THE DEVELOPMENT OF ELECTROCHEMICAL ENZYME-BASED SENSORS FOR CHOLESTEROL

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ABSTRACT

Coronary vascular disease (CVD) is the number one cause of death worldwide. According to a WHO report and global and regional projections of mortality and burden of disease, by 2030, the number of people dying from heart disease and stroke will increase to reach 23.3 million. Non-HDL cholesterol, determined by subtracting the high density lipoprotein cholesterol (HDL-C) concentration from the total cholesterol (TC) content, has been recommended as a target for preliminary CVD prevention. In recent times, single-step homogeneous assays have been developed which allow simple and selective measurement of cholesterol fractions. Electrochemical sensors have also been developed which are based on the electrocatalysis of hydrogen peroxide using low cost printed sensor methodologies and such platforms would have the potential to be used as the basis of fabricating cholesterol biosensors for point of care use.

Here, the development of electrochemical biosensors for the selective measurement of HDL-C, TC, and by subtraction non-HDL-C was explored. A spectrophotometric assay for use at room temperature and with minimal sample dilution was first established in order to optimise the assay reagent components for the development of selective cholesterol assays. Assay chemistries based on polyoxyethylene tribenzylphenyl ethers (Emulgen B-66) and Triton X-100 for the selective measurement of HDL-C and TC, respectively, were developed. The impact of these reagents on the electrocatalytic reduction of hydrogen peroxide at silver paste screen printed electrodes was also evaluated and optimised.

Electrochemical biosensors for HDL-C and TC using externally added assay reagents were developed by combining the homogeneous assay methodologies with the printed electrocatalytic electrodes. The effects of assay reagents such as surfactants, enzymes, HDL-C sample and delipidated serum on the electrode behaviour were assessed amperometrically in the presence of hydrogen peroxide solutions. The electrodes showed increases in their catalytic activity toward hydrogen peroxide in the presence of both selective and non-selective surfactant and decreases in the presence of cholesterol oxidase and HDL-C samples. Despite the negative effects of cholesterol oxidase and sample matrix on electrode behaviour, the electrode response was linear within the clinically relevant ranges of HDL-C and TC. The modified electrodes were evaluated for their ability to

selectively measure HDL-C and TC in clinical serum samples. The resulting HDL-C biosensor yielded a sensitivity of 3.32×10^{-8} A/mM with a linear range of 0 to 4 mM (r^2=0.999), LOD of 0.5 mM and average RSD of 9.5% (n=5) while the TC biosensor had a sensitivity of 2.24×10^{-8} A/mM and a linear range of 0 to 10 mM r^2=0.984), LOD of 2 mM and average RSD of 10.8% (n=3). The correlation between the HDL-C sensor and a commercial laboratory assay in clinical serum samples had a slope of 0.87 and a Pearson correlation coefficient of 0.76 (n=13) while the correlation for TC measurement had a slope of 1.07 and a Pearson correlation coefficient of 0.87.

Finally, in order to develop a disposable biosensor suitable for point of care testing, integrated biosensors for HDL-C and TC were fabricated by inkjet-print deposition of assay reagents on the electrode surface. Integrated biosensors for the measurement of HDL-C were optimised and yielded a sensitivity of 4.55×10^{-8} A/mM with a linear range of 0 to 4 mM (r^2=0.993) with an LOD of 0.25 mM and average RSD of 6.6% (n=3). The integrated TC biosensor had a sensitivity of 9.38×10^{-9} A/mM and linear range of 0 to 9 mM (r^2=0.982), LOD of 0.5 mM and average RSD of 9.5% (n=3).

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To Hassan & Kian

for their unconditional love and support

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Glossary of abbreviations

MOPS	3-(N-morpholino)propanesulfonic acid
ACC	American College of Cardiology
AHA	American Heart Association
apo B-100	Apolipoprotein B-100
apo E	Apolipoprotein E
ASCVD	Atherosclerotic cardiovascular disease
CDC	Centre for disease control and preventation
ChEs	Cholesterol esterase
ChOx	Cholesterol oxidase
CRMLN	Cholesterol Reference Method Laboratory Network
CM	Chylomicron
CV	Coefficient of variation
CHD	Contrary heart disease
CAD	Coronary artery disease
DCM	Designated comparison method
DBSA	Dodecylbenzenesulphonic
EM	Electron microscopy
FC	Free cholesterol
HDL-C	High density lipoprotein cholesterol
HRP	Horseradish peroxidase
IDLs	Intermediate-density lipoproteins
L-B	L-B: Liebermann-Burchard
LCAT	Lecithin-cholesterol acyltransferase
LDL-C	Low density lipoprotein cholesterol
MEMs	Microelectromechanical Systems
NCEP	National Cholesterol Education Program
PBS	Phosphate buffered saline
PLs	Phospholipids
POCT	Point of care testing
PET	Poly ethylene terephthalate
PANI	Polyaniline
PSA	Polyester pressure sensitive adhesive

