>Cl</td>
<td>253</td>
<td>0.049</td>
<td>425$^{77}$</td>
</tr>
</tbody>
</table>

Table 2.1 A summary of the main properties of the four benzidines used in this study, illustrating their differences, in terms of side chain, and solubility.

2.1.3 Surfactants

Surfactants as the name suggests are a group of compounds, which have been found to be surface acting agents. This property originates from the molecular structure of this group of compounds, which have a hydrophobic head group and a hydrophilic tail. This results in them being found in higher concentrations at interfaces (liquid/solid, liquid/liquid and air/liquid), than in the bulk of an aqueous solution, where the tail is in contact with the interface and the head with the aqueous solution, in a similar arrangement to that seen with biological lipid bilayer membranes. When
using low concentrations of surfactant, the majority will be adsorbed at the air/water interface. Only when the concentration is increased will the surfactant begin to adsorb to the surface until saturation is reached, and the surface tension becomes constant, at which point the surfactant will begin to form micelles. This point is referred to as the critical micelle concentration (CMC). The size of the micelles formed differs from surfactant to surfactant. It is measured by the aggregation number, which is the number of surfactant molecules associated with a micelle\textsuperscript{78, 79}.

Here, we have studied the enzymatic oxidation of 3,3'-diaminobenzidine (DAB), 3,3',5,5'-tetramethylbenzidine, and 3,3'-dimethoxybenzidine, in the presence of surfactants in order to improve liquid/surface interactions of the signal species and enzyme activity. Tween 80 (polyoxyethylene sorbitan monooleate), and Triton X-100 (\(t\)-octylphenoxypolyethoxyethanol), two non-ionic hydrophilic surfactants, bis (2-ethylhexyl) hydrogen phosphate (BEP) an anionic surfactant and Aliquat 336s a cationic surfactant were investigated.

### 2.1.4 Polymers

A number of polymers were used with the DAB-HRP-H\textsubscript{2}O\textsubscript{2} reaction with/without Triton X-100. These polymers were poly(vinyl chloride) (PVC), and cellulose acetate, which were prepared by solvent casting; polypyrrole and diaminobenzidine, which were both electropolymerised, with the latter also being fabricated by oxidative polymerisation.

PVC has been used extensively as a membrane, with amperometric oxidase enzyme biosensors\textsuperscript{80-82}. It forms a hydrophobic barrier\textsuperscript{83}, which is perm-selective. This
property is achieved by ion rejection and molecular size exclusion. The authors mentioned above found that these properties resulted in a reduction in the interference from ascorbate and urate, when used with the amperometric biosensor. These substances caused interference with the amperometric detection method because of the oxidation potential applied to the electrode, which oxidised the hydrogen peroxide, resulting in a proportional increase in the current measured. The problem is that at the potentials used, other substances within the biological samples are also oxidised leading to a further increase in the current leading to a false positive, and so to overcome this problem, polymers have been used to reduce these interferences. PVC has been used with the quartz crystal biosensor for the determination of phenol. It was found that the presence of the PVC layer had an effect upon the initial response times, which decreased at low concentrations and increased at high concentrations of the polymer. The same was true for the response size. Reddy explained the difference between the concentrations of the PVC, and its distribution upon the surface of the crystal. At low concentrations, it was deposited at the periphery of the crystal, whereas at higher concentrations it formed a more fibrous layer across the whole crystal surface, and the interaction of the indophenol dye with these surfaces.

Cellulose acetate is a hydrophilic and perm-selective polymer that has been used with amperometric biosensors for the determination of cholesterol, and glucose.

Polypyrrole is a commonly used electropolymerised polymer, which has been used in the fabrication of biosensors for cholesterol, glucose, and hydrogen peroxide. Polypyrrole produces a polymer, which is selective according to size, and
therefore can be used to reduce interference from ascorbate and urate\textsuperscript{87}. The polypyrrole film can be produced by a number of methods e.g. Razola \textit{et al.}\textsuperscript{16}, used cyclic voltammetry between 0.0 and +1.0 V versus Ag/AgCl at 10 mV/s, while Tian \textit{et al.}\textsuperscript{90} used a galvanostatic mode using a current density of 0.06 mA/cm\textsuperscript{2}. In this chapter, the method of Vidal \textit{et al.}\textsuperscript{87} was used, where they maintained a fixed voltage of +0.7 V versus Ag/AgCl for a fixed amount of time, to produce films of different thickness.

The use of diaminobenzidine as a polymer has only been reported twice before, once where it was used to entrap glucose oxidase as part of an amperometric biosensor for glucose\textsuperscript{91}, and the second has part of a polymer-modified electrode for the pre-concentration and anodic stripping voltammetry of selenium\textsuperscript{92}. Zhang \textit{et al.}\textsuperscript{91} used poly(diaminobenzidine) to immobilise glucose oxidase for the amperometric determination of glucose. They found that the resulting polymer was perm-selective for hydrogen peroxide, which increased relative to the electropolymerisation time, thus reducing the interference from ascorbate, urate and paracetamol. Each author used a different method of producing the polymer, Cai \textit{et al.}\textsuperscript{92} used cyclic voltammetry between -0.2 and +0.8 V versus Ag/AgCl at 10 mV/s, while Zhang \textit{et al.}\textsuperscript{91} used a voltage of +0.7 V versus SCE (saturated calomel electrode), and various lengths of time (2 – 30 min). We used a voltage of +0.8 V versus Ag/AgCl, and various lengths of time to produce poly(diaminobenzidine) films.

The polymers were used with the DAB-HRP-H\textsubscript{2}O\textsubscript{2} reaction, both in the absence/presence of Triton X-100. It was thought that the polymers would alter the surface chemistry of the quartz crystal, which would increase the deposition of the
oxidised DAB, and accordingly the change in impedance. Another possible benefit of using polymers could be protection of the quartz crystal surface against the oxidised product, extending the lifetime of the crystal.

2.1.5 Horseradish Peroxidase

Horseradish peroxidase (HRP) is a hydrogen peroxide oxidoreductase (E.C.1.11.1.7, molecular weight of 44 kDa) formed from a single polypeptide with 308 amino acid residues, a haemin prosthetic group and two Ca\(^{2+}\) ions that maintain the enzyme conformation\(^{93}\). HRP was used in the presence of H\(_2\)O\(_2\) to catalyse the oxidation of the benzidines (see equations 2.2 and 2.6). A possible problem is the concentration of H\(_2\)O\(_2\), which if too high will inhibit HRP's catalytic activity\(^{94}\).

2.1.6 Strategy

The aim of this chapter was to develop an optimised hydrogen peroxide biosensor, which could be used to detect other analytes through the addition of the respective oxidase enzyme. A number of strategies were used to optimise the determination of hydrogen peroxide, using the benzidine detection method with the quartz crystal. A number of different benzidines were used to determine the best for detection, and whether any were particularly better at different concentrations. Various surfactants, polymers and oxidised benzidines were used to alter the surface chemistry of the gold electrode in an effort to increase the response size and decrease the response time.
2.2 Experimental

2.2.1 Materials

Phosphate buffer tablets were acquired from Oxoid (Basingstoke, UK), and hydrogen peroxide (60% w/v), N, N dimethylformamide, acetone and isopropanol from Fisher (Loughborough, UK). The enzymes horseradish peroxidase (116PU/mg) (EC. 1.11.1.7), Triton X-100, Tween 80, 3,3',5,5'-tetramethylbenzidine, 3,3'-diaminobenzidine tetrahydrochloride, and 3,3'-dichlorobenzidine dihydrochloride were obtained from Sigma (Poole, UK). The 3,3'-dimethoxybenzidine, Bis (2-ethylhexyl) hydrogen phosphate, Aliquat 336s, cellulose acetate, pyrrole, poly(vinyl) chloride and tetrahydrofuran (THF) were purchased from Aldrich (Poole, UK).

Methods

2.2.2 Crystal Preparation

Before the gold-on-chromium electrodes (100 and 5 nm respectively) were vapour-deposited onto either side of a blank AT-cut quartz crystal (CMAC, UK) (fundamental resonance frequency of 10MHz, and diameter of 8.2 mm), the crystals were cleaned. The mask, into which the crystals were placed for the deposition of the electrodes, was cleaned using acetone, which was allowed to evaporate, and the crystals were cleaned using both acetone and isopropanol. The crystals were then inserted into the mask, after which they were cleaned with isopropanol, followed by the use of a vacuum pump to remove the excess solvent and dust particles. The crystals and mask were then kept in a sealed plastic sachet, prior to the vapour-deposition. Using a vacuum pump, the electrodes were vapour-deposited onto crystal. The vacuum pump was lowered to 10^4 Pa, and the voltage was slowly increased,
allowing the vaporisation of the gold and chromium. After this, the conditions were returned to normal, before the mask was turned over, and the procedure repeated.

2.2.3 Crystal Installation & Measurement Equipment

After the electrodes were deposited to the crystal surface, the crystal was cleaned again with isopropanol, and left to dry. A small length of gold wire was attached to the upper larger electrode, by means of silver paint. The other end of the gold wire was connected onto the gold pin of the cell, using silver paint, so that the crystal sat on top of the brass button. The crystal was now sealed in the sample chamber previously described\(^4\) (see fig. 2.2). The sealing was achieved by gently placing the top half of the cell onto the bottom half. Two fingers were placed inside the well of the top half of the cell, where pressure was applied to lower the top of the cell, onto the spring-loaded bottom half. The clips could then be attached to maintain the position of the crystal, and thus sealing it from leakage by means of an o-ring. A HP4194A impedance analyser coupled to a PC was used to record the crystal’s frequency and impedance (see fig. 2.3). When using the impedance analyser and computer in a continuous scan, readings were taken every ten seconds.
2.2.4 The computer program

The program used to interface the impedance analyser with the computer was written in-house (by Dr S. M. Reddy) using Qbasic. It enables the computer to display the data taken by the analyser in a graphical form using the program, Easyplot (Spiral software). The impedance analyser monitors changes in the impedance and frequency of the crystal’s oscillations. The Qbasic program is capable of presenting the data in two ways, either as a single scan, which is an impedance vs. frequency curve, or a continuous scan, where the change in impedance and frequency is plotted against time. The frequency monitored corresponded to the resonance frequency of the crystal (usually about 9.8 MHz). For the single scan, the impedance analyser measures the parameters at 401 points along the scan, i.e. the frequency range at which the measurements are being taken is split up into 401 equal parts, and one measurement is taken from each part. For all these experiments, the frequency range was 50 kHz, and therefore there are approximately 125 Hz between each measurement. This frequency range included both the series and parallel frequencies, the exact values depending upon the crystal.
2.2.5 Solution preparation

3,3'-diaminobenzidine (DAB), 3,3'-dichlorobenzidine (DCB), 3,3'-dimethoxybenzidine (DMOB), and 3,3',5,5'-tetramethylbenzidine (TMB) were prepared in phosphate buffer (one phosphate buffer tablet dissolved in 100 ml of distilled water), pH 7.4, to give a final concentration of 0.3 mM, 48.6 μM, 0.16 mM, and 0.3 mM respectively. The chemicals were weighed on a piece of aluminium foil, to overcome problems of static. The two carcinogens, DCB and DMOB were both weighed out taking the necessary precautions i.e. glasses, plastic apron, gloves, and mask. The weighing of these two chemicals was completed inside a glove box. Before use, the TMB and DCB solutions were heated to 45°C for 15 minutes, and then cooled to room temperature. This pre-treatment of TMB and DCB was found to be necessary, for the enzyme reaction to occur. The surfactant solutions were prepared in PBS. The horseradish peroxidase (180 PU/mg) was prepared in distilled water, and the hydrogen peroxide solution (10 mM and 5 mM) was prepared using phosphate buffer. When not in use, all solutions were stored at 4 °C, and the horseradish peroxidase and hydrogen peroxide solutions were prepared daily. The benzidines and surfactants were prepared every other day and stored at 4 °C, when not in use.
2.2.6 Measurement procedure

At the beginning of each day a single scan was taken of the dry crystal, this was for the purpose of monitoring the crystals performance over time (days, weeks and months). For all experiments, a single scan was taken of the crystal loaded with distilled water. A continuous scan was then started, with the distilled water being removed at approximately 90 seconds, the water being removed by means of a pasteur pipette. At 210 seconds, 3.5 ml of the reagent were added to the test cell, to which at 240 seconds was then added 18.9 μl horseradish peroxidase. The hydrogen peroxide
was added at about 860-890 seconds, to give a particular final concentration (0 to 400 μM). During all experiments, the solutions were stirred. After each experiment, a single scan of the crystal loaded with distilled water was taken, before cleaning thoroughly with N, N dimethylformamide (added using a pasteur pipette, and left stirring for 10 seconds) and distilled water (left stirring for 10 seconds). The use of the organic solvent removed the dye produced during the experiment, and this was confirmed by the crystal frequency returning to its original level. After cleaning, another single scan of the loaded crystal (with distilled water) was taken. All experiments were performed at room temperature.

2.2.7 Spectrophotometric measurements

All measurements were taken at 492 nm using a 96 well plate reader (Labsystems iEMS Reader MF and Labsystems Genesis Version 3.05 software). For the spectrophotometric measurements, all solutions were prepared as previously described. For the HRP experiments, 300 μl of DAB, 1 μl of HRP, 3 μl of Triton X-100, and a range of H₂O₂ concentrations from 0 to 60 μM were added to each well. The absorbance was measured every 15 seconds, over 10 minutes at 492 nm. For the HRP-ChOx experiments, 300 μl of DAB, 1 μl of HRP, 1 μl of ChOx, and a range of Triton X-100 concentrations were added, after which was added 9 μl of cholesterol (10 mM). Again the absorbance was measured every 15 seconds, over 10 minutes at 492 nm.
2.2.8 Polymer preparation- solvent casting

The two polymers, cellulose acetate and PVC were used at various concentrations (0.05%, 0.01%, and 0.001% (w/v)). Cellulose acetate was prepared in acetone, and PVC in THF (tetrahydrofuran). A 10μl aliquot of the polymer solution was deposited onto the quartz crystal (at t = 60s), during a continuous scan. The continuous scan was continued until steady state conditions were acquired, after which a normal DAB-HRP-H$_2$O$_2$ reaction experiment was completed (as described in section 2.2.5).

2.2.9 Polymer preparation- electropolymerisation

The two monomers, pyrrole and DAB were used in the electropolymerisation experiments. Both solutions of the monomers were prepared at 5 mM in PBS, which was degassed with pure nitrogen gas for 5 minutes. The polymers were electropolymerised directly onto the top electrode of the crystal. For the electropolymerisation, the top electrode of the crystal was the working electrode. A 25 cm length of silver wire was wound into a flat coil, and exposed to a sodium chloride solution (1 M) prepared in water, with a potential of -800 mV vs. Ag/AgCl (controlled by in-house manufactured potentiostat), to produce a Ag/AgCl reference electrode. This electrode was held in position 3 mm above the crystal. A 3 ml sample of the monomer to be electropolymerised was pipetted onto the crystal surface, which also covered the reference electrode. A potential of +800 mV vs. Ag/AgCl was applied for the electropolymerisation, and left for different periods of time (1, 5, 10 and 30 minutes). A single dry scan of the frequency vs. impedance profile was taken before and after the electropolymerisation to determine the mass of the deposited material.
2.2.10 **Statistical analysis**

Correlation coefficients and comparisons were performed using Instat 3 (GraphPad Software Inc, San Diego, CA, USA) software. A one-way ANOVA was used to determine whether there was statistical significance (a P-value <0.05) between different experimental conditions.
2.3 Results & Discussion

2.3.1 The Benzidines

Fig. 2.4 shows the $Z_s$ data obtained, for the benzidine-HRP-H$_2$O$_2$ reaction, when the hydrogen peroxide concentration was increased from 0 to 400 µM. TMB provides the largest $Z_s$ response to the hydrogen peroxide concentration. DMOB reaches a plateau at 200 µM, while DAB and TMB would appear to give a near linear response throughout the concentration range, as seen by their respective correlation coefficient values. The response with DCB is only marginally better than the control at this concentration range.

![Graph showing the changes in the crystal's series impedance measurements](image)

**Fig. 2.4** This figure shows the changes in the crystal's series impedance measurements taken using the quartz crystal, when a range of hydrogen peroxide concentrations (0 – 400 µM) were used with the four benzidine compounds ($n=3 + s.e.m.$).

From the results, it can be seen that when a benzidine is present in the test solution with hydrogen peroxide and horseradish peroxidase, there is a concentration-related
response. The changes in the parameters being measured occur because of the oxidation of the benzidine, which results in the formation of a product having a lower solubility, and a larger size than the benzidine.

At low concentrations of hydrogen peroxide, DMOB would appear to be the best benzidine for the determination of hydrogen peroxide in terms of change in $Z_s$, however at higher concentrations this position is occupied by TMB. This difference in performance of the benzidines is due to the differences in the concentration of the benzidines. During the oxidation of the benzidine, two molecules of hydrogen peroxide are reacted with two benzidine molecules the later then react with each other, forming a more insoluble product. This ratio of hydrogen peroxide and benzidine results in the depletion of the benzidine if it is present at low concentrations and H$_2$O$_2$ at high concentrations. This is the case for DMOB, which is used at a concentration of 160 $\mu$M, giving a corresponding detection limit of 160 $\mu$M hydrogen peroxide, whereas TMB is used at a concentration of 300 $\mu$M, and hence a detection limit of 300 $\mu$M hydrogen peroxide. The existence of a detection limit can be clearly seen in the results, where DMOB begins to plateau between 100 and 200 $\mu$M hydrogen peroxide, and TMB does the same between 300 and 400 $\mu$M hydrogen peroxide. This detection limit is also the cause of the low DCB results, which is used at a concentration of 48.6 $\mu$M due to its low solubility, and therefore can only detect hydrogen peroxide up to a concentration of 48.6 $\mu$M. The DAB results are different; this does not produce a plateau, even though it is only being used at a concentration of 300 $\mu$M. This suggests that this benzidine is being oxidised at more than the one amino group forming oligomers, giving it a greater range of detection$^{95}$. 

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In summary, DCB or DMOB would be the benzidine of choice for the detection of hydrogen peroxide at low concentrations (0-10 μM), at greater concentrations (10-100 μM), it would be DMOB, DAB or TMB, at high concentrations (100-300 μM), DAB or TMB, and above this range, it would be DAB.

2.3.2 Optimisation with Surfactants

As seen in the previous section, in the absence of surfactants, the enzymatic oxidation of the benzidines formed an insoluble suspension. Due to the formation of the suspension, limited impedance and frequency changes were measured (ΔZ = 13.2 ± 0.8 Ω for 300 μM hydrogen peroxide with DAB). These measurements indicated that a limited amount of oxidised benzidine product was adsorbing to the surface of the quartz crystal.

Surfactants were used to increase the solubility of the amphiphilic dimers, in an attempt to increase their interaction with the gold electrodes of the quartz sensor surface. The dimerisation occurs at the amine group of the benzidine, and since DAB possesses more than two amino groups, it is possible that the oxidised dimer may undergo further catalytic oxidation forming tetramers or possibly oligomers. These could be responsible for the larger particles in suspension observed.

BEP and Aliquat solutions in the concentration range 0.001 to 0.1% (v/v) appeared to have an inhibitory effect on the crystal response upon peroxide addition (ΔZ = 2.5 ± 1Ω for 300 μM hydrogen peroxide). However, both surfactants increased the intensity of the coloured solution. It was possible that the anionic surfactant, BEP was forming stable complexes with soluble cationically charged benzidine
intermediates. In contrast, Aliquat 336s, being cationic and surface-active could be repelling like charged species from the surface, and therefore the oxidised benzidine tended to stay in solution. The presence of the red-brown solution suggested that the surfactants were not inhibiting enzyme activity.

Figs. 2.5 shows a clear enhancing effect of the Tween 80 and Triton X-100 concentration respectively on impedance response upon addition of hydrogen peroxide (300 μM) to a solution containing benzidine and peroxidase.

The optimum concentrations of surfactant vary for each benzidine, between each surfactant. It is interesting to note that the concentration of surfactant required for the optimum response is above the CMC, the one exception being TMB with Triton X-100 (see Table 2.2).