PEG	Polyethylene glycol
PET	polyethylene terephthalate
PVP	Polyvinylpyrrolidone
PBNPs	Prussian blue nanoparticles
RM	Reference Method
RSD	Relative standard deviation
RT	Room temperature
Ag SPEs	Silver screen printed electrodes
SD	Standard deviation
TC	Total cholesterol
TGs	Triglycerides
VLDL	Very low density lipoprotein

Chapter 1

Introduction

1.1 Cholesterol and vascular disease

Coronary artery disease (CAD) is the number one cause of death in all developed countries (Heron, 2012). In the 1980s, public concern over the risks of high blood cholesterol levels began to rise. Since then, several studies have demonstrated the increased possibility of cardiovascular diseases including arteriosclerosis in relation to high cholesterol levels. Atherosclerosis is a condition in which the arteries become blocked partly due to the accumulation of cholesterol. When cholesterol deposits on the walls of arteries, plaques form which may lead to blockages and interruption of the circulation, causing angina and myocardial infarction, with their associated morbidity and mortality. Some plaques can burst and release fat into the bloodstream, which can lead to a thromboembolism (Campbell *et al*., 2006). As a result, cholesterol has become one of the main parameters which are measured in routine clinical laboratory testing, accounting for an increase in demand for cholesterol testing technology in the last few years (Marazuela *et al.,* 1997). Treatment of blood cholesterol to reduce atherosclerotic cardiovascular risk in adults was emphasised and considered as the goal of American College of Cardiology (ACC) and the American Heart Association (AHA) in 2013 (Stone *et al.,* 2014). While the new guideline did not introduce any recommendation for or against specific LDL-C or non-HDL-C targets for the primary and secondary prevention of atherosclerotic cardiovascular disease (ASCVD), primary prevention of heart disease by lowering cholesterol levels using statins in individuals with diabetes, or without diabetes with LDL-C between 70 to 189 mg/dL and individuals over 21 years of age with LDL-C more than 190 mg/dL was recommended (Stone *et al.,* 2014).

Cholesterol (including esterified cholesterol), phospholipids (PLs), and triglycerides (TGs) are three major types of lipid present in the plasma. Cholesterol [(3β)-cholest-5-en-3-ol] is by far the most abundant member of a family of polycyclic compounds known as sterols. It occurs in the free form, esterified to long-chain fatty acids (cholesterol esters), and in other covalent and non-covalent linkages in animal tissues, including the plasma lipoproteins (Christie, 2012). The chemical structures of cholesterol and cholesterol ester are shown in Fig. 1.1.

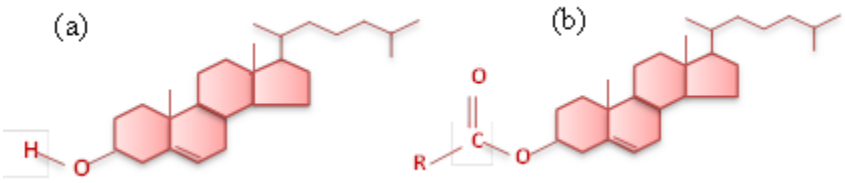

Fig. 1.1. Chemical structure of (a) cholesterol, and (b) cholesteryl ester.

Since lipids are not readily soluble in water, only a small amount is present as free cholesterol (FC). To allow adequate transport of cholesterol and other lipids, lipoproteins form a coat around the lipids in order to suspend them in the plasma. The function of the lipoprotein particle is to transport lipids such as cholesterol or TGs around the body via the blood stream. Based on the relative densities of these species, the four major categories of lipoprotein are chylomicron (CM), very low-density lipoprotein (VLDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) (Christie, 2012). Lipoproteins have unique physical and chemical characteristics, particularly with respect to the types and ratios of lipids and proteins present. These characteristics are used to achieve their separation and identification. Since lipoproteins vary in size and density, ultracentrifugation techniques have been used to separate them from each other (Table 1.1).

Table 1.1. Classification of lipoproteins.[a]

Class[b]	**Density** (kg/L)	**Diameter** (nm)	**Protein** (%)	**FC** (%)	**Cholesteryl ester** (%)
HDL	1.063-1.21	5–15	33	7	40
LDL	1.019–1.063	18–28	25	11	50
VLDL	0.95–1.006	30–80	10	7	18
CM	<0.95	100-1000	<2	2	3

[a]This table was adapted from (Christie, 2012; Garrett and Grisham, 1995; Rifai, Warnick and Dominiczak, 2001). [b] HDL - high density lipoprotein; LDL - low density lipoprotein; VLDL - very low density lipoprotein; CM - chylomicron.