The addition of the enzyme, horseradish peroxidase, to a solution of TMB gave a turquoise solution. This colour change occurred in the absence of the surfactants and hydrogen peroxide. The sonication and heating treatment of TMB may produce a more reactive intermediate, which upon addition of HRP would appear to interact with the latter, resulting in the colour change observed. There was no observable change in colour upon the addition of surfactant, though it is possible this could be concealing any colour changes due to the formation of a microemulsion, which would explain the lower amounts of surfactant required for optimisation. Upon measurement of the absorbance at 492 nm (this wavelength was chosen because it was the nearest filter to 465 nm, the λmax of TMB), there is a small change in absorbance between the TMB with horseradish peroxidase solution (0.135), and the
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TMB with horseradish peroxidase and 0.002 % Tween 80 solution (0.162). This result would indicate that there is a possibility that microemulsion is occurring.

Fig. 2.5a

![Graph showing effect of Triton X-100 on impedance](image)

**Fig. 2.5a**

**Fig. 2.5b**

![Graph showing effect of Tween 80 on impedance](image)

**Fig. 2.5 a & b** Effect on 300 μM H₂O₂ response in impedance (Zₘ), upon varying (a) Triton X-100 and (b) Tween 80 concentrations. The benzidines studied were DAB, DMOB, and TMB.
Chapter 2- Hydrogen Peroxide Optimisation

Optimum [surfactant] (mM)

<table>
<thead>
<tr>
<th>Surfactant</th>
<th>Tween 80</th>
<th>Triton X-100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aggregation No.</td>
<td>60</td>
<td>140</td>
</tr>
<tr>
<td>CMC</td>
<td>0.012</td>
<td>0.24</td>
</tr>
<tr>
<td>DAB</td>
<td>0.098 (70.6 Ω)</td>
<td>1.71 (32.3 Ω)</td>
</tr>
<tr>
<td>DMOB</td>
<td>0.041 (86.2 Ω)</td>
<td>3.42 (36.5 Ω)</td>
</tr>
<tr>
<td>TMB</td>
<td>0.016 (38.9 Ω)</td>
<td>0.10 (14.6 Ω)</td>
</tr>
</tbody>
</table>

Table 2.2. The major properties of Tween 80 and Triton X-100, and the optimal concentration required for each benzidine used, in brackets are the crystal impedance responses for the various benzidines at these concentrations, with 300 μM H₂O₂. In the absence of any surfactants at 300 μM H₂O₂, the responses of the benzidines were 21.6, 11.5 and 12.1 Ω for TMB, DMOB, and DAB respectively.

For DMOB and DAB, the solution remained colourless upon the addition of the surfactant, and therefore it can be suggested that the result was a solubilisation of the benzidines rather than emulsion. The DMOB and DAB responses are optimised at similar concentrations of surfactant, implying that the mechanism of interaction between the surfactant and the benzidine is the same. There is a substantial difference in the optimal concentrations of surfactant required for DMOB, DAB and TMB (see Table 2.2). The enzymatic oxidation of TMB is optimised at a significantly lower concentration of surfactant than the other two benzidines, but the response is similar in size to the response in the absence of surfactant. This would indicate that the surfactants are having no positive effect upon the TMB reaction.

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The presence of the surfactant in the reaction solution is having two possible effects, which lead to the improved signal generated. The surfactant is most likely to be altering the solubility of the oxidised benzidine produced. If DAB forms tetramers upon oxidation, this will increase the hydrophobicity reducing the ability of the surfactant to solubilise the oxidised product. Hence this could be a possible reason for it not producing large signals on a scale with DMOB. The other possibility is an increase in the activity of horseradish peroxidase due to the presence of surfactants. There have been numerous examples of where surfactants have been used to enhance enzyme activity. Equally, there are examples where the presence of surfactants has had a negative effect upon the enzyme activity. It has been demonstrated previously that Triton X-100 at a concentration range of 0.05-0.1% (v/v), increases the activity of cholesterol oxidase. At concentrations above this, Triton X-100 was found to inhibit the activity of the enzyme. Tween 80 has been found to enhance the activity of protease and α-amylase.

Triton X-100 has a larger CMC than Tween 80, and hence a larger surfactant concentration is required before solubilisation of the oxidised benzidine occurs. Another key difference is the aggregation number, where Triton X-100 has a larger number of molecules per micelle than Tween 80.

It was noted that the oxidation of the benzidine in the presence of the Triton X-100 resulted in quicker response times, than seen in the absence of the surfactant (Table 2.3). This was possibly due to the surfactants improving the enzyme activity, and also improving the dimerisation reaction. The other possibility was that the detergents
increased the solubility of the substrate. These observations could explain the improved response time for Triton X-100 in the present study.

<table>
<thead>
<tr>
<th></th>
<th>No surfactant</th>
<th>With Triton X-100</th>
<th>With Tween 80</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$t_{50}$</td>
<td>$t_{95}$</td>
<td>$t_{50}$</td>
</tr>
<tr>
<td>DAB</td>
<td>272</td>
<td>1266</td>
<td>275</td>
</tr>
<tr>
<td>DMOB</td>
<td>348</td>
<td>4100</td>
<td>413</td>
</tr>
<tr>
<td>TMB</td>
<td>1016</td>
<td>4533</td>
<td>687</td>
</tr>
</tbody>
</table>

Table 2.3. The various reaction times for the oxidation of the benzidines by hydrogen peroxide, and the effect of the surfactant at $t_{50}$ and $t_{95}$ (the $t_{95}$ value is the time lapsed between the addition of the hydrogen peroxide and the stabilisation of the impedance, and $t_{50}$ is the time taken from addition of hydrogen peroxide to when the impedance value is half of the final impedance value).

The extended response time for the oxidation of DMOB and TMB with Tween 80 compared with Triton X-100 could be due to the partitioning of the substrate into Tween micelles, as a result of their higher hydrophobicity than DAB. This could lead to a slow release of benzidine between the micelles and aqueous environment, via a concentration gradient.

Figs. 2.6 (a) and (b) show calibration curves for hydrogen peroxide at the optimised surfactant concentration for each of the benzidines, when using Tween 80 and Triton X-100 respectively.
Fig. 2.6 The impedance ($Z_s$) response, to the hydrogen peroxide calibration in the presence of the optimal concentrations of (a.) Triton X-100, and (b.) Tween 80 ($n=3 \pm \text{s.e.m.}$).
With increasing hydrogen peroxide concentration, there is a corresponding change in the measured impedance. The changes occur because of the oxidation of the benzidine, which results in the formation of a product having a lower solubility, and a larger size than the benzidine. The response measured is a result of two factors, the adsorption of oxidised product to the surface of the quartz crystal, and the increase in surface activity of the oxidised benzidine. The surfactant can form a layer on the crystal surface, which leads to increased adsorption of oxidised benzidine. The other possibility is that surface interaction occurs by the oxidised benzidine being incorporated into the micelles of the surfactant.

Hydrogen peroxide cannot be detected at low concentrations, due to the limited solubility of the oxidised product. At concentrations of hydrogen peroxide below 50 μM, there is generally a lack of deposition of the oxidised benzidine, and consequently no measurable response.

It was found that the DMOB response began to plateau at 150-200 μM hydrogen peroxide. One molecule of hydrogen peroxide will oxidise one molecule of DMOB, and therefore at concentrations of hydrogen peroxide above 160 μM, all the DMOB will have been oxidised to its dimer form. For this reason DMOB cannot be used to detect hydrogen peroxide concentrations above 160 μM, as an endpoint assay, but could possibly be used at higher concentrations, if the concentration of analyte was determined through the use of rate of change in the parameters measured. DAB is present at a concentration of 300 μM, which would suggest that this benzidine could be used to detect hydrogen peroxide concentrations up to this concentration. However, it is capable of producing a significant signal at 400 μM, and this suggests
that it is indeed forming tetramers or oligomers. The calibration curves for TMB (present at 300μM) began to plateau at 300 μM, as observed with the DMOB system. Overall, DMOB gave a linear response for each non-ionic surfactant between 50 – 150 μM H₂O₂ (R² = 0.949 and 0.964 for Triton X-100 and Tween 80 respectively). DAB produced a linear response throughout the range studied (25 – 400 μM H₂O₂), as is demonstrated by the correlation coefficient values (R² = 0.957 and 0.918 for Triton X-100 and Tween 80 respectively).

2.3.3 Optimisation with Polymers

A number of polymers were prepared by different methods for use with the DAB-HRP-H₂O₂ reaction with H₂O₂ at 300 μM, in the presence/absence of Triton X-100. The results are summarised in table 2.4. The two polymers, polyvinyl chloride and cellulose acetate were fabricated by solvent casting, which produces a polymer whose deposition is uncontrollable in terms of thickness and surface coverage, so reproducibility could be a problem, however according to the results obtained (table 2.4) demonstrate this does not seem to have been the case. This method will also lead to a non-uniform polymer layer, which may be thicker in some areas, and possibly absent in others, especially at the lower concentrations used.

Electropolymerisation was used to fabricate a polypyrrole and polyDAB film on the quartz crystal. This method of production is more controllable, since the voltage applied and the time of production can be better controlled, leading to better reproducibility and a more uniform polymer layer. This latter point is especially true for polyDAB, which is probably a non-conducting polymer. This conclusion was drawn from the frequency changes measured before and after polymer fabrication, at
the various electropolymerisation times. These results show that the amount of polyDAB being deposited begins to plateau after 5 min (see fig. 2.7). This conclusion agrees with that of Zhang et al.21.

![Graph showing relationship between deposited mass and electropolymerisation time](image)

**Fig. 2.8.** The relationship between the deposited mass (determined from dry frequency measurements) of the polyDAB with the electropolymerisation time, showing that with time the rate of deposition begins to decrease indicating that polyDAB is a non-conducting polymer producing an insulating layer on the electrode surface (n=6 ± s.e.m.).

Further work was completed using DAB, where a polyDAB polymer was fabricated by oxidative polymerisation. This would produce a film, the nature of which could not be controlled as with the solvent casting, hence it would be a non-uniform layer in terms of surface coverage and thickness.
<table>
<thead>
<tr>
<th>Polymer</th>
<th>Conc./time used</th>
<th>Mass of polymer (μg)</th>
<th>Mass before polymerisation (μg)</th>
<th>Δ[Z] (Ω)</th>
<th>Response time (min)</th>
<th>With Triton</th>
<th>Response time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bare</td>
<td>-</td>
<td>1.1 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>13.2 ± 0.8</td>
<td>21.1 ± 1.2</td>
<td>-</td>
<td>9.9 ± 0.3</td>
</tr>
<tr>
<td>Polypyrrole</td>
<td>1.5, 10, 30 min</td>
<td>3.3 ± 0.2</td>
<td>3.3 ± 0.2</td>
<td>23.5 ± 0.4</td>
<td>20.0 ± 1.4</td>
<td>13.8 ± 1.5</td>
<td>16.9 ± 2.4</td>
</tr>
<tr>
<td>Electropoly</td>
<td>1.5, 10, 30 min</td>
<td>0.7 ± 0.2</td>
<td>0.7 ± 0.2</td>
<td>35.0 ± 1.6</td>
<td>16.9 ± 2.4</td>
<td>17.6 ± 2.9</td>
<td>17.6 ± 2.9</td>
</tr>
<tr>
<td>polyDAB Oxidised</td>
<td>10, 30, 50, 100</td>
<td>0.05% (w/v)</td>
<td>0.05% (w/v)</td>
<td>34.6 ± 1.6</td>
<td>20.6 ± 0.1</td>
<td>17.6 ± 2.9</td>
<td>13.8 ± 1.0</td>
</tr>
<tr>
<td>polyDAB PVC</td>
<td>300 μM H₂O₂</td>
<td>0.05% (w/v)</td>
<td>0.05% (w/v)</td>
<td>38.7 ± 4.6</td>
<td>20.7 ± 2.3</td>
<td>8.9 ± 0.8</td>
<td>12.0 ± 2.2</td>
</tr>
<tr>
<td>Cellulose</td>
<td>0.001, 0.005 %</td>
<td>0.05% (w/v)</td>
<td>0.05% (w/v)</td>
<td>38.5 ± 2.6</td>
<td>20.3 ± 3.1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>0.001, 0.005 %</td>
<td>0.05% (w/v)</td>
<td>0.05% (w/v)</td>
<td>20.3 ± 2.2</td>
<td>20.3 ± 2.2</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Nitrocellulose</td>
<td>0.001, 0.005 %</td>
<td>0.05% (w/v)</td>
<td>0.05% (w/v)</td>
<td>-</td>
<td></td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 2- Hydrogen Peroxide Optimisation

Table 2.4. A summary of the main results from the use of the polymers with the DAB-HRP-H$_2$O$_2$ reaction, in the presence/absence of Triton X-100. This table shows the changes in response time and response size with the various polymers. The mass of polymer deposited on the crystal surface was determined through the use of dry frequency measurements taken before and after deposition to give a corresponding frequency change, which using the Sauerbrey equation could be equated to mass.

The use of cellulose acetate has a polymer had no positive effect upon the crystal impedance produced from the reaction DAB-HRP-H$_2$O$_2$, either in the absence or presence of Triton X-100. In the absence of Triton X-100, the response was diminished. In the absence of Triton X-100, the optimal concentration of cellulose acetate was found to be 0.05 % (w/v), where it gave an impedance response of 8.2 ± 2.4 Ω, and a response time of 38.5 ± 2.6 min. The impedance response was not significantly diminished (p > 0.05) at the lower concentrations of cellulose acetate used (at 0.001 % (w/v), 6.0 ± 1.7 Ω and a response of 30.5 ± 2.2 min), though the response time was less. The results indicate that the presence of any amount of cellulose acetate is interfering with the deposition of the oxidised DAB rather than aiding, and this is further supported by the longer response time at the higher cellulose acetate concentration used. The polymerisation of cellulose acetate would produce a polymer backbone with attached COCH$_3$ groups, which do not provide good nucleation for oxidised DAB precipitation.

When the DAB-HRP-H$_2$O$_2$ reaction with Triton X-100 was performed on a crystal with a cellulose acetate polymer film, it was found that there was little variation between the different cellulose acetate concentrations in terms of impedance change (19.0 ± 2.8 and 20.3 ± 3.1 Ω at 0.001 and 0.05 % (w/v), respectively) or response time.
(8.2 ± 0.2 and 8.9 ± 0.8 min at 0.001 and 0.05 % (w/v), respectively). This is due to the surface chemistry of the crystal being dominated by the presence of a Triton X-100 monolayer, which would result from the addition of this surfactant at a concentration above its CMC.

The use of PVC membranes with DAB without Triton X-100 had a significant effect upon the crystal’s impedance response in comparison to a bare electrode (p<0.01), though the response time was not improved. The impedance response did not differ greatly between the concentrations of PVC used (24.3 ± 3.1, 21.8 ± 6.4, and 24.7 ± 1.6 Ω at 0.001, 0.01 and 0.05 % (w/v) respectively), and a response time that was not significantly different (p>0.05). These results from the use of PVC to provide a nucleation site for oxidised DAB deposition corresponds to the results of Reddy, who used PVC for phenol detection, and also found that it provided an improved surface for deposition of a precipitate. It would appear the same would be true in the case of oxidised DAB deposition onto the PVC film. It should be noted that the deposition of DAB is not always reproducible as is clearly demonstrated by the large standard errors of mean, and this can be attributed to the irreproducibility of the PVC membrane.

The use of PVC film with the DAB-HRP-H$_2$O$_2$ reaction in the presence of Triton X-100 did not improve upon the bare crystal with Triton X-100, and it is probably due to the same reason as with cellulose acetate, namely that Triton X-100 is dominating the surface chemistry of the crystal.
When polypyrrole was used as a polymer upon the crystal surface, it was used with the DAB-HRP-H₂O₂ reaction in the presence of Triton X-100. It was found that as with the PVC and cellulose acetate under the same conditions, the impedance response of the crystal (18.5 - 20.0 Ω) and the response time (13.0 - 17.0 min) did not vary much with the different electropolymerisation times used. As with the other polymers, this is due to the presence of Triton X-100 in the reaction solution.

The electropolymerised polyDAB polymer gave an optimal response with an electropolymerisation time of 1 min, and the corresponding response of 6.95 ± 1.78 Ω, in the absence of Triton X-100. With this electropolymerisation time, the response time was 33.2 ± 4.0 min. The response time between the different electropolymerisation time varied little from 25 to 33 min (10 and 1 min respectively, p>0.05). The size of the impedance response decreased with increasing electropolymerisation time, plateauing at 10 min (2.75 ± 0.48 Ω). The reason for these differences can be explained in terms of the polymers coverage of the electrode surface. The peak response is obtained after 1 min of electropolymerisation, this is probably because the polymer has not completely covered the surface, whereas at 5 min the surface is nearly covered, and beyond there is a uniform film covering the surface. From these results and this explanation, it can be seen that polyDAB does not provide a good surface for oxidised DAB deposition. According to Cai & Khoo⁹², polyDAB will take the form of aromatic o-diamine groups attached to the polymer backbone. It could be the case that these groups are too closely packed to allow the oxidised DAB to interact, and hence the lack of response.

The use of polyDAB polymer coated quartz crystals gave a different response to that previously seen with the other polymers discussed when used for hydrogen peroxide
determination with Triton X-100 present in the reaction solution. The impedance response was significantly decreased in comparison to a bare crystal result (p>0.01). The response time did not vary much between the different electropolymerisation times (11.6 - 13.3 min), and that of a bare crystal (p<0.05). There is the possibility that the decreased impedance response is due to the side groups of the polymer layer penetrating beyond the surfactant layer, and hence exerting an effect like that seen in the absence of Triton X-100. This will be further investigated through the use of surface characterisation techniques such as Atomic Force Microscopy (AFM).

An oxidised polyDAB layer did not provide a surface that encouraged an increase in the deposition of oxidised DAB for the determination of hydrogen peroxide. In the presence of Triton X-100 in the reaction solution, there was a very significant difference (p<0.01) between the impedance responses to the various surfaces with the deposited membranes. The impedance response peaked at 50 μM H₂O₂ (20.6 ± 0.1 Ω), whereas at higher and lower values the response was less (15.1 ± 1.4 Ω and 16.1 ± 0.6 Ω, at 30 and 100 μM H₂O₂ respectively). This would indicate that as with the electropolymerised DAB, the density of the o-diamine side groups of the polymer affects the deposition of the oxidised DAB. At 50 μM H₂O₂, the density of these groups would appear to be sufficient to allow direct interaction with the oxidised DAB, whereas at lower concentrations, they are in insufficient quantity, and at higher concentrations, the density is becoming too great to allow this interaction. This possible explanation is supported by the frequency measurements taken before and after the initial deposition of the oxidised polyDAB, where the change in frequency (Δf) was 700, 1060 and 1630 Hz for 30, 50 and 100 μM H₂O₂, respectively. The response times did not vary with the different polyDAB films used or from the bare
crystal (p>0.05), indicating that response time is dependent upon the presence of Triton X-100 rather than the polymer layer.

Fig. 2.8 A summary of the main findings from the use of surfactants and polymers with the benzidine-HRP-H2O2 reaction. Sequence (a.) shows the original experiment in the absence of both polymers and surfactants, where poor adsorption was seen and hence low Zg responses. (b.) shows the change due to the introduction of the non-ionic surfactants, which led to greater adsorption onto the top electrode of the quartz crystal, giving the increased Zg responses. (c.) shows the presence of both the non-ionic surfactant and a polymer layer, where a monolayer of surfactant is formed above the surfactant, and hence why the response does not change, and finally (d.) which shows the use of polymer without surfactant, where poor adsorption occurred, because the surface chemistry was not compatible with the oxidised benzidine.
2.4 Conclusion

The main findings from the experiments described in this chapter are summarised in fig 2.8.

It was found that the range over which the benzidines could be used to detect hydrogen peroxide in an end-point assay was pre-determined by two factors, the concentration of the benzidine used, which was limited according to its solubility, and the solubility of the end-product, the oxidised benzidine. The latter determined the lower concentration detected, and the former the upper limit. The lower concentration detected was found to be around 25 pM H$_2$O$_2$, below this concentration, the oxidised benzidine was soluble. The concentration range of DAB is wider than these conditions would usually permit, because it has the ability to polymerise. It is true that the problem of benzidine solubility could be overcome by rather than using an endpoint assay, the rate of change in the crystal’s impedance was used to determine the concentration of hydrogen peroxide. This would have the added advantage of reducing assay time.

When Triton X-100 was added to the reaction solution, it was found that this increased the response size and decreased the response time, whereas Tween 80 increased the response size, but had no effect upon the response time. These improvements were due to the increased adsorption of the oxidised benzidine to the surface. This phenomenon will be further investigated through the use of surface characterisation techniques, such as Atomic Force Microscopy (AFM) and Ellipsometry. The decreased response time found in the presence of Triton X-100 is probably due to the increased HRP activity. The ionic surfactants used were found to
have the opposite effect due to their effects on the intermediate products of the oxidised benzidine, and the charged repulsion of the oxidised benzidine for anionic BEP and cationic Aliquat 336, respectively.

Overall the use of polymers did not demonstrate any beneficial effects in the absence of Triton X-100. Only PVC improved the response size, though it did not have any effect upon the response time. The PVC provided a favourable surface coating for the nucleation of the oxidised DAB and the subsequent proliferation along the surface. When the polymers were used with Triton X-100 in the reaction solution, it was found that they had neither beneficial nor detrimental effect on the response size or time, and therefore they could be used to protect the top electrode of the quartz crystal from the oxidised benzidine, resulting in a crystal with a extended life-time, and an element of re-usability.

To conclude, it was found the best benzidines to be used in future experiments were DAB and DMOB, and that Triton X-100 improved both the response time and size for the hydrogen peroxide assay.
Chapter 3 - Enzyme-based determination of Cholesterol

using the Quartz Crystal Acoustic Wave Sensor

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And presented (posters) at Biosensors 2002, and QCM 2002
Chapter 3- Cholesterol determination

3.1 Introduction

3.1.1. Background

Cholesterol is routinely measured for the risk assessment of cardiovascular conditions, such as atherosclerosis and hypertension, which can develop into coronary heart disease, myocardial and cerebral infarction (stroke). In conditions such as hypothyroidism, nephrosis, diabetes mellitus, myxedema, and obstructive jaundice, the patient will have increased levels of cholesterol and its esters above the physiological norm. Decreased levels are found in patients suffering from hyperthyroidism, anaemia, malabsorption and wasting syndromes.

![Structure of Cholesterol](image)

**Fig. 3.1** The structure of Cholesterol

The desired total plasma cholesterol for an individual is less than 5.2 mM (200 mg/dl), and a high level being considered as greater than 6.2 mM (240 mg/dl)\(^\text{102}\). Plasma cholesterol levels increase with age, and are generally less in women than men, until menopause, when the values in women exceed those in men\(^2\). Cholesterol is carried in plasma by a series of protein-containing micelles known as lipoproteins. The lipoproteins are classified into distinct subtypes according to their density, very low density lipoprotein (VLDL), low density lipoprotein (LDL), intermediate density lipoprotein (IDL) and high density lipoprotein (HDL). About 70% of the total
Chapter 3- Cholesterol determination

Cholesterol are contained within lipoproteins is esterified by fatty acids. Hence the concentration of free cholesterol within lipoproteins is approximately 1.0-2.2 mM (40-85 mg/dl)\(^{103}\).

3.1.2 Previous Cholesterol Assays

Historically, cholesterol was measured using non-enzymatic spectrometry, via the production of a coloured substance. In this reaction, cholesterol behaves as an alcohol, when it reacts with acetic acid, in the presence of concentrated sulphuric acid, which results in a coloured product (a cholesterol ester), the absorbance of which can be measured at 540 nm (see equation 3.1)\(^2\). The reaction maybe further enhanced by the presence of iron (III) chloride\(^{104}\). This is known as the two-step iron-salt-acid method\(^{105}\).

\[
\text{H}_2\text{SO}_4 \\
\text{Cholesterol} + \text{Acetic acid} \rightarrow \text{Cholesterol ester (green-blue product)} (3.1)
\]

This method suffered from poor specificity, instability of the colour reagent, standardisation difficulties, the variable reactivity of esters and the unstable and corrosive nature of the reagents used\(^{106, 107}\). Consequently this method was not suitable for automated analysis\(^{105}\).

Richmond\(^{106}\) improved the selectivity of the chemical reaction with the introduction of the enzymes, cholesterol esterase (ChE) and cholesterol oxidase (ChOx). The cholesterol esterase (EC 3.1.1.13) was used to hydrolyse the cholesterol esters to free cholesterol, and cholesterol oxidase (EC 1.1.3.6) to oxidise the free cholesterol,
producing hydrogen peroxide, and cholest-4-en-3-one. The reaction sequence is shown in equations 3.2 and 3.3.

\[ \text{Cholesterol Esters} + H_2O \xrightarrow{\text{ChE}} \text{Cholesterol} + \text{Fatty acid} \]  
(3.2)

\[ \text{Cholesterol} + O_2 \xrightarrow{\text{ChOx}} \text{Cholest-4-en-3-one} + H_2O_2 \]  
(3.3)

The cholest-4-en-3-one can be reacted with 2,4-dinitrophenylhydrazine (known as Brady's reagent) to produce a coloured hydrazone\(^{108}\) (see equation 3.4). Alternatively the consumption of \(O_2\)\(^{109}\) or the production of \(H_2O_2\)\(^{110}\) (during the ChOx reaction) are the easier methods of quantifying cholesterol spectrophotometrically, with the latter being the preferred method, since it is less problematic\(^{111}\). The reason for this is that molecular oxygen in clinical samples will result in false positives in any method measuring consumption of oxygen unless steps are taken to remove it. Equally, the oxygen produced will be consumed by other substances, which are present in clinical
samples, such as ascorbic acid, as found by Marazuela et al., rather than being measured leading to an incorrect measurement. The same is true for other optical and amperometric determination of cholesterol.

![Reaction diagram](image)

The first method of enzymatic determination of cholesterol involved the use of Nocardia sp. cells, which had been used to produce ChOx. The cholesterol oxidase was isolated and purified from the bacterium. For the measurement of total cholesterol, the serum sample was mixed with KOH, which leads to the release of cholesterol from lipoproteins. The KOH was then neutralised with HCl, and the resulting sample was treated with mercuric ions, which removed any interfering reducing substances generated during the saponification step. The cholesterol was oxidised to cholest-4-en-3-one and hydrogen peroxide by the ChOx. The former product was then chelated with xylenol orange and quadrivalent titanium, producing a complex, the absorbance of which was measured at 550 nm. This method of cholesterol determination did not suffer from interference due to haemoglobin or
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bilirubin, though it did involve the use of corrosive compounds, and is a complex method, involving a number of steps.

Allain et al.103, improved upon the method of Richmond106. They used ChE to hydrolyse the lipoproteins rather than the more complex alkali method. This enzyme along with ChOx produced hydrogen peroxide, which was reacted with 4-aminoantipyrine and phenol, in the presence of horseradish peroxidase (HRP), to produce a quinoneimine dye (see reaction 3.5), the absorbance of which could be measured at 500 nm. The reaction was completed within 10 minutes at 37 °C, and the colour product was stable for 90 minutes. This method did not suffer interference from bilirubin, haemoglobin, ascorbic acid or urea. This was the first all enzymatic method for determining total cholesterol in a serum sample.

\[
2\text{H}_2\text{O}_2 + \text{4-aminoantipyrine} + \text{phenol} \xrightarrow{\text{HRP}} \text{quinoneimine dye} + 4\text{H}_2\text{O}
\]

(3.5)

Another example of an enzymatic-spectrophotometric method to determine cholesterol in lipid extracts from tissue was developed by Sale et al.113. This was a modification of the method used by Allain et al.103. The authors extracted lipids from
brain tissue using the solvents chloroform and methanol. They used ChE and ChOx
to produce hydrogen peroxide from the lipid extract, which was reacted with the
chromogen, 3,3'-dimethoxybenzidine (DMOB, o-dianisidine), in the presence of
HRP, to yield a coloured product (see reaction 3.6). The sample was incubated for 30
minutes at 37 °C, before the absorbance at 450 nm was measured. The authors found
that they could detect cholesterol at concentrations as low as 3 µg (8 nM), though they
did find that complete removal of the solvents used to extract the lipids was necessary
prior to the assay, which involved additional steps including heating at 95 °C for 30
minutes.

\[ \text{3,3'-dimethoxybenzidine} + \text{H}_2\text{O}_2 \xrightarrow{\text{HRP}} \text{4,4'-diimino-3,3'-dimehtoxybiphenyl} \]

\[ \text{bis(3,3'-dimethoxy-4-amino)azobiphenyl} \]

(3.6)
A fluorometric method for the determination of cholesterol was developed by Huang et al. They used the enzymes ChE and ChOx, as in the previously described methods, but then used homovanillic acid with the hydrogen peroxide produced, which in the presence of hydrogen peroxide, produces the fluorescent compound, 2,2'-dihydroxy-3,3'-dimethoxy-biphenyl-5,5'-diacetic acid. The reaction solution was incubated for 30 minutes at 37 °C, before measuring the fluorescence. The effect of possible interferents was not explored.

3.1.2 Biosensor Methods

Trettnak & Wolfbeis developed a fluorescent method for the determination of free cholesterol, the fluorescence being measured using a fibre optic. This worked on the principle that during the cholesterol oxidase reaction, molecular oxygen is consumed, and that this could be followed through the use of a dye (decacyclene), whose fluorescence is quenched by molecular oxygen. The decacyclene was dissolved into a layer of silicone, while the cholesterol oxidase was immobilised on a nylon membrane. The decacyclene has excitation and emission maxima at about 415 and 480/506 nm, respectively. The authors found the analytical range of the sensor was 0.2 to 3 mM, with a response time ranging from 7 to 12 minutes. The sensor was not tested using real samples, such as serum, in which case the sensor may suffer from interference due to the presence of bilirubin.

Gaus & Hall have used surface plasmon resonance to measure the degree of interaction between LDL/oxidised LDL (oxLDL) and modified 1-mercaptundecanoic acid monolayers. The monolayer was modified with amino acids. The negatively charged amino acids aspartate and glutamate, coupled to the -COOH of the
monolayer, showed an increased LDL adsorption and a better distinction between the adsorption of LDL and oxLDL, than the unmodified monolayer. It was found that small chains of coupled amino acids were best for measuring adsorption of LDL and oxLDL. The Glycine-Cystine-Serine-Aspartate-Glutamate gave the best surface for LDL adsorption, and Glycine-Lysine-Lysine-OH for oxLDL. Both surfaces were able to detect their respective analyte at 1 µg/ml, and gave a linear response up to 20 µg/ml, and a fast response time of approximately 100 seconds.

A potentiometric method for the determination of cholesterol has been developed\textsuperscript{117}. The enzymes ChE, ChOx, and HRP were used to produce hydrogen peroxide from the cholesterol and cholesterol esters. The hydrogen peroxide was used to oxidise ferrocyanide, [Fe (CN)\textsubscript{6}]\textsuperscript{4−}, in a flow injection system (see reaction 3.7).

\[
\text{HRP} \quad \begin{array}{c}
\text{H}_2\text{O}_2 + 2[\text{Fe (CN)}_6]^{4+} + 2\text{H}^+ \rightarrow 2[\text{Fe (CN)}_6]^{3-} + 2\text{H}_2\text{O}
\end{array}
\] (3.7)

The change in the redox potential was related to the change in concentration of the ferrocyanide. The serum sample was incubated with the enzymes for 15 min, before being injected into the flow system, which could measure the change in redox potential within a minute. The biosensor gave a linear relationship between the log of the cholesterol concentration, and the change in redox potential, between the concentrations of 0.05 to 3 mM cholesterol. It was found that at normal physiological concentrations, interferents such as ascorbic acid, bilirubin, and proteins had negligible effects upon this method.

Srivastava \textit{et al.}\textsuperscript{118} have developed a conductive biosensor, which uses membrane permeability to determine cholesterol (see fig. 3.2). This method exploits
enhancement in conductivity of liquid membranes generated on hydrophobic supporting membranes. The authors used prostaglandin E\textsubscript{1} saturated, and nystatin (which increases membrane permeability) saturated lecithin (a phospholipid in the brain) liquid membranes. These generate hydrophilic pathways, which manifest as an enhancement of electrical conductance of the liquid that is detected by the platinum electrodes (P\textsubscript{1} and P\textsubscript{2}), on either side of the cellulose acetate membrane (M). The response time to the analyte (lecithin) was less than 60 s. The sensor was successfully used to measure free cholesterol in serum. This sensor did not suffer from enzyme instability, because none are used, however the lecithin and prostaglandin E\textsubscript{1} solutions were unusable after 10 days, due to microbial growth.

Fig. 3.2. The arrangement used by Srivastava et al.\textsuperscript{118}, for their biosensor, where M refers to the cellulose acetate membrane, and P\textsubscript{1} and P\textsubscript{2} are platinum electrodes.
Amperometric biosensors for the determination of cholesterol have been developed. Motonaka & Faulkner\textsuperscript{19} developed an amperometric biosensor for the determination of cholesterol concentrations. They immobilised the enzymes, ChOx and ChE with a redox mediator (osmium complex) onto a porous Nafion-modified carbon. With this kind of immobilisation, no membrane was required to retain the enzymes. The cholesterol concentration was determined by measuring the anodic current of the hydrogen peroxide. The sensor gave a linear response for cholesterol determination between the concentrations of 0.02 and 1 mM, when used with a cholesterol ester as the analyte. The response time was 2 minutes. It was found that ascorbic acid, uric acid, and retinol caused interference during the cholesterol determination.

Another example is the biosensor developed by Gilmartin & Hart\textsuperscript{85}. They produced an amperometric biosensor for single determination use. An outer membrane of cellulose acetate was used, to retain the ChOx, and improve selectivity of the biosensor by reducing the effect of interferents such as ascorbic and uric acid. The ChOx was immobilised on the inner surface of the cellulose acetate membrane, so the hydrogen peroxide was produced in close proximity to the modified electrode. The electrode (a screen-printed carbon electrode (SPCE)) was modified with cobalt phthalocyanine (CoPC, a redox mediator), to accelerate the oxidation of the $\text{H}_2\text{O}_2$. With cholesterol standard solutions prepared in ethanol and 1% Triton X-100, the biosensor gave a plateaued response in under 2 minutes, and gave a linear response between 1.13 and 3.68 mM cholesterol, which covers the range of free cholesterol concentrations in serum. The authors found that ascorbic acid, paracetamol, glutathione and uric acid were major interferents, when using this biosensor, because they participated in the electrodis process. Upon storage of the prepared biosensor, it
was found to give similar responses over five days, if it was used once, otherwise the performance deteriorated over time.

Nakaminami et al.\textsuperscript{120}, used an amperometric biosensor with artificial electron mediators to determine free cholesterol. They used a number of redox dyes, which behaved as electron acceptors for the reduced cholesterol oxidase, replacing the usual $\text{O}_2/\text{H}_2\text{O}_2$ (see reactions 3.8-3.10).

\begin{align*}
\text{ChOx (ox)} + S & \leftrightarrow \text{ChOx-S} \rightarrow \text{ChOx (red)} + P \quad (3.8) \\
\text{ChOx (red)} + M_{\text{ox}} & \leftrightarrow \text{ChOx-M} \rightarrow \text{ChOx(ox)} + M_{\text{red}} \quad (3.9) \\
M_{\text{red}} & \rightarrow M_{\text{ox}} + n\text{e}^- \quad (3.10)
\end{align*}

In the above reactions, ChOx (ox) and ChOx (red) are ChOx whose active redox centre is oxidised (FAD) and reduced (FADH\textsubscript{2}), respectively, $M_{\text{ox}}$ and $M_{\text{red}}$ are the redox compounds in the oxidised and reduced states, S and P are the substrate (cholesterol) and product (cholest-4-en-3-one), and ChOx-S and ChOx-M are the intermediate complexes between the substrate and mediator with ChOx, respectively.

The response time for the biosensor was less than five minutes, it was not tested with different concentrations of cholesterol. The response was seriously influenced by dissolved oxygen within the cholesterol samples, and therefore before electrochemical measurements could be carried out, the system must be purged of dissolved oxygen. Urea also had a negative effect upon the cholesterol measurement.

Wang & Mu\textsuperscript{121} used electropolymerisation to immobilise ChOx in a polyaniline film. Triton X-100 is also involved in the stabilisation of activity of ChOx, however at high
concentrations it will inhibit the activity of ChOx. At 5% Triton X-100, a linear range was gained in the cholesterol concentration range of 0.05-0.5 mM at 0.45V vs. SCE (Saturated Calomel Electrode) and 0.05-0.2 mM at 0.60V, and with Triton X-100 at 1 %, the result was a linear range between 0.01-0.1 mM at both potentials of 0.45 and 0.60V. The difference at the two Triton X-100 concentrations, they believed to be due to its effect on the active site of the enzyme, and the amount adsorbed onto the polyaniline film. The current reached a steady state within two minutes after addition of the analyte, but the activity of the biosensor dropped to 51 % of its initial value after 11 days, during which time it had been used for 230 assays. The response of the biosensor suffered serious interference due to ascorbic and uric acid.

In another amperometric biosensor, an overoxidised polypyrrole membrane was used for the immobilisation of the ChOx. Polypyrrole was used because the authors found that it had size-exclusion properties, improving the selectivity against interferents such as ascorbic and uric acid. By overoxidising the film, they also removed the polymer's ability to conduct, but retaining the size-exclusion properties. The biosensor was constructed in a flow-injection system to allow numerous cholesterol determinations. The biosensor exhibited a linear response for cholesterol determination over the concentration range of 0.025 - 0.3 mM, and fast response time of less than 30 seconds. The polypyrrole film did reduce the interference from ascorbic and uric acid. The biosensor gave a constant response for cholesterol determination over 15 days (550 assays).

An enzymatic thermistor based flow injection analysis method for cholesterol determination was developed by Raghavan et al. They immobilised the enzymes ChOx, ChE and catalase by crosslinking with glutaraldehyde onto glass beads, which
were packed a column, and one column without immobilised ChE for the
determination of free cholesterol. The free cholesterol determination gave a linear
response from 1.0 to 8.0 mM, and the total cholesterol a linear response from 1.0 to
4.0 mM. The assay time was 4 minutes.

3.13 The enzymes

Cholesterol oxidase is a FAD-dependent oxidase. It catalyses the oxidation of 3β-
hydroxysteroids at the trans double bond at \( \Delta^5-\Delta^6 \) of the steroid ring, resulting in the
production of hydrogen peroxide and a \( \Delta^5 \)-ketosteroid, which is isomerised by the
enzyme to a \( \Delta^4 \)-3-ketosteroid. The 3β-hydroxy group on the substrate is essential for
enzyme activity. The enzyme has a molecular mass of 55 kDa, a pI of 4.4 – 5.1, and
contains a FAD prosthetic group. The optimum pH is between 5.5 and 8.0.

Cholesterol esterase is a FAD-dependent esterase, which hydrolyses cholesterol esters
to free cholesterol and fatty acids. It has a molecular mass of 400kDa, with a pI of
5.95, and contains a FAD prosthetic group. The optimum pH for ChE is between
6.0 and 8.0, with a maximum at 7.6. This enzyme suffers from thermal instability at
temperatures above 20 °C.

3.1.4 Aims

In this chapter, we describe our method for using the quartz crystal acoustic biosensor
for the determination of free cholesterol (using the enzymes ChOx and HRP) and total
cholesterol (using the enzymes ChOx, ChE and HRP). In doing so, we have
determined the optimal concentration of ChOx, ChE and Triton X-100 for this assay,
at the volumes used. We also determined total cholesterol in human LDL and HDL sub-fractions and in human serum using this assay.

3.2 Experimental

3.2.1 Materials

Phosphate buffer tablets were purchased from Oxoid (Basingstoke, UK), Propan-2-ol from BDH (Poole, UK), and potassium bromide from Fisher (Loughborough). Horseradish peroxidase (HRP type I, EC. 1.11.1.7, 180 PU/mg), Cholesterol oxidase (ChOx, EC 1.1.3.6. from *Streptomyces* sp., 19 U/mg), Cholesterol esterase (ChE, EC 3.1.1.13 from *Pseudomonas* sp.), 3,3'-diaminobenzidine tetrahydrochloride (D5637), Cholesterol (C8667), Triton X-100, Infinity™ Cholesterol reagent (401-25P) and cholesterol calibrators (C0534) were obtained from Sigma (Poole, UK).