LDL and HDL are the two major lipoproteins found in humans, and are responsible for carrying cholesterol in the blood. The lipid core of HDL and LDL contains cholesteryl esters and TGs surrounded by PLs and specialized proteins known as apolipoproteins (Fig. 1.2). The cholesteryl esters are enriched in linoleate, reflecting their biosynthetic origin. Apolipoproteins are a group of proteins which are specialized to facilitate several biochemical steps associated with plasma lipid metabolism. Lipoproteins have unique physical and chemical characteristics which relate to their relative amounts of lipids, protein/lipid ratios and specific protein

species present (Table 1.1). Since lipoproteins vary in size and density, centrifugation techniques have been used to separate them and distinguish them from each other. Apolipoproteins are amphipathic in nature which can interact with lipid moieties of lipoproteins and the aqueous environment and are specialized to facilitate several biochemical steps associated with plasma lipid metabolism (Segrest *et al.,* 2001). Plasma apolipoproteins can be classified as the non-exchangeable apolipoproteins (e.g., apo B-100) and the exchangeable apolipoproteins (eg. apo A-I, apo A-II).

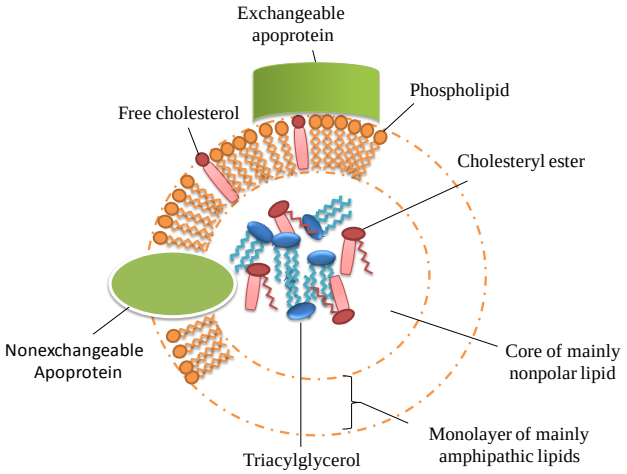

Fig. 1.2. General structure of lipoproteins. Adapted from Griffin (2009).

LDL particles with a density range between 1.019 and 1.063 kg/L carry 60–70% of the total serum cholesterol. LDL is the major atherogenic lipoprotein and has long been identified by the National Cholesterol Education Program (NCEP) as the primary target of cholesterol lowering therapy. The importance of reducing the risk of coronary heart disease (CHD) by lowering LDL-C has been shown by clinical trials (Adult Treatment Panel III (ATP III), 2002; Eleni, Bairaktari and Seferiadis, 2005). However, in recent years, both LDL-C and non-HDL-C (cholesterol contained in lipoproteins other than HDL) have been considered as a target for preliminary and secondary prevention of ASCVD (Stone *et al.,* 2014; Talwalkar *et al.,* 2013). Fig. 1.3 is a schematic of the LDL consensus model summarizing the proposed organization of lipids: Hydrophobic core lipid including cholesterol ester and TGs, hydrophilic shell of phospholipid and unesterified cholesterol (Segrest *et al.,* 2001).

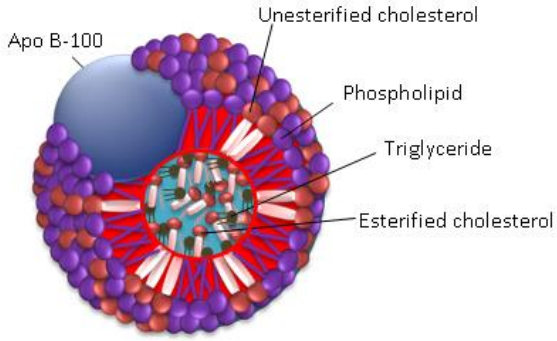

Fig. 1.3. LDL consensus model.

Intermediate-density lipoproteins (IDLs) have a hydrated density between LDL and VLDL of 1.006-1.019 and have a very similar structure and functionality to LDL. LDL, ILDL and VLDL with apolipoprotein B-100 (apo B-100) as their single apolipoprotein are classified as non-HDL-C.