Methods

Crystal preparation and experimental set-up have been previously described in section 2.2.

3.2.2 Solution preparation

Diaminobenzidine was prepared in phosphate buffer (0.01 M, pH 7.4) to give a final concentration of 0.3 mM. Cholesterol (10 mM) was prepared in isopropanol; the latter solvent has been shown to have no effect upon the activity of the enzymes at the final concentration used. All enzymes were prepared in phosphate buffer, giving a final activity of 360, 50 and 70 U/ml for HRP, ChE and ChOx, respectively. Triton
X-100 was prepared in phosphate buffer, to give a final concentration of 1% (v/v). When not in use, all solutions were stored at 4°C. The diaminobenzidine and HRP solutions were prepared daily; and the Triton X-100 and Cholesterol solutions were prepared weekly. The ChOx and ChE were prepared in PBS and stored at -80°C until 1 hr before use (the remaining enzyme being discarded after that day’s experiments).

3.2.3 Measurement procedure

For all experiments, 3.5 ml DAB containing 0.2 U/ml HRP was added to the test cell. Various concentrations of Triton X-100 (0.01-0.2 % (v/v)), cholesterol (0-400 μM), LDL (0-400 μM), HDL (300 μM) or serum (300 μM), ChOx (0-0.5 U/ml) and if applicable, ChE (0.5-2 U/ml) were then added. The test solution was stirred, during all experiments. After each experiment, the crystal was cleaned thoroughly with N,N-dimethylformamide (DMF) and deionised water. The use of DMF removed the oxidised DAB adsorbed to the crystal during the experiment, and was confirmed by the crystal frequency returning to its original value. All experiments were performed at room temperature.

3.2.4 Spectrophotometric measurements

All measurements were taken at 492 nm using a 96 well plate reader (Labsystems iEMS Reader MF and Labsystems Genesis Version 3.05 software). For the spectrophotometric measurements, all solutions were prepared as previously described. Into each well of a 96 well plate were added 300 μl of DAB, 1 μl of HRP, 1 μl of ChOx, and a range of Triton X-100 concentrations, after which 9 μl of
cholesterol was pipetted into each well. The absorbance was measured every 15 seconds, over 10 minutes at 492 nm.

3.2.5 Isolation of HDL/LDL

The method of isolation used has been previously described by Rankin et al.\textsuperscript{125} 100 ml of venous blood from healthy volunteers was collected into 2 ml of Na\textsubscript{2}EDTA (150 mM, pH 7.4, prepared in water and filter sterilised) using a 20 gauge butterfly catheter (Abbott, Sligo, Rep. Ireland). The blood was centrifuged at 800g for 30 minutes at 4°C in a Beckman GPR centrifuge (High Wycombe, Buckinghamshire) to obtain plasma. The density of the plasma was adjusted to 1.019 g/ml, by the addition of a "high density" (\(\rho = 1.32 \text{ g/ml}\)) potassium bromide (KBr) solution. The plasma was then transferred into 11 ml ultracentrifugation tubes (ultra clear, 16 x 76 mm) from Beckman Instruments (High Wycombe, Buckinghamshire) and centrifuged at 108 000 g for 18 hours at 4 °C in a Beckman 70Ti rotor, and Beckman Optima XL-100K ultracentrifuge (Beckman Instruments, High Wycombe, Buckinghamshire). Following centrifugation, the fraction containing LDL and HDL was recovered, its density adjusted to 1.063 g/ml by the addition of KBr and then dialysed against a PBS solution containing KBr for four hours, to give a final density of 1.063 g/ml. Following this, it was centrifuged as previously described under the same conditions. The LDL and HDL fractions were recovered and dialysed against 4 changes of PBS to remove any KBr. The separate LDL and HDL sub-fractions were filter sterilised (pore size, 0.2 \(\mu\text{M}\) from Sartorius Group, Epsom, Surrey), to remove lipoprotein aggregates and stored at 4°C.
3.2.6 Spectrophotometric determination of cholesterol concentration

This method is a modification of the Sigma Diagnostics procedure No. 401. Three microlitres of standard (cholesterol calibrator 100, 200, and 400 g/l and 6 μl of 400 g/l to obtain 800 g/l), LDL, HDL, or serum was pipetted per well in replicate on a 96 well plate. Three hundred microlitres of Sigma Infinity™ Cholesterol reagent was added per well and incubated at room temperature for 30 minutes. The absorbance at 525 nm was measured using a 96 well plate reader (Labsystems iEMS Reader MF, and Labsystems Genesis Version 3.05 software). The cholesterol concentration was then calculated from the standard curve.

3.2.7 Preincubation method

In terms of volumes and concentrations these were the same as used in the previously described experiments. The difference was that the HDL and LDL were incubated with the enzymes, ChOx and ChE, with Triton X-100 in an eppendorf vial for 30 minutes. Only 18 μl of the 1% solution of Triton X-100 was added, so the concentration was maintained at 0.1%. After incubation, this solution mixture was added to a DAB solution, with HRP, and 332 μl of Triton X-100. The experiment then proceeded as normal.

3.2.8 Use of polymer membranes

Cellulose acetate membranes with/without Triton X-100 were prepared. To make a 5% cellulose acetate membrane, 500 mg of cellulose acetate was dissolved in 10 ml of acetone, and stirred vigorously. To prepare a cellulose acetate membrane with 2 or 4% Triton X-100, 200 μl or 400 μl of Triton X-100 was added to the cellulose acetate solution. These solutions were then poured into a covered glass Petri dish (90 mm in
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diameter), and left in the fume cupboard overnight, to allow the slow evaporation of
the solvent. After the membrane had been formed, a circle of it was cut out, and laid
in the lid of a sterilin bottle, and washer placed on top. The lid of the sterilin bottle
had a hole through it. Into the cell was pipetted 1.0 ml DAB, 9.5 µl HRP, and 100 µl
Triton X-100. This volume of reaction solution filled the well above the quartz
crystal, onto which was placed the polymer membrane in its holder (see fig. 3.3).
After placement of the polymer membrane, 2.5 ml of PBS, 7 µl of ChOx, 70 µl of
ChE, and 250 µl of Triton X-100 were pipetted into the cell on top of the polymer,
and a stirring bar added to allow mixing of the solutions. At which point, the
experiment was performed as normal, with the addition of LDL as the analyte, to give
a concentration of 300 µM.

Fig. 3.3 The quartz crystal cell with the polymer membrane arrangement.

3.3 Results and Discussion

3.3.1 Optimisation of Triton X-100 with Cholesterol Oxidase

The Triton X-100 concentration required for the optimal response for the
measurement of free cholesterol was determined. A range of Triton X-100
concentrations were used (0.001 to 0.2 %, v/v), while the ChOx concentration was
maintained at 0.1 U/ml, and the cholesterol concentration at 300µM (fig. 3.4).
Increasing concentrations of Triton X-100 increased the change in crystal impedance ($Z_s$) reaching a maximum at around 0.1% (v/v) (fig. 3.4).

We have previously found that the optimal concentration of Triton X-100 for the determination of hydrogen peroxide using DAB was 0.1% (v/v). Indeed, the data shows (fig. 3.4) that this is also the optimal concentration of Triton X-100 for the determination of cholesterol. We believe this is due to the surfactant improving the enzyme activity, and also improving the dimerisation process, which is supported by spectrophotometric analysis. When measuring the absorbance at 492 nm, we found that for the hydrogen peroxide determination, the $V_{\text{max}}$ increased from 1.06 ΔA/min (no surfactant present) to 2.46 ΔA/min in the presence of Triton X-100. The $K_m$ remained reasonably constant (0.68 mM and 0.66 mM in the absence and presence of surfactant respectively). Another possibility is the Triton is increasing the solubility of the substrate. We found previously that Triton X-100 at 0.1% (v/v), led to increased adsorption of the oxidised DAB product at the crystal surface. It has also been demonstrated previously that Triton X-100 at a concentration range of 0.05-0.1% (v/v), increases the activity of ChOx. At concentrations above this, Triton X-100 was found to inhibit the activity of the enzyme. Therefore, Triton X-100 is not only increasing adsorption of the oxidised DAB, but also ChOx activity, and the activity profile may result from a combination of these two effects. Upon measuring the change in absorbance at 492 nm, for various concentrations of Triton X-100 with the ChOx-HRP reaction, we found that the change in absorbance peaked at about 0.1-0.05% (v/v) Triton X-100 (0.11 ΔA/min), compared to 0.2 and 0% (v/v) Triton X-100 (0.08 and 0.09 ΔA/min respectively) (data not shown).
Fig. 3.4 Triton X-100 concentration profile for the optimum response to 300μM cholesterol in the impedance (Zs). Each value is the mean ± s.e.m. (n=3).

3.3.2 Optimisation of Cholesterol Oxidase

To determine the optimal ChOx concentration, a range of ChOx concentrations were used (0.01 to 0.5 U/ml), using Triton X-100 at 0.1 % (v/v) and cholesterol at 300 μM. Increasing concentrations of ChOx (0.1-0.01 U/ml) resulted in a decrease in the observed $Z_s$ (Fig. 3.5).

However, increasing concentrations of ChOx decreased the response time from 34.5 ± 1.5 min for 0.01 U/ml, to 22.3 ± 0.3 min using 0.2 U/ml (data not shown). It was decided that the optimal concentration of ChOx for the determination of cholesterol was 0.2 U/ml, when both response size and time were considered. This concentration of ChOx is similar to optimal concentration used by others\textsuperscript{137}.
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Fig. 3.5 The optimisation of ChOx, using 0.1 % (v/v) Triton X-100, and 300 μM cholesterol.

Each value is the mean ± s.e.m. (n=3).

3.3.3 Calibration with cholesterol

Using the previously optimised Triton X-100 and ChOx concentrations, the response to cholesterol was determined over the range, 0 to 400 μM. A linear relationship between the cholesterol concentration (0-300 μM) and change in $Z_s$ was observed (fig. 3.6). For clinical measurement of free cholesterol in serum, a 1:10 sample dilution would be required, so that the cholesterol concentration would fall inside the linear range of our biosensor.
3.3.4 Optimisation of Cholesterol Esterase

We isolated LDL from human blood as a substrate for the optimisation of ChE, since cholesterol esters occur naturally in aqueous solution within LDL, whilst cholesterol esters e.g. cholesteryl oleate, require preparation in various solvents, the presence of which may have unforeseen effects on enzyme activity and stability. The cholesterol concentration within LDL was established by means of the cholesterol determination spectrophotometrically.

The optimal concentration of the enzyme, ChE was determined, using the previously optimised concentrations of Triton X-100 and ChOx. The ChE was used at 0.2, 1, and 2 U/ml, and it was found that 1 U/ml gave the best response in terms of response size and reproducibility (see fig 3.7). For all further experiments, 1 U/ml ChE was used.
Fig. 3.7 The optimisation of ChE, using 0.1 % Triton X-100, 0.2 U/ml ChOx, and 300 μM LDL. Each value is the mean ± s.e.m. (n=3).

3.3.5 Calibration curve with LDL

Using the isolated LDL, a series of experiments were performed to produce a calibration curve over the concentration range of 25-400 μM LDL cholesterol (fig. 3.8). This produced a linear relationship between the concentration of LDL, and the corresponding change in Zr. For clinical measurement of total cholesterol in serum, a 1:20 sample dilution would be required, so that the total cholesterol concentration would fall within the linear range of our biosensor.
Fig. 3.8 The calibration curve for LDL determination, between the concentrations of 0 and 400 μM, when using 0.1 % Triton X-100, 0.2 U/ml ChOx, and 1 U/ml ChE. Each value is the mean ± s.e.m. (n=3).

3.3.6 Determination of Cholesterol in serum

Figure 3.9, shows the Z\textsubscript{s} responses to the use of LDL, HDL and serum at 300 μM cholesterol, as the substrates for determination. Experiments with all three substrates were performed, with the enzymes, triton X-100, and DAB being used at the optimal concentrations. It was found that isolated LDL gave an enhanced response and HDL gave a poor response, in comparison to serum, which was found to be statistically significant (p<0.001).
Fig. 3.9 The $Z_a$ responses for the use of serum, HDL, and LDL as the substrates at 300 $\mu$M. Each value is the mean ± s.e.m. (n=3).

To identify possible causes of this reduced response to HDL, the LDL and HDL subfractions were re-isolated by centrifugation at the end of the experiment. It can be seen from the photographs of the two tubes (fig. 3.10), the oxidised DAB was distributed differently. In the LDL tube, the LDL fraction is at the top of the tube, and the oxidised DAB pelleted at the bottom. However, the HDL and the oxidised DAB co-localised at the top of the HDL tube. This would suggest that the oxidised DAB is dissolving within HDL, leading to a lack of adsorption to the crystal surface, and the correspondingly small change in $Z_a$. 

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Fig. 3.10 The results from the ultracentrifugation of LDL and HDL, after a normal assay for the determination of cholesterol content. The photos show the regions of oxidised DAB accumulation in the two tubes, in relation to the areas of LDL and HDL (M = meniscus, H = HDL, L = LDL, D = oxidised DAB).

It will be noted that in figure 3.9, there is a difference between the $Z_s$ response for LDL and serum. This measured difference is perhaps due to the negative interference caused by the HDL in the serum sample as previously discussed, but also may in part be due to the activity of catalase, which will be present in serum. Catalase will compete with HRP for the hydrogen peroxide produced, but will not oxidise the DAB, thereby reducing the response. This problem may be overcome by the addition of sodium azide, though at high concentrations this may inhibit the activity of HRP$^{128,97}$. 
3.3.7 Preincubation of LDL and HDL

In order to try and overcome the problem of not being able to measure HDL directly, preincubation of the analyte with the enzymes ChOx, and ChE for 30 minutes, before being added to the normal concentrations of HRP, and DAB was experimented with. It was thought that incubation with ChE before the addition of DAB, would remove the interference due to HDL. However, as can be seen in fig. 3.11, even after preincubation HDL proved to be still a problem, so preincubation with ChE was not the answer, since not all the HDL was removed.

![Figure 3.11](image)

**Fig. 3.11** The results for the preincubation of HDL and LDL with the enzymes ChOx and ChE, before the addition of DAB, and HRP. Each value is the mean ± s.e.m. (n=3).

3.3.8 Use of polymers with LDL determination

Polymers were used to separate the reaction solution into two halves, with on one side DAB, and HRP, and on the other ChOx, ChE and LDL, with a constant concentration of Triton X-100 maintained on both sides of the polymer. Through this segregation,
H₂O₂ was produced in the top half of the cell, and then diffused through the membrane to react with the DAB and HRP, producing the insoluble product. The use of the polymer membrane in this way could reduce interference from possible interferents, in the solution (e.g. plasma proteins in serum), which might adhere to the crystal surface affecting the measurement, but it could have been an avenue to overcome the problem of HDL solubilising the dimerised DAB. Cellulose acetate produces a membrane, which is highly selective against anions⁸⁰, and does allow diffusion of hydrogen peroxide as a result⁸⁶. The incorporation of a surfactant as a plasticiser increases the pliability of the membrane reducing its brittleness⁸⁰, but also as been found to have an effect upon the permeability of the membrane⁸⁶.

In this study, Triton X-100 was used as the plasticiser within the polymer membrane, because of its solubilising effect on cholesterol, and it was already in solution, which would reduce its leaching from the membrane, as seen by Reddy and Vadgama¹²⁹. The presence of plasticisers has been shown to increase the permeability of cellulose acetate to hydrogen peroxide, but with a loss of selectivity⁸⁶. As can be seen in figure 3.12, there is an improved response for the membrane consisting of cellulose acetate, with 2 % (v/v) Triton X-100, when compared to cellulose acetate with no plasticiser. This difference is due to the increased permeability to hydrogen peroxide. However, with an increased Triton X-100 concentration (4 % v/v) in the polymer membrane, the response dropped. This is due to a further increase in the permeability of the membrane, which leads to DAB and HRP crossing the membrane, and reacting with the hydrogen peroxide produced enzymatically. The product of which then precipitated onto the membrane, rather than the crystal surface. This result accounts
for the slight discoloration of the cellulose acetate, with 4 % (v/v) membrane, when removed after the experiment.

Fig. 3.12 The use of the polymer, cellulose acetate (CA, in w/v) with Triton X-100 (TX, in % v/v) as a plasticiser, for the determination of LDL-Cholesterol (at 300 μM). Each value is the mean ± s.e.m. (n = 3).

3.4 Conclusions.

We have applied our optimised hydrogen peroxide detection system using the quartz crystal acoustic wave sensor to the determination of both free and total cholesterol in biological samples. We found that the optimal concentration of Triton X-100 was 0.1 % (v/v), for ChOx was 0.2 U/ml, and 1 U/ml for ChE. Using the reagents at these concentrations, we found that there was a linear range for LDL Cholesterol determination up to 400 μM. For clinical measurement of total cholesterol using this method, 1 in 20 dilution of the serum would be required.
The response time of the quartz crystal cholesterol biosensor is poor in comparison to the other biosensors listed in table 3.1. The amperometric methods particularly have fast response times. It is possible that the response time may be decreased by not determining cholesterol using an end-point assay, but rather using rates of change in crystal impedance. Using the latter would reduce the response time to less than 3 min, which is more comparable to the other methods discussed.

<table>
<thead>
<tr>
<th>Author</th>
<th>Detection Method</th>
<th>Response Time (mins)</th>
<th>Linear Range (mM)</th>
<th>Detection Limit (µM)</th>
<th>Known Interferents</th>
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<td>0.025 to 0.4 for TC</td>
<td>25</td>
<td>HDL and other proteins</td>
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<td>Ascorbic acid, bilirubin and proteins</td>
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<tr>
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<td>0.58 to 3.68 for FC</td>
<td>60</td>
<td>Ascorbic acid, paracetamol, glutathione, uric acid</td>
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<td>1</td>
<td>Not linear for TC</td>
<td>500</td>
<td>Not tested</td>
</tr>
<tr>
<td>Allain et al., (1974)</td>
<td>Absorption</td>
<td>5</td>
<td>2.6 to 15.6 for TC</td>
<td>2.6</td>
<td>ascorbic acid, uric acid and haemoglobin</td>
</tr>
</tbody>
</table>

Table 3.1 Comparison of some recently reported biosensors for cholesterol determination, and the strategy discussed here. FC refers to the fact that the biosensor was used to measure free cholesterol only, while TC means total cholesterol was determined.
The range of the linear response is similar in magnitude to the other technologies listed. The concentration range over which the quartz crystal biosensor could determinate cholesterol would necessitate a dilution of the biological sample, to place the cholesterol concentration within the linear range of the biosensor. Since a dilution of the biological sample is required, the lower detection limit will have little effect upon biosensor performance. The dilution of the biological sample would reduce the amount of interference suffered by the assay, and this will be discussed further.

The biosensor described here is not a true biosensor, in respect to the enzymes are being used in solution. It is possible that the enzymes can be immobilised, possibly through crosslinking with glutaraldehyde (or other crosslinking agent), and entrapment within a membrane. The immobilisation of the enzymes would create a true “biosensor” with the possibility of being used for repeated measurements.

The real problem with this strategy for the determination of cholesterol is the interference which the measurements suffer from. It was found that one of the analytes, HDL would solubilise the signal-producing oxidised benzidine. Further in Chapter 5, it will be reported that the use of proteins with the hydrogen peroxide determination had differing effects upon the response size and time. This resulted in both positive and negative interference. Due to the latter sources of interference, this method of cholesterol determination cannot be used to accurately measure cholesterol in biological samples. To overcome this difficulty, experiments were performed where a cellulose acetate membrane was added to partition the reaction solution. The benzidine and HRP were placed on the crystal side of the membrane, with ChOx and
ChE and the analyte on the other side (see fig 3.3). It was thought that by removing the analyte (biological sample) from the benzidine, it would diminish the interference suffered. The preliminary results are encouraging, the response size for LDL was reduced, but this was due to the diffusion of the benzidine across the membrane, and its deposition onto the polymer surface rather than the crystal surface. It is possible that the use of a different polymer e.g. PVC, may give a better response. PVC may provide a better barrier, because it is perm-selective which is achieved by ion rejection and molecular size exclusion. This requires further work.

In summary, the response time of the biosensor is too long in comparison to the methods listed in table 3.1, though this is a problem, which can be overcome. It also suffers from interference, which is a problem suffered by all the methods listed to one extent or another, so this is not a unique problem. In all, this is a promising method, which requires further development.
Chapter 4 - Determination of Creatinine using the Quartz Crystal Biosensor
4.1.0 Introduction

4.1.1 Background

The determination of creatinine is important for the diagnosis of a number of conditions and diseases; these include renal, thyroid and muscular disorders. Creatinine is a by-product from the breakdown of the high-energy compound, creatine phosphate, which is an energy source for muscle tissue. Creatine phosphate is an energy storage compound found in the muscle, because sufficient energy for the muscles cannot be stored as ATP.

\[
\text{Creatine} + \text{ATP} \xrightarrow{\text{Creatine kinase}} \text{Creatine Phosphate} + \text{ADP}
\]

\[
\begin{align*}
\text{Creatine} & \quad \text{Creatine Phosphate} \\
\text{CH}_3 & \quad \text{CH}_3 \\
\end{align*}
\]

Creatine Phosphate

\[
\begin{align*}
\text{Creatine Phosphate} & \rightarrow \text{Creatinine} + P_i \\
\text{NH} & \quad \text{NH} \\
\end{align*}
\]

Creatinine

In serum, the normal reference range for a child is 36-88 µM (0.4-1.0 mg/dl), and for an adult 53-106 µM (0.6-1.2 mg/dl). In urine, for an adult creatinine should be around ~88 mM (~1 mg/min). The serum creatinine concentration is not affected by factors such as sepsis, trauma, fever, state of hydration or dietary changes.
Table 4.1 Typical correlation of serum creatinine concentrations with the creatinine clearance and patient status (taken from Kaplan et al.²).

Creatinine is traditionally measured using the Jaffré reaction (reaction 4.1), where the active methyl group of creatinine reacts with alkaline sodium picrate resulting in a red-yellow complex, the concentration of which is determined spectrophotometrically. The problem with this method is its specificity for creatinine; it suffers from interference, due to other metabolites such as α-ketoacids or amines, which react with the sodium picrate³³.
4.1.2 Creatinine Biosensors

There are two methods of determining creatinine enzymatically. These methods have been applied to the development of amperometric and potentiometric biosensors for creatinine.

One method uses the enzyme, creatinine deiminase (creatinine iminohydrolase, CIH), which catalyses the hydration of creatinine, leading to the production of ammonium and \( N \)-methylhydantoin (reaction 4.2). However this method can suffer from interference due to endogenous ammonium and several cationic substances, unless steps are taken to overcome this.\(^{134; 135}\)

\[
\text{Creatinine} + 2\text{H}_2\text{O} \rightarrow \text{N-Methylhydantoin} + \text{NH}_4^+ + \text{OH}^-
\]

\(^{(4.2)}\)

Shih & Huang\(^{136}\) used creatinine deiminase immobilised within a modified polyaniline-Nafion membrane on a carbon electrode, for amperometric determination
of creatinine. They reported that their biosensor had a lower detection limit of 0.5 µM, a linear response between 0.5 and 500 µM, and a response time of 60 s. There was one problem, they found that the electrode was more sensitive to NH₄⁺ ions than creatinine. This was overcome through an alkalisation/heating pre-treatment, which removed the endogenous ammonium ions.

This method was used for the determination of creatinine by Ho et al. They used a modified poly(methyl vinyl ether)/maleic anhydride polymer membrane deposited upon the electrode. The membrane is sensitive to changes in pH, so when the pH increased from 7, due to the production of ammonium, the polymer began to degrade resulting in a change in potential difference being measured. The problem of endogenous ammonium is overcome, since the pH change occurs due to the production of ammonium.

The other method of creatinine determination is based upon the enzyme cascade introduced by Tsuchida et al. The enzymes, creatininase (CA), creatinase (CI), and sarcosine oxidase (SO) are used to convert creatinine to hydrogen peroxide (see reactions 4.3 - 4.5), which is usually determined amperometrically, though here we report upon an acoustic method. The major problem with this method is the interference from creatine during the measurement of creatinine, which can be overcome by the separate measurement of creatine, and the difference between the two values is the creatinine response.
This enzymatic system has been used with a rapid colorimetric creatinine assay\textsuperscript{139}. The colour reagent used was dimethoxybenzidine (DMOB), which is oxidised by the hydrogen peroxide produced in reaction 4.5, in the presence of HRP. The assay time was 6 min, and gave a linear relationship between the creatinine concentrations of 3 \(\mu\)M and 70 \(\mu\)M. They found that the oxidised DMOB was affected by the presence of the three enzymes CA, Cl, and SO, where side reactions between the enzymes and the oxidised DMOB were occurring. Reaction 4.4 was found to be the rate-limiting step for creatinine determination.
Next, Nguyen et al.\textsuperscript{140} used the same three enzymes immobilised onto a polypropylene membrane, with a Clark oxygen gas-sensitive electrode. They were able to measure the consumption of oxygen during the sarcosine oxidase reaction. A linear calibration curve was obtained between 3 $\mu$M and 300 $\mu$M, with the lower value being the detection limit. The response time was 60 s.

Madaras et al.\textsuperscript{141} used the three enzyme system with an amperometric transducer for the determination of creatinine. An outer layer of polyurethane was used to protect and retain the enzymes, with an inner membrane of cellulose acetate to protect the electrode. The polyurethane produces a layer which acts as a diffusion barrier, reducing interference from ascorbic acid and the like, while the cellulose acetate is a permselective membrane for hydrogen peroxide, so reducing fouling of the electrode surface, plus reducing interference. They immobilised the enzymes using three different techniques, entrapment within a polyurethane hydrogel, entrapment in a polymer matrix, and crosslinking with glutaraldehyde. Crosslinking with glutaraldehyde was found to give a superior immobilisation method providing better sensitivity and long-term stability. The end result was a sensor with a detection limit of 30 $\mu$M, a linear range up to 2 mM, and a response time of 5 min, for creatinine determination in serum samples.

A totally different method of determining creatinine is an ELISA (Enzyme-Linked-Immunoassay), used with amperometric transducer\textsuperscript{142}. A monoclonal antibody against creatinine was developed, which would allow biorecognition of the analyte. The ELISA was used with an amperometric transducer, which would allow measurement of the creatinine concentration in the sample. The basis of this assay is
a competitive reaction, as will be explained. A cellulose membrane with immobilised creatinine was placed above the electrode. A creatinine solution was then introduced, with mouse anti-creatinine antibodies, and glucose oxidase-labelled anti-IgG (mouse) antibodies. This was then washed out, and a glucose solution added, and the production of hydrogen peroxide determined, by measuring the current increase. The degree of reduction in the current produced is proportional to an increase in the concentration of creatinine.

4.1.3 The enzymes

Sarcosine oxidase is a FAD-dependent oxidase, which oxidises sarcosine to glycine, formaldehyde and hydrogen peroxide. It has a molecular weight of 40 kDa, with an isoelectric point of 4.7. The optimum pH for SO is 8.0, with increased activity at temperatures greater than 20 °C, particularly above 35 °C.

Creatinase (or creatine amidinohydrolase) is a hydrolase, which acts on carbon-nitrogen bonds, other than peptide bonds within a linear amide. In this case, creatine is hydrolysed producing sarcosine and urea. It has an isoelectric point of 4.7, an optimum pH of 8.0, and a molecular weight of 91 kDa.

Creatininase (or creatinine amidohydrolase) is also a hydrolase, but it acts on carbon-nitrogen bonds, which are part of a cyclic amide, but are not a peptide bond. It has an optimum pH of 7.8, a molecular weight of 160 kDa, and an isoelectric point of 4.7. The reactivity of the enzyme increases with temperature, plateauing at 35 °C. The enzyme is stable between pH 7.5 and 9.0; it is also photosensitive.
4.1.4 Aims

The aim is to create a new creatinine biosensor, which does not suffer from interference due to endogenous ammonium as with the creatinine deiminase reaction, and bilirubin for the spectrophotometric or amperometric methods. These interferences have been overcome, but through the use of various strategies, such as polymer films. This leads to a complication of the method, which reduces the ability for untrained personnel to use the biosensor. Using the quartz crystal, we hope to produce a biosensor for creatinine determination, which will be more accurate than the current Jaffré method used in clinical chemistry laboratories, and provide a rapid measurement. The creatinine level will be determined using the enzymatic sequence of sarcosine oxidase, creatinase, and creatininase (see equations 4.3-4.5), with the optimised hydrogen peroxide method described in chapter 2, which will not suffer from interference in the same manner as the Jaffré method.

4.2 Experimental

4.2.1 Materials

Phosphate buffer tablets were purchased from Oxoid (Basingstoke, UK). Horseradish peroxidase (HRP type I, EC 1.11.1.7, 180 PU/mg), Sarcosine oxidase (SO, EC 1.5.3.1 from Bacillus sp., 42 U/mg), Creatinase (Cl, EC 3.5.3.3 from Pseudomonas sp., 15 U/mg), Creatininase (CA, EC 3.5.2.10 from Flavobacterium sp., 250 U/mg), 3,3'-diaminobenzidine tetrahydrochloride (D5637), Creatinine hydrochloride (C6257), Creatine hydrate (C3630), Sarcosine (S7672), and Triton X-100 were obtained from Sigma (Poole, UK).
Methods

The methods of crystal preparation and installation, and the details of the experimental set-up are described in section 2.2.

4.2.2 Solution Preparation

Diaminobenzidine, creatinine, creatine, and sarcosine were prepared in phosphate buffer (0.01 M, pH 7.4) to give a final concentration of 0.3, 10, 10 and 10 mM, respectively. All enzymes were prepared in phosphate buffer, giving a final activity of 360, 70, 200 and 30 U/ml for HRP, SO, Cl and CA, respectively. Triton X-100 was prepared in phosphate buffer, to give a final concentration of 1% (v/v). When not in use, all solutions were stored at 4 °C. The diaminobenzidine and HRP solutions were prepared daily; and the Triton X-100, sarcosine, creatine and creatinine solutions were prepared weekly. The CA, Cl and SO were prepared in PBS and stored at -80 °C until 1 hr before use (the remaining enzyme being discarded after that day’s experiments).

4.2.3 Measurement procedure

For all experiments, 3.5 ml DAB containing 0.2 U/ml HRP was added to the test cell. Various concentrations of Triton X-100 (0.01-0.2 % (v/v)), sarcosine (300 μM), creatine (300 μM), or creatinine (300 μM), SO (0.1-5 U/ml) and if applicable, Cl (5 or 10 U/ml) and CA (5 U/ml) were then added. The test solution was stirred, during all experiments. After each experiment, the crystal was cleaned thoroughly with N, N-dimethylformamide (DMF) and deionised water. The use of DMF removed the oxidised DAB adsorbed to the crystal during the experiment, and was confirmed by
the crystal frequency returning to its original value. All experiments were performed at room temperature.

### 4.3 Results & Discussion

#### 4.3.1 Optimisation of Sarcosine Oxidase

A range of SO concentrations was used between 0.1 and 5 U/ml, to determine the optimal for the maximum response (see fig. 4.1). It was found that the higher concentration of SO produced a significantly larger response, than the lower concentrations. A concentration of SO of 5 U/ml produced a response of 39.5 ± 0.7 Ω, compared with a response of 5.7 ± 0.3 Ω at 0.1 U/ml. The response time in all the experiments was found to be around 45 minutes. The concentration of 5 U/ml was used in all subsequent experiments.

![Graph showing the optimisation of Sarcosine Oxidase](image)

**Fig. 4.1.** The optimisation of Sarcosine Oxidase, using a range of SO concentrations, with HRP at 0.2 U/ml, Triton X-100 at 0.1 % (v/v), and 300 μM sarcosine (n= 3 ± s.e.m).
4.3.2 Optimisation of Triton X-100

A range of Triton X-100 concentrations was used to determine the optimal concentration, which would produce the largest impedance change in the shortest period of time. It was determined that 0.1% (v/v) gave the optimal conditions for the determination of 300 μM sarcosine, with a response time of 45 min. The possible reason for the difference between this response time, and that for hydrogen peroxide determination could be due to the interference commented upon by Nguyen et al.\textsuperscript{139}, when they used DMOB with SO, which is probably similar to the interference suffered in the determination of serum cholesterol from HDL (i.e. solubilisation of the oxidised benzidine), but may be in part due to the increased number of steps in the production of the oxidised benzidine from the analyte.

![Bar chart](image)

**Fig 4.2.** The optimisation of Triton X-100 for the determination of 300μM sarcosine, using 0.2 U/ml HRP, and 5 U/ml SO (n= 3 ± s.e.m).
4.3.3 Determination of Creatine

Two concentrations of Cl were used during this study (5 and 10 U/ml). The results are shown in figure 4.3. It can be seen that during the time of the experiment (up to 3 hours), it does not reach an endpoint. The initial plateau period after the addition of creatine changes between the two different concentrations, with the 5 U/ml being longer than 10 U/ml (76.5 min and 29.6 min respectively). There is a small difference in rate of change of the impedance after the plateau period (0.22 Ω/min at 5 U/ml, and 0.28 Ω/min at 10 U/ml).

![Figure 4.3](image.png)

**Fig. 4.3.** The use of two different concentrations of Cl, for the measurement of 300 μM creatine, using SO at 5 U/ml and Triton X-100 at 0.1 % (v/v).

The change in response time between the sarcosine and creatine determination, is probably due to increased interaction between the enzymes and DAB, as mentioned
by Nguyen et al. It is possible also that creatine and DAB or oxidised DAB are interacting because of the presence of the amino group on creatine, which may become involved during the oxidation of the benzidine, i.e. when two amino groups react to form a dimer, except the presence of another amino group containing species may complicate matters. Identification of the products in the reaction solution will be undertaken using mass spectrometry.

4.3.4 Determination of Creatinine

Fig. 4.4. The measured impedance response for the determination of 300 µM creatinine, using SO at 5 U/ml, Cl at 10 U/ml, CA at 5 U/ml, and Triton X-100 at 0.1 % (v/v). This figure shows three repeats of the experiment under the same conditions.

The creatinine determination was completed using SO at 5 U/ml, Cl at 10 U/ml, and CA at 5 U/ml (see fig 4.4). An interesting difference between the creatine and
creatinine determination results is the endpoint of the creatinine measurements is achieved in less than 3 hours. This observation would support the earlier conclusion that creatine is interacting with DAB, causing the slower rate of change in the measured impedance.

4.4 Conclusions

In terms of response time, the quartz crystal biosensor described here does not meet the requirements of a fast response (<2min) device. With a response time of 3 hr for an endpoint assay, there are far quicker methods available (see table 4.2). This response time could be reduced by changing from an endpoint assay to a rate of change assay, where the concentration of creatinine is determined by measuring the rate of change in the crystal's impedance. An investigation is needed to find out whether the method herein is a viable alternative to the conventional techniques.

It will be noted that during the experiments with CI, it was found that increasing the concentration of this enzyme had a positive effect upon the rate of production of the response. It has been commented by previous authors\textsuperscript{139}, that this is the rate limiting step in the enzyme cascade for creatinine determination. Therefore it is possible that a further substantial increase in the concentration of this enzyme could reduce the assay time.

The problem of interference could be reduced through the immobilisation of the enzymes to the surface of the crystal, since one of the sources of interference occurs between these enzymes and the oxidised benzidine. The interference due to these
enzymes is similar to that due to HDL i.e. the oxidised benzidine is solubilised, producing a reduced change in the crystal’s impedance. The other source of interference which has been identified in Chapters 3 and 5, will be that occurring due to the presence of proteins in the biological sample, but as previously mentioned this can be diminished through the use of a polymer membrane partitioning the reaction mixture.

<table>
<thead>
<tr>
<th>Author</th>
<th>Detection Method</th>
<th>Response Time (min)</th>
<th>Linear range (μM)</th>
<th>Known Interferents</th>
</tr>
</thead>
<tbody>
<tr>
<td>My method</td>
<td>Quartz crystal</td>
<td>&lt;180</td>
<td>Not tested</td>
<td>Proteins</td>
</tr>
<tr>
<td>Shih &amp; Huang (1999)</td>
<td>Amperometric</td>
<td>1</td>
<td>0.5 - 500</td>
<td>NH₄⁺ ions</td>
</tr>
<tr>
<td>Nguyen et al., (1990)</td>
<td>Colorimetric</td>
<td>6</td>
<td>3 - 70</td>
<td>Proteins</td>
</tr>
<tr>
<td>Madaras et al., (1996)</td>
<td>Amperometric</td>
<td>5</td>
<td>30 - 2000</td>
<td>Negligible effects from ascorbic acid</td>
</tr>
<tr>
<td>Benkert et al., (2000)</td>
<td>Amperometric</td>
<td>30</td>
<td>0.09 - 90</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>(ELISA)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.2 Comparison of a number of creatinine biosensors which have been reported using different methods.

Rather than using the enzyme cascade used in the experiments discussed here, it might be the case that the use of the enzyme, creatinine deiminase (sec reaction 4.2) with a pH sensitive polymer membrane deposited onto the quartz crystal surface may
provide a better method of creatinine determination. This would be similar to the work described earlier by Ho et al.\textsuperscript{137} For the quartz crystal, a pH sensitive polymer such as the poly(methyl vinyl ether)/maleic anhydride polymer would be deposited onto the crystal surface, and then the creatinine deiminase and creatinine would be added. From the reaction of these two, would be produced ammonium (reaction 4.2), which would degrade the polymer due to a net increase in the pH of the reaction solution. The degradation of the polymer would lead to a frequency shift as polymer is removed from the crystal surface, and this would be proportional to the original concentration of creatinine. This alternative method would remove the need to perform two separate measurements of creatinine and creatine, to determine the former concentration, plus it would not suffer from protein interference. The other alternative strategy is the use of anti-creatinine antibodies, which are not commercially available, so this could be an expensive method, but would improve the specificity of the quartz crystal response for creatinine.

In summary, the determination of creatinine using the quartz crystal has been shown to be possible, but further development is required before it can compete with the other methods listed. Further investigation of using creatinine deiminase with a pH sensitive polymer as a different creatinine determining strategy is a potential way forward.
Chapter 5 - Enhancement of the Hydrogen Peroxide 

Quartz Crystal Biosensor using Proteins 

Presented orally at Eurosensors XVII, University of Minho, 

Guimaraes, Portugal (on 21st September 2003)
5.1 Introduction

It has been demonstrated previously that chemical modification or the presence of particular "cofactors" can have a beneficial effect upon the activity and stability of an enzyme. An approach has been recently described in which the surfactants, Triton X-100 and Tween 80 were used to enhance the detection of hydrogen peroxide, using the quartz crystal acoustic wave sensor\(^{126}\). This was shown to be due in part to an increase in enzyme activity as a result of the presence of the non-ionic surfactants (see section 2.3.2).

During the immobilisation of enzymes using glutaraldehyde, it has been frequently reported that the result is a decrease in the activity of the enzyme\(^{149}\). In a recent report, it was found that the use of protein stabilising agents could result in an enhanced activity of the immobilised enzyme\(^{150}\). The authors used three proteins, BSA, gelatin and lysozyme with an electrochemical biosensor for glucose and sucrose. They found that lysozyme enhanced the operational stability of the multienzyme biosensor, and they proposed that this be due to a "complimentarity of surface properties between the desired enzyme and lysozyme and also the ionic interactions involved".

Another example of a modified enzyme was reported by Garcia et al.\(^{151}\), where they covalently modified HRP with \(p\)-nitrophenylchloroformate methoxymethyl-PEG (mPEG), which was thought to reduce the electrostatic repulsion between surface charges, resulting in increased stability at pH and thermal extremes.
In other work, it was found that the addition of nanometre-scale particles improved the enzymatic activity of a GOx membrane. Hydrophilic gold and hydrophobic silica nanoparticles of various sizes were used. They proposed that since Hecht et al. had suggested that GOx has a hydrophobic shell consisting of an α-helix and β-sheet, and a hydrophilic oxidised FAD (Flavin adenine dinucleotide) interior, so the GOx molecule binds to a hydrophilic particle at the hydrophilic part, thus exposing the hydrophobic part. The changes in activity they thought was due to enzyme deformation caused by adsorption of the nanoparticle, exposing the FAD to the substrate when using SiO₂, and Au leads to improved communication of FAD with the electrode.

Liu et al. reported upon increased thermostability of HRP by chemical modification. They treated HRP with phthalic anhydride or glucosamine, which demonstrated greater thermostability, by 10 and 9-fold respectively, with no decrease in activity. It was concluded that this improvement was due to the neutralisation of the lysine positive charge on HRP. The modification also increased in the rate of reaction compared to the native HRP.

Here is described a study of the effects of a variety of proteins upon the DMOB/DAB-H₂O₂ reaction, measured using the quartz crystal acoustic wave sensor. This work was initiated when during a DMOB-HRP-H₂O₂ experiment, in the presence of GOx, a larger and faster response was gained than in the absence of GOx. This was discovered by accident, but is further investigated in this Chapter. It is thought that the addition of the protein having either a positive or negative effect upon the response time and size will achieve this by influencing the enzyme activity and
interaction with the benzidine. The proteins used during this study are listed in Table 1, along with a number of their key characteristics. The proteins chosen were readily available in the lab, but were used because they had different pI and molecular weight, so giving a range of both properties. Most of the proteins are enzymes, which have a FAD active centre (GOx, ChOx, β-amylase, α-amylase and SO), while pepsin is an aspartic protease, which means that its active site contains two aspartate residues one of which is ionised and the other unionised for the enzyme to be active. BSA is a protein with no enzymatic activity.

<table>
<thead>
<tr>
<th>Protein</th>
<th>MW (kDa)</th>
<th>PI</th>
</tr>
</thead>
<tbody>
<tr>
<td>GOx</td>
<td>160\textsuperscript{156}</td>
<td>4.2\textsuperscript{157}</td>
</tr>
<tr>
<td>BSA</td>
<td>66\textsuperscript{158}</td>
<td>4.7\textsuperscript{159}</td>
</tr>
<tr>
<td>ChOx</td>
<td>55\textsuperscript{99}</td>
<td>4.4 - 5.1\textsuperscript{99}</td>
</tr>
<tr>
<td>Pepsin</td>
<td>35\textsuperscript{160}</td>
<td>&lt;1.0\textsuperscript{155}</td>
</tr>
<tr>
<td>α-Amylase</td>
<td>50\textsuperscript{161}</td>
<td>5.2\textsuperscript{162}</td>
</tr>
<tr>
<td>β-Amylase</td>
<td>210\textsuperscript{163}</td>
<td>4.8\textsuperscript{164}</td>
</tr>
<tr>
<td>Sarcosine oxidase</td>
<td>44\textsuperscript{143}</td>
<td>4.7\textsuperscript{144}</td>
</tr>
</tbody>
</table>

Table 5.1 Molecular weight and pI of the proteins used to enhance the DMOB-HRP-H\textsubscript{2}O\textsubscript{2} reaction.
Chapter 5 — Protein enhancement

5.2 Experimental

5.2.1 Materials

Phosphate buffer tablets were acquired from Oxoid (Basingstoke, UK), and hydrogen peroxide (60 % w/v) and N,N dimethylformamide from Fisher (Loughborough, UK). The enzymes horseradish peroxidase (116 PU/mg) (EC. 1.11.1.7), Cholesterol oxidase (ChOx, E.C. 1.1.3.6. from Streptomyces sp., 19 U/mg), β-amylase (E.C. 3.2.1.2 from Sweet potato), α-amylase (E.C. 3.2.1.1. from porcine pancreas), Sarcosine oxidase (SO, E.C. 1.5.3.1 from Bacillus sp., 42 U/mg, and Glucose oxidase (GOx, EC. 1.1.3.4, from Aspergillus niger), FAD, bovine serum albumin (BSA) and 3,3'-diaminobenzidine tetrahydrochloride, were obtained from Sigma (Poole, UK). The 3,3'-dimethoxybenzidine was purchased from Aldrich (Poole, UK), and Pepsin (E.C. 3.4.23.1), from Merck (Lutterworth, UK).

Methods

All methods of crystal preparation and experimental set-up have been described in Section 2.2.

5.2.2 Measurement procedure.

For all experiments, 3.5 ml of a 300 μM DMOB solution was added to the test cell, to which was added 18.9 μl of a 360 U/ml HRP solution, giving a final concentration of 0.2 U/ml, and an appropriate concentration of protein to give a concentration between 0 and 0.14 % (w/v). All protein and FAD solutions were prepared in PBS, daily. A concentration of 300 μM hydrogen peroxide was used throughout all experiments.
The test solution was stirred, during all experiments. After each experiment, the crystal was cleaned thoroughly with N, N dimethylformamide and deionised water. The use of the organic solvent removed the film adsorbed to the crystal during the experiment, and this was confirmed by the crystal frequency returning to its original value. All experiments were performed at room temperature.

5.3 Results & Discussion

A variety of proteins and enzymes were studied with the DMOB-HRP-H$_2$O$_2$ reaction. Some were tried with DAB instead of DMOB, but it was found that they had no enhancing effect upon this benzidine reaction. This is probably due to the proteins solubilising the oxidised DAB in a similar manner as HDL, which was reported in Chapter 3. There is probably an interaction between the additional amino groups of the DAB and the carboxyl groups of the protein, as reported by Emmett et al. This possibly interaction will be investigated using mass spectrometry to identify the products of the reaction.
Fig. 5.1. The use of β-amylase with the DMOB-HRP-H$_2$O$_2$ reaction, with H$_2$O$_2$ at 300 μM. Each value is the mean ± s.e.m. (n=3).

In the absence of any additional proteins, DMOB gave an impedance response of 14.75 ± 0.6 Ω at 300 μM hydrogen peroxide, in the presence of horseradish peroxidase, with a response time of 68 min. When the same experiment was performed with the proteins, a different response was seen. All the enzymes gave a reduced response time of 15 – 20 minutes (see table 5.2), while BSA had a limited beneficial effect upon the response time. BSA may not have produced a beneficial effect because it will solubilise DMOB, as suggested by Emmett et al.\textsuperscript{165}, where they studied serum protein binding of benzidine and derivatives, and reported that the benzidine dyes they were using were binding to albumin. This would explain the optimal BSA concentration being the lowest concentration used. Only GOx and β-amylase had a positive effect upon the response size, as well as the response time.

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GOx gave an improved response of 138.4 ± 26.3 Ω at 0.056 % (w/v), and β-amylase a response of 26.7 ± 2.7 Ω at 0.005 % (w/v). It is possible that β-amylase would give a similar sized response to GOx at 0.056 % (w/v), as is apparent from the similar size response at the similar concentrations used. However, β-amylase is a considerably more expensive enzyme and so this would not be economically viable. When GOx was denatured by heating at 70 °C for 15 min according to Malikkides & Weiland\textsuperscript{166}, and then used with the DMOB-HRP-H\textsubscript{2}O\textsubscript{2} reaction, the response was significantly reduced (ΔΩ = 3.7 ± 1.6 Ω), and a response time of 20.3 ± 3.4 min. This loss of response is probably due to the deformation of the enzyme, plus the loss of the covalently bound FAD from the enzyme. During these experiments, particles were observed in solution, so it is possible that the denatured enzyme and the oxidised DMOB were complexing in solution, rather than adsorbing to the crystal surface. It is interesting that ChOx at its optimal concentration (0.05 % w/v) gave a response similar to that of DMOB with no protein present, but at the decreased response time of 15.5 ± 3.0 min. This enzyme has a similar pI to GOx and β-amylase, but approximately a quarter of the molecular weight.
**Fig. 5.2.** The use of GOx with the DMOB-HRP-H$_2$O$_2$, with H$_2$O$_2$ at 300 μM. Each value is the mean ± s.e.m. (n=3).

**Fig. 5.3.** The use of FAD with the DMOB-HRP-H$_2$O$_2$, with H$_2$O$_2$ at 300 μM. Each value is the mean ± s.e.m. (n=3).
<table>
<thead>
<tr>
<th>Protein</th>
<th>Conc. range studied (% w/v)</th>
<th>Optimal Conc. (% w/v)</th>
<th>Response size ± s.e.m. (Ω)</th>
<th>Response time ± s.e.m. (min)</th>
<th>In Summary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Only HRP</td>
<td>-</td>
<td>-</td>
<td>14.76 ± 0.64</td>
<td>68.0</td>
<td>-</td>
</tr>
<tr>
<td>GOx</td>
<td>0.003 - 0.140</td>
<td>0.056</td>
<td>138.36 ± 26.25</td>
<td>19.5 ± 2.2</td>
<td>Increased response, decreased time</td>
</tr>
<tr>
<td>BSA</td>
<td>0.001 - 0.1</td>
<td>0.001</td>
<td>8.80 ± 1.19</td>
<td>35.6 ± 0.1</td>
<td>Reduced response, decreased time</td>
</tr>
<tr>
<td>ChOx</td>
<td>0.001 - 0.1</td>
<td>0.05</td>
<td>14.21 ± 1.69</td>
<td>15.5 ± 3.0</td>
<td>Same response, decreased time</td>
</tr>
<tr>
<td>Pepsin</td>
<td>0.001 - 0.1</td>
<td>0.005</td>
<td>8.85 ± 2.71</td>
<td>19.5 ± 2.2</td>
<td>Reduced response, decreased time</td>
</tr>
<tr>
<td>α-Amylase</td>
<td>0.001 - 0.1</td>
<td>0.1</td>
<td>9.08 ± 1.10</td>
<td>11.7 ± 0.7</td>
<td>Reduced response, Decreased time</td>
</tr>
<tr>
<td>β-Amylase</td>
<td>0.0001 - 0.005</td>
<td>0.005</td>
<td>26.70 ± 2.68</td>
<td>17.2 ± 0.1</td>
<td>Increased response, Decreased time</td>
</tr>
<tr>
<td>SO</td>
<td>0.001 - 0.1</td>
<td>0.001</td>
<td>9.52 ± 0.98</td>
<td>16.6 ± 1.8</td>
<td>Reduced response, Decreased time</td>
</tr>
<tr>
<td>FAD</td>
<td>0.0005 - 0.01</td>
<td>0.01</td>
<td>6.76 ± 0.56</td>
<td>15.7 ± 0.2</td>
<td>Reduced response Decreased time</td>
</tr>
</tbody>
</table>
Table 5.2 (overleaf). A summary of the results obtained from the use of the various proteins used in this study, with the DMOB-HRP-H$_2$O$_2$ reaction. Each value is the mean ± s.e.m. (n=3).

The two enzymes (GOx and β-amylase), that gave better response times and enhanced response size have a similar pI and a higher molecular weight than the rest of the proteins used, while ChOx and SO that have similar pI, gave better response times. A further experiment was performed to study the effect of pure FAD with this reaction, and it was found that this decreased the response time, but did not increase the response size, which corresponds to the results obtained from SO, α-amylase, ChOx and pepsin. So, it would appear the response time was reduced due to the FAD component of the enzymes, and the response size due the molecular weight, rather than the pI. The results from using ChOx, SO and BSA would suggest that maybe the pI is not the major factor in influencing the response size, but rather than the molecular weight. It is also likely that that the protein is adding to a mass/viscoelastic effect by surface-entrapment within the adsorbed precipitate, and the degree of interaction between the oxidised benzidine and the protein depends upon the molecular weight, and therefore the size of the protein, though the pI of these larger proteins may have a part to play.

5.4 Conclusions

In the present work, it was found that the presence of GOx and β-amylase had a beneficial effect upon the crystal impedance response to the DMOB-HRP-H$_2$O$_2$ reaction, and the time taken to reach the end of this response. The decreased response time is due to the presence of the FAD or amino acids within the active site of the
enzymes, which are behaving as cofactors to HRP. The increased response size is mainly due to the molecular weight, and to a lesser extent the pI of the enzyme, which leads to increased deposition of oxidised DMOB at the crystal surface.

Further work will involve the use of enzymes with larger molecular weights and a range of pI, to establish that the conclusion that the molecular weight, rather than the pI is the contributing factor to the increased impedance change of the crystal. It may be the fact that the two proteins used here (GOx and β-amylase) have a surface charge distribution, which is optimal for complexing with the oxidised benzidine. If this is the case, this will be established through the use of proteins with different pI, but with substantial molecular weights.

As discussed in the previous two chapters, the potential for proteins to interfere with the sensing strategy has serious consequences for the accurate determination of any analyte using this method.
Chapter 6 – General Discussion

& Conclusions
6.1 General Discussion & Conclusions

The AT-cut quartz crystal sensor was used to detect the adsorption of an insoluble precipitate, on the upper electrode. The precipitate was formed from benzidine oxidation, in the presence of horseradish peroxidase and hydrogen peroxide. By monitoring changes in the series impedance \( Z_s \) of the crystal, the adsorption of the precipitate could be followed. The degree of change in \( Z_s \) was directly proportional to the amount of precipitate produced, which in turn was a reflection of the amount of hydrogen peroxide present in the test solution. So through the use of a calibration curve of \( Z_s \) versus hydrogen peroxide concentration, an unknown concentration of the later could be determined.

Four benzidines were used with the hydrogen peroxide determination, as part of an endpoint assay. These were 3,3'-diaminobenzidine (DAB), 3,3'-dichlorobenzidine (DCB), 3,3'-dimethoxybenzidine (DMOB) and 3,3',5,5'-tetramethylbenzidine (TMB). It was found that DCB was limited in terms of the hydrogen peroxide concentrations it could be used to determine because of its relatively low solubility (49 \( \mu \)M), so it was not used beyond this stage. The benzidine, DMOB was similarly limited to detection of hydrogen peroxide at concentrations below 160 \( \mu \)M. Of the two remaining benzidines, it was found that 3,3'-diaminobenzidine was the most promising for future work, because of its ability to polymerise, and measure hydrogen peroxide concentrations over a larger range, than the other benzidines used. If the assay method was altered from an endpoint to a rate of change in impedance, this would remove the problem of solubility, with the added advantage of reducing response time. Further work will be undertaken to see whether this is a viable alternative.
To enhance the degree of adsorption of the oxidised benzidine, surfactants were added to the test solution. The non-ionic surfactant, Triton X-100 was found to give the greatest enhancement of both response time (decreased by more than 50%), and response size (doubled). Tween 80 increased the response size, but had no positive effect upon the response time. The positive effects of Triton X-100 were probably due to an increase in enzyme activity (hence the decreased response time), and increased adsorption at the electrode surface (hence increased response size). The mechanism of interaction between surfactant-benzidine and the quartz crystal surface will be further investigated using Atomic Force Microscopy (AFM) and ellipsometry.

A number of polymers were used with the hydrogen peroxide determination in the presence/absence of Triton X-100. The polymers used were PVC, cellulose acetate and nitrocellulose fabricated by solvent casting, polypyrrole and poly(diaminobenzidine) prepared by electropolymerisation, and poly(diaminobenzidine) produced by oxidative polymerisation. All were found to have no beneficial effect in the presence of Triton X-100, which dominated the surface chemistry at the upper electrode of the quartz crystal. This does suggest that these polymers could be used to protect the upper electrode against the detrimental build-up of oxidised benzidine with time, if the sensor was used for more than one assay. In the absence of Triton X-100, the use of PVC resulted in an increased crystal response, but had no effect upon the response time. The PVC provides a more hydrophobic surface at the crystal, which proves to have chemical properties more favourable for the nucleation of the oxidised DAB adsorption.
Chapter 6 — General Discussion

The hydrogen peroxide detection was also enhanced by the presence of some proteins in the test solution. It was found that the inclusion of the enzymes, β-amylase and glucose oxidase increased the response size, and decreased the response time. All the enzymes used were found to decrease the response time, and this was due to the presence of the FAD or charged amino acids groups within the active centre, as was demonstrated through the use of a solution of FAD with the test solution. It was concluded that the active sites (i.e. the FAD or amino acids) are acting as cofactors for HRP. The increased response size was determined to be probably due to the molecular weight of the protein and it complexing with the oxidised DMOB, then adsorbing to the surface, resulting in the increased impedance changes. These results would suggest the detection strategy used would suffer from interference from endogenous proteins in the biological sample, and proteins would need to be removed or segregated from parts of the reaction mixture, probably by use of polymer membrane within the chamber.

The optimised DAB-HRP-H$_2$O$_2$ with Triton X-100 reaction was used for the determination of cholesterol, both free and total, through the addition of the enzymes, cholesterol oxidase and cholesterol esterase. The optimal concentrations of both enzymes were determined, and applied to the determination of total cholesterol in isolated LDL samples from human sera. It was found that this gave a linear response for LDL cholesterol between the concentrations of 25 and 400 μM, and a response time of approximately 20-25 min. For clinical measurements of total cholesterol, a 1 in 20 dilution would be required before determination, but with the added advantage that smaller volumes of serum can be used. It was found that HDL interfered with this reaction, as it solubilised the oxidised DAB, so leading to a decreased response.
The response time for this assay is slow compared to other biosensor methods, but if a rate of change assay was adopted, this would reduce the time to a few minutes.

Further to the cholesterol determination, the optimised DAB-HRP-H$_2$O$_2$ with Triton X-100 reaction was used for creatinine measurement. The enzymes, sarcosine oxidase (SO), creatinase (Cl) and creatininase (CA) were added to the test solution to produce hydrogen peroxide from creatinine. The determination of sarcosine through the use of SO gave a response time of 45 min, which is an increase upon the response time for hydrogen peroxide determination. This was believed to be due to the interference of the enzyme, SO upon the DAB-HRP-H$_2$O$_2$ reaction, as was previously described by Nguyen et al\textsuperscript{139}. With the addition of Cl for the creatine determination, the crystal response did not reached an endpoint after 3 hr. There was an initial plateau period after addition of creatine, the length of which appeared to be affected by the concentration of Cl, and the rate of impedance change was also affected by this. More work is required to determine whether these two features could be used for creatine determination. Creatinine determination gave a response time of less than 3 hr, indicating that creatine may be a possible interferent in this reaction sequence. Overall, it was concluded that unless rates of change in the impedance could be used for creatinine determination, then the quartz crystal did not provide a very competitive biosensor for this analyte.

6.2 Further Work

In the work presented in chapter 2, the DAB-HRP-H$_2$O$_2$ reaction with Triton X-100, this reaction has been optimised at 300 µM. However, DAB can be used at concentrations greater than this, since it has a good solubility in PBS as proven by it
being used at 5 mM. If this reaction was optimised at this concentration then the need for dilution of clinical samples for cholesterol determination could be avoided. However this might require the optimisation of HRP and Triton X-100 concentrations to allow for this greater concentration of DAB, especially if the DAB is being solubilised by the surfactant to enhance the enzymatic activity as has been suggested. The larger amount of oxidised DAB will require more micelles if it is being incorporated as discussed. The concentration of HRP may need to be higher to increase the oxidation of DAB, and hence reduce the response time. This would in turn require a higher concentration of all the enzymes within the test solution. To understand the nature of the relationship between Triton X-100 and DAB with the quartz crystal surface, the techniques AFM and ellipsometry will used.

It has been established that the protein enhancement of the hydrogen peroxide detection is due in part to the presence of FAD as a constituent part of the added protein, and this reduces the response time. The increased crystal response seen has been linked to the molecular weight of the protein. This requires more work through the use of more proteins with similar or higher molecular weights than those of glucose oxidase and β-amylase with a range of pl to fully establish whether this is indeed the case.

Cholesterol determination in serum has been carried out at a known concentration of total cholesterol in the serum. It was found that there was a difference between the LDL cholesterol and the serum cholesterol responses, at the same concentrations. This difference may be wholly or partially due to the presence of HDL, which has been found to act as an interferent in our detection strategy. To determine the
proportion of the difference being due to the HDL, a study of possible interferents needs to be conducted. Possible interferents will include paracetamol, ascorbate and urate, which are known to interfere with amperometric methods of detection, but not thought to have any effect upon quartz crystal measurements. This would have the added bonus of demonstrating the advantage of using the quartz crystal biosensor over the amperometric biosensor. Another possible source of interference may be the plasma proteins, including albumin. In chapter 5, bovine serum albumin was used with the DMOB-HRP-H$_2$O$_2$ reaction, and was found to have a detrimental effect on biosensor performance. The albumin used in this study is at a substantially higher concentration than that found in serum, so the effect seen may well be reduced. The other proteins used in this study do suggest that the presence of proteins may have both negative and positive effects upon the determination of cholesterol, and these effects will need to be overcome to allow accurate measurements. When the problem of interference has been diminished, a total cholesterol calibration curve will be produced, to establish whether a linear response is seen between the concentration of 25 and 400 μM. Following this, serum samples where the cholesterol concentration is not known could be used, and the cholesterol concentration measured compared to an established method such as the spectrophotometric method used initially to determine the cholesterol concentration in the isolated LDL.

Creatinine determination using the quartz crystal biosensor was found to give poor results. It is possible that using higher concentrations of the enzymes, sarcosine oxidase, creatinase and creatininase are needed, to increase the rate of hydrogen peroxide production from this enzyme cascade. If a higher concentration of creatinase was used, it might reduce the interferent effects of creatine. Equally
though a higher concentration of sarcosine oxidase may lead to more interference, so a balance needs to be established. If the response time is not reduced by these means, it may be possible to determine creatinine concentration by studying the rate of impedance change at different concentration or the length of initial plateau period after addition of creatinine. Through these avenues of investigation, it may be found that creatinine can be determined using the quartz crystal biosensor. The alternative method of determining creatinine using creatinine deiminase with a pH sensitive polymer needs investigating.
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Appendix : Published Papers
Optimisation of the enzyme-based determination of hydrogen peroxide using the quartz crystal microbalance

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Abstract

The benzidines, 3,3'-diaminobenzidine (DAB), 3,3'-dimethoxybenzidine (DMOB) and 3,3',5,5'-tetramethylbenzidine (TMB) were enzymatically oxidised to detect hydrogen peroxide, using the quartz crystal. The oxidised product mainly remains in suspension, resulting in a limited quartz sensor signal. We have used two non-ionic surfactants, Tween 80 and Triton X-100 to interact with the oxidised amphiphilic products to increase their solubility and surface activity, and their ability to adsorb to the crystal surface. Tween 80 exhibits optimised response effects for DAB, DMOB and TMB at 0.012, 0.005, and 0.002% (v/v), respectively, whereas Triton X-100 is optimum at 0.1, 0.2, and 0.006% (v/v), respectively. As a result, we have improved the quartz crystal sensor sensitivity to peroxide. The use of Triton X-100 gave an improved response time. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Quartz crystal microbalance; Surfactant; Hydrogen peroxide; Biosensor

1. Introduction

The operation of the quartz crystal microbalance (QCM) as described by the Sauerbrey equation (Sauerbrey, 1959), provides a relationship between a shift in the resonant frequency and a corresponding change in mass on the crystal surface. The relationship is only applicable if crystal measurements are made under dry conditions. Developments in quartz crystal theory (Rodahl et al., 1996; Ferrante et al., 1994) have now allowed the application of the QCM for measuring analytes in liquid media. This is possible due to the oscillating crystal's ability to detect mass and viscoelastic changes in the liquid phase. By monitoring changes in the resonant frequency (due to mass adsorption), it is possible to attribute changes in the solution conductivity, viscosity and density (Rodahl et al., 1996), as well as mass and viscoelasticity (Ferrante et al., 1994) to events occurring at the liquid–crystal interface.

The QCM has been recently used as an immunosensor (Su and Li, 2001; Attili and Sulciman, 1996; Park et al., 2000), a DNA sensor (Su et al., 1995; Tombelli et al., 2000), and an enzyme-based sensor (Reddy et al., 1998; Patolsky et al., 1999; Alfonta et al., 2000). We have previously used the QCM for the enzymatic determination of glucose (Reddy et al., 1998). The detection of glucose was accomplished by means of the two enzyme (glucose oxidase (GOx) and horseradish peroxidase (HRP)) catalysed oxidation and dimerisation of the benzidine, 3,3'-dimethoxybenzidine (DMOB). The ensuing precipitation led to a concentration-dependent frequency ($f_0$) and impedance ($Z_0$) shift at series resonance (the parameter $f_0$ refers to the frequency when the phase angle of the oscillation wave is zero). A similar QCM biosensor has been developed for the determination of acetylcholine (Alfonta et al., 2000).

The use of the quartz crystal to measure oxidase-catalysed production of hydrogen peroxide offers an alternative approach to the determination of low molecular weight solutes, such as glucose, lactate and cholesterol. The quartz crystal technique also offers advantages over the more conventional electrochemical and optical biosensing strategies; by virtue of its mass/viscoelasticity sensing mode, it is not affected by electrochemically active species (e.g. ascorbic acid, uric acid) and optical interferents.
The signal species are amphiphilic in nature, which increase in hydrophobicity due to dimerisation and oligomerisation, i.e. the end-product has a reduced water solubility leading to precipitation. Here, we have studied the enzymatic oxidation of 3,3′-diaminobenzidine (DAB), 3,3′,5,5′-tetramethylbenzidine (TMB), and DMOB, in the presence of surfactants in order to improve liquid/surface interactions of the signal species and enzyme activity. Tween 80 (polyoxyethylene sorbitan monooctanoate), and Triton X-100 (tetraoctylphenoxypolyethoxyethanol), two non-ionic hydrophilic surfactants, were investigated.

A major property of surfactants is that they are present in higher concentrations at interfaces (liquid/solid, liquid/liquid and air/liquid), than in the bulk of the solution. When using low concentrations of surfactant, the majority will be adsorbed at the air/water interface. Only when the concentration is increased will the surfactant begin to adsorb to the surface until saturation is reached, and the surface tension becomes constant, at which point the surfactant will begin to form micelles. This point is referred to as the critical micelle concentration (CMC). The size of the micelles formed differs from surfactant to surfactant. It is measured by the aggregation number, which is the number of surfactant molecules associated with a micelle (Porter, 1991; Rosen, 1989).

2. Experimental

2.1. Materials

Phosphate buffer tablets were purchased from Oxoid (Basingstoke, UK), and hydrogen peroxide (30% w/v) from Fisher (Loughborough, UK). HRP (180 PU/mg), DAB, TMB, Triton X-100, and Tween 80 were obtained from Sigma (Poole, UK). DMOB was purchased from Aldrich (Poole, UK).

2.2. Methods

2.2.1. Solution preparation

Phosphate buffer tablets were dissolved in deionised water giving a solution concentration of 0.01 M, pH 7.4. The benzidine solution was prepared in phosphate buffer to give a final concentration of 0.3 mM (0.16 mM for DMOB, due to its limited solubility). The TMB solution was heated and sonicated at 45 °C for 15 min, then cooled to room temperature, before being sonicated at room temperature. This pre-treatment of TMB was found necessary to increase the reactivity of TMB. The hydrogen peroxide solution (10 mM) was prepared in phosphate buffer. The HRP was prepared using deionised water, giving a final activity of 180 PU/ml. The surfactant solutions were prepared in phosphate buffer, to give a final concentration of 1% (v/v). When not in use, all solutions were stored at 4 °C, and fresh solutions were prepared daily.

2.2.2. Crystal preparation

Gold-on-chromium electrodes (100 and 5 nm, respectively) were vapour-deposited onto either side of a blank AT-cut quartz crystal (IQD, Crewkerne, UK). The crystals had a fundamental resonance frequency of 10 MHz, and diameter of 8.2 mm. The crystals were cleaned with acetone and isopropanol, and dried with vacuum suction, prior to electrode deposition. The crystal was then sealed in the sample chamber as previously described (Reddy, 2000). A HP4194A impedance analyser coupled to a PC was used to record resonant frequency and impedance changes.

2.2.3. Measurement procedure

For all experiments, 3.5 ml of the benzidine solution was added to the test cell, to which was then added 18.9 μl HRP solution, and an appropriate volume of surfactant to give a concentration between 0 and 0.5% (v/v). Various concentrations of hydrogen peroxide were added, which was increased stepwise (from 0 to 400 μM) over time. The test solution was stirred, during all experiments. After each experiment, the crystal was cleaned thoroughly with N,N-dimethylformamide and deionised water. The use of the organic solvent removed the film adsorbed to the crystal during the experiment, and this was confirmed by the crystal frequency returning to its original value. All experiments were performed at room temperature.

2.2.4. Spectrophotometric measurements

All measurements were taken at 490 nm. A 3.5 ml solution of TMB was used as the blank, to which was then added 18.9 μl HRP. This was mixed thoroughly, and the absorbance measured. To this was then added a volume of Tween 80, which gave the optimum concentration of surfactant for the oxidation of TMB. The solution was mixed thoroughly, before the absorbance was measured again.

3. Results and discussion

In the absence of surfactants, the enzymatic oxidation of the benzidines formed an insoluble suspension. Due to the formation of the suspension, limited impedance and frequency changes were measured (Δf = 150, 450, 450 Hz for 300 μM hydrogen peroxide with DAB, TMB, and DMOB, respectively). These measurements indicated that a limited amount of oxidised benzidine product was adsorbing to the surface of the quartz crystal. The oxidation of the benzidine is given in Eq. (1), where R1 = NH2 and R2 = H for DAB, R1 = OCH3, buffer, to give a final concentration of 1% (v/v). When not in use, all solutions were stored at 4 °C, and fresh solutions were prepared daily.
and $R_2 = H$ for DMOB, and $R_1$ and $R_2 = CH_3$ for TMB.

Surfactants were used to increase the solubility of the amphiphilic dimers, in an attempt to increase their interaction with the gold electrodes of the quartz sensor surface. The dimerisation occurs at the amine group of the benzidine, and since DAB possesses more than two amino groups, it is possible that the oxidised dimer may undergo further catalytic oxidation forming tetramers or possibly oligomers. These could be responsible for the larger particles in suspension observed.

Figs. 1 and 2 show a clear enhancing effect of Tween 80 and Triton X-100 concentration, respectively, on the frequency response upon addition of hydrogen peroxide (300 μM) to a solution containing benzidine and peroxidase.

The optimum concentrations of surfactant vary for each benzidine, between each surfactant. It is interesting to note that the concentration of surfactant required for the optimum response is above the CMC, the one exception being TMB with Triton X-100.

The addition of HRP to a solution of TMB gave a turquoise solution. This colour change occurred in the absence of the surfactants and hydrogen peroxide. The sonication and heating treatment of TMB may produce a more reactive intermediate, which upon addition of HRP would appear to interact with the latter, resulting in the colour change observed. There was no observable change in colour upon the addition of surfactant. Upon measurement of the absorbance at 490 nm, there is a small change in absorbance between the TMB with HRP solution (0.135), and the TMB with HRP and 0.002% Tween 80 solution (0.162). This result would indicate that there is the formation of micelles occurring leading to a spectral shift (Biswas et al., 1999; Patist et al., 2001).

For DMOB and DAB, the solution remained colourless upon the addition of the surfactant, and therefore it can be suggested that the result was a solubilisation of the benzidines rather than emulsion. The DMOB and DAB responses are optimised at similar concentrations of surfactant (see Table 1), implying that the mechanism of interaction between the surfactant and the benzidine is the same. There is a substantial difference in the optimal concentrations of surfactant required for DMOB, DAB and TMB. The enzymatic oxidation of TMB is optimised at a significantly lower concentration of surfactant than the other two benzidines, but the response is similar in size to the response in the absence of surfactant. This would indicate that the surfactants has no positive effect upon the TMB reaction.

Table 1
The surfactant concentration required for the observed optimised response for each benzidine

<table>
<thead>
<tr>
<th>Optimum [surfactant] with DAB (mM)</th>
<th>Tween 80</th>
<th>Triton X-100</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMC</td>
<td>0.012</td>
<td>0.24</td>
</tr>
<tr>
<td>DAB</td>
<td>0.098</td>
<td>1.71</td>
</tr>
<tr>
<td>DMOB</td>
<td>0.041</td>
<td>3.42</td>
</tr>
<tr>
<td>TMB</td>
<td>0.016</td>
<td>0.10</td>
</tr>
</tbody>
</table>
The presence of the surfactant in the reaction solution has two possible effects, which lead to the improved signal generated. The surfactant is most likely to be altering the solubility of the oxidised benzidine produced. If DAB forms tetramers upon oxidation, this will increase the hydrophobicity, reducing the ability of the surfactant to solubilise the oxidised product. Hence this could be a possible reason for it not producing large signals on a scale with DMOB. The other possibility is an increase in the activity of HRP due to the presence of surfactants (Kamiya et al., 2000). It was found that the increased response in the presence of surfactants is due to adsorption of the oxidised benzidine at the surfactant layer, rather than a bulk solution effect. Indeed the bulk solution was tested again on the sensor after thorough washing of the crystal to remove any precipitate. The response (viscosity effect) was negligible compared to the precipitate response, further confirming that the majority of the signal was due to the surfactant-enhanced adsorption of the oxidised benzidine.

Triton X-100 has a larger CMC (0.24 mM) than Tween 80 (0.012 mM), and hence a larger surfactant concentration is required before solubilisation of the oxidised benzidine occurs. Another key difference is the aggregation number, where Triton X-100 has a larger number of molecules per micelle (140 molecules) than Tween 80 (60 molecules).

It was noted that the oxidation of the benzidine in the presence of the Triton X-100 resulted in quicker response times than seen in the absence of the surfactant (Table 2). This was possibly due to the surfactants improving the enzyme activity, and also improving the dimerisation reaction. The other possibility was that the detergents increased the solubility of the substrate. These observations could explain the improved response time for Triton X-100 in the present study.

The extended response time for the oxidation of DMOB and TMB with Tween 80 compared with Triton X-100 could be due to the partitioning of the substrate into Tween micelles, as a result of their higher hydrophobicity than DAB. This could lead to a slow release of benzidine between the micelles and aqueous environment, via a concentration gradient. The partitioning of the substrate could also explain the poor responses with TMB and Triton X-100, where this substrate's high hydrophobicity would lead to an equilibrium being established where the majority of the substrate is partitioned into micelles, and as a consequence a small response occurs.

Figs. 3 and 4 show calibration curves for hydrogen peroxide at the optimised surfactant concentration for each of the benzidines, when using Tween 80 and Triton X-100, respectively.

With increasing hydrogen peroxide concentration, there is a corresponding change in the measured parameter ($f_\text{m}$). The changes occur because of the oxidation of the benzidine, which results in the formation of a product having a lower solubility, and a larger size than the benzidine. The response measured is a result of two factors, the adsorption of oxidised product to the surface of the quartz crystal, and the increase in surface activity of the oxidised benzidine. The surfactant can form a layer on the crystal surface, which leads to increased adsorption of oxidised benzidine. The other possibility is that surface interaction occurs by the oxidised benzidine being incorporated into the micelles of the surfactant.

Hydrogen peroxide cannot be detected at low concentrations, due to some solubility of the oxidised product. At concentrations of hydrogen peroxide below 30 µM, there is generally a lack of deposition of the oxidised benzidine, and consequently no measurable response.

It was found that the DMOB calibration curve tends to plateau around 150–200 µM hydrogen peroxide. One molecule of hydrogen peroxide will oxidise one molecule of DMOB, and therefore at concentrations of hydrogen peroxide above 160 µM, all the DMOB will have been oxidised to its dimer form. For this reason DMOB cannot be used to detect hydrogen peroxide concentrations above 160 µM. DAB is present at a concentration of 300 µM, which would suggest that this benzidine could be used to detect hydrogen peroxide concentra-

### Table 2

<table>
<thead>
<tr>
<th>Benzidine</th>
<th>No surfactant</th>
<th>With Triton X-100</th>
<th>With Tween 80</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$t_{50}$</td>
<td>$t_{50}$</td>
<td>$t_{50}$</td>
</tr>
<tr>
<td>DAB</td>
<td>272</td>
<td>1413</td>
<td>275</td>
</tr>
<tr>
<td>DMOB</td>
<td>348</td>
<td>4100</td>
<td>413</td>
</tr>
<tr>
<td>TMB</td>
<td>1016</td>
<td>4533</td>
<td>687</td>
</tr>
<tr>
<td></td>
<td>$t_{0.5}$</td>
<td>$t_{0.5}$</td>
<td>$t_{0.5}$</td>
</tr>
<tr>
<td>DAB</td>
<td>1870</td>
<td>135</td>
<td>1833</td>
</tr>
<tr>
<td>DMOB</td>
<td>1312</td>
<td>2323</td>
<td>5468</td>
</tr>
<tr>
<td>TMB</td>
<td>2247</td>
<td>2883</td>
<td>5089</td>
</tr>
</tbody>
</table>

Fig. 3. The frequency ($f_\text{m}$) response, to the hydrogen peroxide calibration in the presence of Tween 80.
Fig. 4. Hydrogen peroxide calibration in the presence of Triton X-100 and the frequency (f) response.

ions up to this concentration. However it is capable of producing a significant signal at 400 µM, and this suggests that it is indeed forming tetramers or oligomers. TMB did not display any linear response in the detection of hydrogen peroxide with either surfactant, and consequently these results are not shown.

4. Conclusions

The oxidation of DMOB and DAB by hydrogen peroxide, in the presence of HRP is enhanced by the use of non-ionic surfactants, however this was not the case for TMB, where there was no beneficial effect. The use of Tween 80 generally extends the response time, while Triton X-100 has a more beneficial effect on response time. This has major implications for the optimisation of oxidase enzyme-based biosensor strategies using the quartz crystal.

Acknowledgements

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References


Enzyme-based determination of cholesterol using the quartz crystal acoustic wave sensor

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Abstract

We have used the AT-cut quartz crystal sensor to measure in real-time the total cholesterol concentration in buffer and serum, using the trienzyme system of cholesterol esterase (ChE), cholesterol oxidase (ChOx) and horseradish peroxidase (HRP). The hydrogen peroxide produced from the ChE-ChOx reaction oxidises diaminobenzidine (DAB), in the presence of HRP. The response of the sensor to cholesterol is optimal in the presence of 0.1% (v/v) Triton X-100 at 0.2 U/ml ChOx, and 1 U/ml ChE. A response is obtained in less than 25 min. Using the optimal concentrations of the reagents, the linear range for free cholesterol and low density lipoprotein (LDL) cholesterol determination was between 50 and 300 µM, and 25 and 400 µM, respectively. It was found that the concentration of high density lipoprotein (HDL) cholesterol could not be determined because it solubilised the oxidised DAB, leading to poor adsorption at the crystal surface. We obtained a response to the use of cholesterol in serum at 300 µM, demonstrating that this biosensor could be used for cholesterol determination in clinical samples.

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Keywords: Cholesterol; Quartz crystal acoustic wave sensor; Cholesterol oxidase; Diaminobenzidine

1. Introduction

Cholesterol is routinely measured for the risk assessment of cardiovascular conditions, such as atherosclerosis and hypertension, which can develop into coronary heart disease, myocardial and cerebral infarction (stroke). In conditions such as hypothyroidism, nephrosis, diabetes mellitus, myxedema, and obstructive jaundice, the patient will have increased levels of cholesterol and its esters above the physiological norm. Decreased levels are found in patients suffering from hyperthyroidism, anaemia, malabsorption and wasting syndromes.

The desired total plasma cholesterol for an individual is less than 5.2 mM (200 mg/dl), and a high level being considered as greater than 6.2 mM (240 mg/dl) [1]. Plasma cholesterol levels increase with age, and are generally less in women than men, until menopause, when the values in women exceed those in men [2]. Cholesterol is carried in plasma by a series of protein-containing micelles known as lipoproteins. The lipoproteins are classified into distinct subtypes according to their density, very low density lipoprotein (VLDL), low density lipoprotein (LDL), intermediate density lipoprotein (IDL) and high density lipoprotein (HDL). About 70% of total plasma cholesterol contained within lipoproteins is

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esterified by fatty acids. Hence, the concentration of free cholesterol within lipoproteins is approximately 1.0–2.2 mM (40–85 mg/dl) [3].

Historically, cholesterol was measured using non-enzymatic spectrometry, via the production of a coloured substance, chiefly via cholestapolyenes and cholestapolyene carbonium ions (Liebermann–Burckhard reaction). This method suffered from poor specificity, instability of the colour reagent, standardisation difficulties, the variable reactivity of esters and the unstable and corrosive nature of the reagents used [4,5]. The selectivity of the chemical reaction was improved with the introduction of the enzymes, cholesterol esterase (ChE) and cholesterol oxidase (ChOx):

\[
\text{chsterol esters} + \text{H}_2\text{O} \rightarrow \text{cholesterol} + \text{fatty acids}
\]

(1)

\[
\text{cholesterol} + \text{O}_2 \rightarrow \text{cholest-4-en-3-one} + \text{H}_2\text{O}
\]

(2)

The cholest-4-en-3-one can be reacted with 2,4-dinitrophenylhydrazine to produce a coloured hydrazone [6], although the consumption of \( \text{O}_2 \) [7], or the production of \( \text{H}_2\text{O}_2 \) [3,8-10] are the easier methods of quantifying cholesterol spectrophotometrically, with the latter being the preferred method. The presence of molecular oxygen in clinical samples will result in false positives in any method measuring consumption of oxygen unless steps are taken to remove it [11]. The oxygen will be consumed by other substances, which are present in clinical samples, such as ascorbic acid, as found by Marazuela et al. [12].

A number of cholesterol biosensors have been developed over the past 30 years. Examples of optical biosensors, which determine cholesterol enzymatically have been developed [12-15]. Some of these methods suffer from interference from other substances found in the serum, as has been previously commented upon [12] (see Table 1). Amperometric and potentiometric methods have been researched to determine cholesterol [16-20]. The major disadvantages of these methods include the need for calibration of the sensor both before and after the measurement, the lifetime of the sensor is short, and the oxidation of other electrochemically active species (known as interferents) present in the test sample may lead to false positive signals. The major interferents are ascorbic and uric acid (see Table 1). This problem can be overcome through the use of polymer layers, which are more selective for the analyte of interest and eliminate or reduce the interferences, but this requires more time for preparation, and increases the complexity of the biosensor. If the interferents are

| Table 1 |
| A comparison of previous methods for determining cholesterol |

<table>
<thead>
<tr>
<th>Reference</th>
<th>Detection method</th>
<th>Response time (min)</th>
<th>Linear range (mM)</th>
<th>Detection limit (µM)</th>
<th>Lifetime (days)</th>
<th>Known interferents</th>
</tr>
</thead>
<tbody>
<tr>
<td>[14] Fibre-optic fluorescence</td>
<td>7-12</td>
<td>Not linear for FC</td>
<td>200</td>
<td>–</td>
<td>Not tested</td>
<td></td>
</tr>
<tr>
<td>[12] Fibre-optic luminescence</td>
<td>&lt;0.5</td>
<td>0.15-3 for FC</td>
<td>500</td>
<td>&gt;60</td>
<td>Ascorbic acid</td>
<td></td>
</tr>
<tr>
<td>[20] Potentiometric</td>
<td>16</td>
<td>0.05-3 for TC</td>
<td>10</td>
<td>–</td>
<td>Ascorbic acid, bilirubin and proteins had negligible effects</td>
<td></td>
</tr>
<tr>
<td>[19] Amperometric</td>
<td>&lt;2</td>
<td>0.58-3.68 for FC</td>
<td>60</td>
<td>5</td>
<td>Ascorbic acid, uric acid, paracetamol, glutathione</td>
<td></td>
</tr>
<tr>
<td>[18] Amperometric</td>
<td>5</td>
<td>No data for FC</td>
<td>–</td>
<td>–</td>
<td>Oxygen, urea</td>
<td></td>
</tr>
<tr>
<td>[17] Amperometric</td>
<td>1</td>
<td>Not linear for TC</td>
<td>500</td>
<td>1</td>
<td>Not tested</td>
<td></td>
</tr>
<tr>
<td>[15] Fluorometric</td>
<td>&lt;30</td>
<td>0.005-0.05 for TC</td>
<td>5 pM</td>
<td>–</td>
<td>Minimal interference from bilirubin</td>
<td></td>
</tr>
<tr>
<td>[3] Spectrophotometric</td>
<td>5</td>
<td>2.6-15.6 for TC</td>
<td>2.6</td>
<td>–</td>
<td>Negligible interference from ascorbic acid, uric acid and haemoglobin</td>
<td></td>
</tr>
</tbody>
</table>

FC refers to the fact that the biosensor was used to measure free cholesterol only, while TC means total cholesterol was determined.
not reduced, this can lead to an overestimation of the analyte concentration.

It has been clearly demonstrated by Chang and Shih [21] that neither ascorbic acid nor uric acid interfere with the operation of the quartz crystal sensor. Bilirubin proved to act as an interferent in absorption measurements, because it absorbed light at the same wavelength as the analyte being measured, and therefore will not be an interferent for the quartz crystal.

There is the possibility that ascorbic acid and uric acid act as hydrogen donors in the peroxidase reaction, which was raised by Nguyen et al. [22]. However in other peroxidase reactions such as Allain et al. [3], using 4-aminophenylamine and phenol or the Sigma Infinity™ kit which uses 4-aminophenylamine and hydroxybenzoic acid, there was no reported interference at normal physiological concentrations. Therefore, the same would be true for the cholesterol quartz crystal sensor, since all three assays are peroxidase reactions.

The operation of the quartz crystal acoustic wave sensor as described by the Sauerbrey equation [23] provides a relationship between a shift in the resonant frequency and a corresponding change in mass on the crystal surface. The relationship is only applicable if crystal measurements are made under dry conditions. Developments in quartz crystal theory [24,25] have now allowed the application of the quartz crystal for measuring analytes in liquid media. This is due to the oscillating crystal’s ability to respond to mass and viscoelastic changes in the liquid phase. The acoustic wave sensor operates in the thickness shear mode resulting in the propagation of a shear wave into the interfacing liquid. By monitoring changes in the resonant frequency and impedance, it is possible to attribute changes in the solution conductivity, viscosity and density [24], as well as mass and viscoelasticity [25] at the liquid-crystal interface.

The quartz crystal has been recently used as an immunoassay [26-28], a DNA sensor [29-31], and an enzyme-based sensor [31—33]. We have also previously used the quartz crystal for the enzymatic determination of glucose [31]. This was accomplished by means of the two enzyme (glucose oxidase (GOx) and HRP) catalysed oxidation and dimerisation of the benzidine, DMOB. The ensuing precipitation led to a concentration-dependent frequency ($f_0$) and impedance ($Z_0$) shift at series resonance (the parameter, $f_0$ refers to the frequency when the phase angle of the oscillation wave is zero, and $Z_0$ refers to the corresponding impedance at this point). We monitored changes in $Z_0$ for the same reason as previously mentioned by Reddy et al. [31], where they found it was difficult to establish $f_0$ when the crystal load is dissipative. The changes in both parameters are directly proportional to the other, so in effect we are still measuring changes in the viscosity and density of the solution, as well as any additional mass loading due to adsorption [34]. The biosensor was used to determine glucose in the range 60–160 μM.

The use of the quartz crystal to measure oxidase-catalysed production of hydrogen peroxide offers an alternative approach to the determination of low molecular weight solutes, such as glucose, lactate and cholesterol. Indeed, we have optimised the hydrogen peroxide response with different benzidines (diaminobenzidine (DAB), DMOB, and tetramethylbenzidine (TMB)) through the use of the non-ionic surfactants, Triton X-100, and Tween 80 [35]. In this paper, we describe our method for using the quartz crystal microbalance for the determination of free cholesterol (using the enzymes ChOx and HRP) and total cholesterol (using the enzymes ChOx, ChE and HRP). In doing so, we have determined the optimal concentration of ChOx, ChE and Triton X-100 for this assay. We also determined total cholesterol in human LDL and HDL sub-fractions and in human serum using this assay.

2. Experimental

2.1. Materials

Phosphate buffer tablets were purchased from Oxoid (Basingstoke, UK), propan-2-ol from BDH (Poole, UK), and potassium bromide (KBr) from Fisher (Loughborough). Horseradish peroxidase (HRP type I, EC 1.11.1.7, 180 PU/mg), Cholesterol oxidase (EC 1.1.3.6. from Streptomyces sp., 19 U/mg), Cholesterol esterase (EC 3.1.1.13 from Pseudomonas sp.), 3,3'-diaminobenzidine tetrahydrochloride (D5637), Cholesterol (C8667), Triton X-100, Infinity™ cholesterol reagent (401-25P) and cholesterol calibrators (C0534) were obtained from Sigma (Poole, UK).
3. Methods

3.1. Solution preparation

Diaminobenzidine was prepared in phosphate buffer (0.01 M, pH 7.4) to give a final concentration of 0.3 mM. Cholesterol (10 mM) was prepared in isopropanol; the latter solvent has been shown to have no effect upon the activity of the enzymes at the final concentration used [36]. All enzymes were prepared in phosphate buffer, giving a final activity of 360, 50 and 70 U/ml for HRP, ChE and ChOx, respectively. Triton X-100 was prepared in phosphate buffer, to give a final concentration of 1% (v/v). When not in use, all solutions were stored at 4°C. The diaminobenzidine and HRP solutions were prepared daily; and the Triton X-100 and Cholesterol solutions were prepared weekly. The ChOx and ChE were prepared in PBS and stored at — 80°C until 1 h before use (the remaining enzyme was discarded after that day’s experiments).

3.2. Crystal preparation

Gold-on-chromium electrodes (100 and 5 nm, respectively) were vapour-deposited onto either side of a blank AT-cut quartz crystal (IQD, Crewkerne, UK). The crystals had a fundamental resonance frequency of 10 MHz, and diameter of 8.2 mm. Crystals were cleaned with acetone and isopropanol, and dried with vacuum suction, prior to electrode deposition. One crystal piece was then sealed in the sample chamber as previously described [37]. A HP4194A impedance analyser coupled to a PC was used to record resonant frequency and impedance changes at series resonance.

3.3. Measurement procedure

For all experiments, 3.5 ml DAB containing 0.2 U/ml HRP was added to the test cell. Various concentrations of Triton X-100 (0.01–0.2%, v/v), cholesterol (0–400 μM), LDL (300 μM) or serum (300 μM), ChOx (0–0.5 U/ml) and if applicable, ChE (0.5–2 U/ml) were then added. The test solution was stirred, during all experiments. After each experiment, the crystal was cleaned thoroughly with N,N-dimethylformamide (DMF) and deionised water. The use of DMF removed the oxidised DAB adsorbed to the crystal during the experiment, and was confirmed by the crystal frequency returning to its original value. All experiments were performed at room temperature.

3.4. Spectrophotometric measurements

All measurements were taken at 492 nm using a 96-well plate reader (Labsystems iEMS Reader MF and Labsystems Genesis Version 3.05 software). For the spectrophotometric measurements, all solutions were prepared as previously described. For the HRP experiments, 300 μl of DAB, 1 μl of HRP, 3 μl of Triton X-100, and a range of H2O2 concentrations from 0 to 60 μM were added to each well. The absorbance was measured every 15 s, over 10 min at 492 nm. For the HRP–ChOx experiments, 300 μl of DAB, 1 μl of HRP, 1 μl of ChOx, and a range of Triton X-100 concentrations were added, after which was added 9 μl of cholesterol. Again, the absorbance was measured every 15 s, over 10 min at 492 nm.

3.5. Isolation of HDL/LDL

The method of isolation used has been previously described by Rankin et al. [38]. One hundred millilitres of venous blood from healthy volunteers was collected into 2 ml of Na2EDTA (150 mM, pH 7.4, prepared in water, and filter sterilised) using a 20 gauge butterfly catheter (Abbott, Sligo, Rep., Ireland). The blood was centrifuged at 800 × g for 30 min at 4°C in a Beckman GPR centrifuge (High Wycombe, Buckinghamshire) to obtain plasma. The density of the plasma was adjusted to 1.019 g/ml, by the addition of a “high density” (η = 1.32 g/ml) potassium bromide solution. The plasma was then transferred into 11 ml ultracentrifugation tubes (ultra clear, 16 mm × 76 mm) from Beckman Instruments (High Wycombe, Buckinghamshire) and centrifuged at 108,000 × g for 18 h at 4°C in a Beckman 70Ti rotor, and Beckman Optima XL-100K ultracentrifuge (Beckman Instruments, High Wycombe, Buckinghamshire). Following centrifugation, the fraction containing LDL and HDL was recovered, its density adjusted to 1.063 g/ml by the addition of KBr and then dialysed against a PBS solution containing KBr for 4 h, to give a final density of 1.063 g/ml. Following this, it was centrifuged as previously described under the same conditions. The LDL and HDL fractions
were recovered and dialysed against four changes of PBS to remove any KBr. The separate LDL and HDL sub-fractions were filter sterilised (pore size, 0.2 μM from Sartorius Group, Epsom, Surrey), to remove lipoprotein aggregates and stored at 4°C.

3.6. Spectrophotometric determination of cholesterol concentration

This method is a modification of the Sigma Diagnostics procedure No. 401. Three microlitres of standard (cholesterol calibrator 100, 200 and 400 g/l, and 6 μl of 400 g/l to obtain 800 g/l), LDL, HDL, or serum was pipetted per well in replicate on a 96-well plate. Three hundred microlitres of Sigma Infinity™ cholesterol reagent was added per well and incubated at room temperature for 30 min. The absorbance at 525 nm was measured using a 96-well plate reader (Labsystems iEMS Reader MF, and Labsystems Genesis Version 3.05 software). The cholesterol concentration was then calculated from the standard curve.

4. Results and discussion

4.1. Optimisation of Triton X-100 with cholesterol oxidase

The Triton X-100 concentration required for the optimal response for the measurement of free cholesterol was determined. A range of Triton X-100 concentrations were used (0.001–0.2%, v/v), while the ChOx concentration was maintained at 0.1 U/ml, and the cholesterol concentration at 300 μM (Fig. 1). Increasing concentrations of Triton X-100 increased the change in crystal impedance (Zcr) reaching a maximum at around 0.1% (v/v) (Fig. 1).

We have previously found that the optimal concentration of Triton X-100 for the determination of hydrogen peroxide using DAB was 0.1% (v/v) [35]. Indeed, the data shows (Fig. 1) that this is also the optimal concentration of Triton X-100 for the determination of cholesterol. We believe this is due to the surfactant improving the enzyme activity, and also improving the dimerisation of oxidised DAB, which is supported by spectrophotometric analysis. When measuring the absorbance at 492 nm, we found that for the hydrogen peroxide determination, the Fmax increased from 1.06ΔA min⁻¹ (no surfactant present) to 2.46ΔA min⁻¹ in the presence of Triton X-100 (data not shown). The Km remained reasonably constant (0.68 and 0.66 mM in the absence and presence of surfactant, respectively). Another possibility is the Triton is increasing the solubility of the substrate [14,20,39]. We found previously that Triton X-100 at 0.1% (v/v) led to increased adsorption of the oxidised DAB product at the crystal surface. It has also been demonstrated previously that Triton X-100 at a concentration range of 0.05–0.1% (v/v), increases the activity of ChOx [36,40–42]. At concentrations

![Fig. 1. Triton X-100 concentration profile for the optimum response to 300 μM cholesterol in the crystal impedance (Zcr) at 10 MHz. Each value is the mean ± S.E.M. (n = 3).](image-url)
above this, Triton X-100 was found to inhibit the activity of the enzyme [40]. Therefore, Triton X-100 is not only increasing adsorption of the oxidised DAB, but also ChOx activity, and the activity profile may result from a combination of these two effects. Upon measuring the change in absorbance at 492 nm, for various concentrations of Triton X-100 with the ChOx-HRP reaction, we found that the change in absorbance peaked at about 0.1–0.05% (v/v) Triton X-100 (0.11ΔA and 0.12ΔA min⁻¹, respectively), compared to 0.2 and 0% (v/v) Triton X-100 (0.08ΔA and 0.09ΔA min⁻¹, respectively) (data not shown).

4.2. Optimisation of cholesterol oxidase

To determine the optimal ChOx concentration, a range of ChOx concentrations were used (0.01–0.5 U/ml), using Triton X-100 at 0.1% (v/v) and cholesterol at 300 μM. Increasing concentrations of ChOx (0.1–0.01 U/ml) resulted in a decrease in the observed Zₜ (Fig. 2). However, increasing concentrations of ChOx decreased the response time from 34.5 ± 1.5 min for 0.01 U/ml to 22.3 ± 0.3 min using 0.2 U/ml (data not shown). It was decided that the optimal concentration of ChOx for the determination of cholesterol was 0.2 U/ml, when both response size and time were considered. This concentration of ChOx is similar to optimal concentration used by others [43].

4.3. Calibration with cholesterol

Using the previously optimised Triton X-100 and ChOx concentrations, the response to cholesterol was determined over the range, 0–400 μM. A linear relationship between the cholesterol concentration (0–300 μM) and change in Zₜ was observed (Fig. 3). For clinical measurement of free cholesterol in serum, a 1:10 sample dilution would be required, so that the cholesterol concentration would fall inside the linear range of our biosensor.

4.4. Optimisation of cholesterol esterase

We isolated LDL from human blood as a substrate for the optimisation of ChE, since cholesterol esters occur naturally in aqueous solution within LDL, whilst cholesterol esters, e.g. cholesteryl oleate, require preparation in various solvents, the presence of which may have unforeseen effects on enzyme activity and stability. The cholesterol concentration within LDL was established by means of the cholesterol determination spectrophotometrically.

The optimal concentration of the enzyme, ChE was determined, using the previously optimised concentrations of Triton X-100 and ChOx. The ChE was used at 0.2, 1, and 2 U/ml, and it was found that 1 U/ml gave the best response in terms of response size and reproducibility (see Fig. 4). For all further experiments, 1 U/ml ChE was used.

4.5. Calibration curve with LDL

Using the isolated LDL, a series of experiments were performed to produce a calibration curve over the concentration range of 25–400 μM LDL cholesterol (Fig. 5). This produced a linear relationship between the concentration of LDL, and the
Fig. 3. The calibration curve for cholesterol determination, using 0.2 U/ml ChOx, and 0.1% (v/v) Triton X-100. Each value is the mean ± S.E.M. (n = 3).

Fig. 4. The optimisation of ChE, using 0.1% Triton X-100, 0.2 U/ml ChOx, and 300 µM LDL. Each value is the mean ± S.E.M. (n = 3).

Fig. 5. The calibration curve for LDL determination, between the concentrations of 0 and 400 µM, when using 0.1% Triton X-100, 0.2 U/ml ChOx, and 1 U/ml ChE. Each value is the mean ± S.E.M. (n = 3).
corresponding change in \(Z_s\). For clinical measurement of total cholesterol in serum, a 1:20 sample dilution would be required, so that the total cholesterol concentration would fall within the linear range of our biosensor.

### 4.6. Determination of cholesterol in serum

Fig. 6 shows the \(Z_s\) responses to the use of LDL, HDL and serum at 300 \(\mu\)M cholesterol, as the substrates for determination. Experiments with all three substrates were performed, with the enzymes, Triton X-100, and DAB being used at the optimal concentrations. It was found that isolated LDL gave an enhanced response and HDL gave a poor response, in comparison to serum, which was found to be statistically significant (\(P < 0.001\)).

To identify possible causes of this reduced response to HDL, the LDL and HDL subfractions were re-isolated by centrifugation at the end of the experiment. It can be seen from the photographs of the two tubes (Fig. 7), the oxidised DAB was distributed differently. In the LDL tube, the LDL fraction is at the top of the tube, and the oxidised DAB pelleted at the bottom. However, the HDL and the oxidised DAB co-localised at the top of the HDL tube. This would suggest that the oxidised DAB is dissolving within HDL, leading to a lack of adsorption to the crystal surface, and the correspondingly small change in \(Z_s\).

It will be noted that in Fig. 6, there is a difference between the \(Z_s\) response for LDL and serum. This measured difference is perhaps due to the negative interference caused by the HDL in the serum sample.
as previously discussed, but also may in part be due to the activity of catalase, which will be present in serum. Catalase will compete with HRP for the hydrogen peroxide produced, but will not oxidise the DAB, thereby reducing the response. This problem may be overcome by the addition of sodium azide, though at high concentrations this may inhibit the activity of HRP [44,45]. The influence of other possible interferents may be overcome by constructing a calibration curve using serum with known cholesterol concentrations.

5. Conclusions

We have successfully applied our optimised hydrogen peroxide detection system using the quartz crystal acoustic wave sensor to the determination of both free and total cholesterol in biological samples. We found that the optimal concentration of Triton X-100 was 0.1% (v/v), for ChOx was 0.2 and lU/ml for ChE. Using the reagents at these concentrations, we found that there was a linear range for LDL cholesterol determination up to 400 μM. For clinical measurement of total cholesterol using this method, a dilution (1/20) of the serum would be required. The quartz crystal technique offers advantages over the more conventional electrochemical and optical biosensing strategies; by virtue of its mass/viscoelasticity sensing mode, it is not affected by electrochemical and optical interferents.

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