HDL-C is known as the 'good' cholesterol, capable of removing cholesterol from atherosclerotic plaques and transporting it back to the liver for excretion or re-utilization. HDL is the smallest lipoprotein, which normally carries 20–30% of the total serum cholesterol known as HDL-C. Apo A-I and apo A-II are the two major apolipoproteins of HDL. They are both classified as exchangeable amphipathic apolipoproteins and are soluble in aqueous solutions. Apo A-I is the main protein component of HDL, which is synthesized within the liver (70%) and intestine (30%) (Christie, 2012). It has been shown that a 1 mg/dL increase in HDL-C concentration decreases the risk of CHD by 2-3% (Chapman *et al.,* 2004). However, recent studies have shown that while HDL-C is an important factor in cardiovascular risk, the relationship between HDL-C and ASCVD is not yet fully understood. HDL consists of two major subclasses known as HDL_2 and HDL_3. HDL_2 – with a hydrated density between 1.063 and 1.125 kg/L – is the larger particle and is enriched with apolipoprotein E (apo E), while HDL_3 – with a hydrated density between 1.125 and 1.210 kg/L – is the smaller particle and more important in predicting cardiovascular disease. The metabolic origin of HDL-C has been discussed by Rye *et al.* (2012). While some studies reported the importance of the measurement of HDL-C subfractions as a better option which reflect CHD risk compared to total HDL-C (Ashmaig *et al.,* 2013; Ito *et al.,* 2014; Okada *et al.,* 2001), this differentiation has not yet been reported for general clinical use. Although HDL-C levels are inversely correlated with the risk of CHD, the value of treating low HDL-C is not as well established as treating high LDL-C. Most treatment options for lowering high LDL-C

levels such as physical exercise, weight loss and even some of the cholesterol lowering drugs such as statins, have demonstrated a beneficial effect on HDL-C concentration (Adult Treatment Panel III (ATP III), 2002; Warnick, Nauck and Rifai, 2001). Levels of HDL-C above 60 mg/dL (1.55 mM) are considered to have a positive protective role in heart disease, while low HDL-C levels (less than 40 mg/dL or about 1 mM) are linked to an increase in heart attack risk. For this reason, the importance of measurement of HDL-C has been emphasized by NCEP since the late 1980s (Bethesda, 1995; Warnick, 1995).

The measurement of HDL-C is also important for two other purposes (Adult Treatment Panel III (ATP III), 2002; Contois, Warnick and Sniderman, 2011; Rifai, Warnick and Dominiczak, 2001):

- The calculation of (low density lipoprotein cholesterol) LDL-C using the Friedewald formula: LDL-C = Total cholesterol (TC) - (HDL-C + TG/5)
- The calculation of non-HDL cholesterol, determined by subtracting the HDL cholesterol concentration from the TC content. Non-HDL-C has been recommended as a target for preliminary ASCVD prevention.

1.2 Importance of standardisation of cholesterol measurement

Since low HDL-C and high LDL-C levels are linked to an increase in heart attack risk, the importance of accurate measurement of both HDL-C and LDL-C has been emphasized by NCEP (Bethesda, 1995; Caudill *et al.,* 1998; Warnick, 1995). NCEP is a program managed by the US National Heart, Lung and Blood Institute which established the laboratory standardisation panel on blood cholesterol measurement. (Rifai, Warnick and Dominiczak, 2001). The main reason for standardisation is to ensure the agreement of reported results across measurement systems, laboratories and over time (Warnick *et al.,* 2009). Table 1.2 shows the classification of serum TC, LDL-C and HDL-C as summarised in the third report of the expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (Adult Treatment Panel III, or ATP III) presenting the NCEP's updated recommendations for cholesterol testing and management (ATP III, Adult treatment panel III, 2001). 30 mg/dL above the LDL-C treatment target is considered as the treatment goal for non-HDL-C. Based on the clinical need to reliably categorise patients, NCEP established analytical performance goals for measurement of the TC, HDL-C and LDL-C (Table 1.3) (Bachorik, 1995; Warnick, 1995).

Table 1.2. ATP III Classification of TC, LDL-C and HDL-C.

Analyte	Concentration (mM)	Classification
TC	<5.2	Desirable
	5.2-6.2	Borderline high
	≥6.2	High
LDL-C	<2.6	Optimal
	2.6-3.3	Near or above optimal
	3.3-4.1	Borderline high
	4.1-4.9	High
	≥4.9	Very high
HDL-C	<1.0	Low
	≥1.6	High

Table 1.3. NCEP criteria for TC, HDL-C and LDL-C testing.

Analyte	Inaccuracy	Imprecision	Total error
TC	≤ ±3%RM	CV<3%	≤8.9%
HDL-C	≤ ±5%RM	SD≤1.7 at (<1.1 mM) CV≤4.0% at (≥1.1 mM)	≤13%
LDL-C	≤ ±4%RM	CV≤4%	≤12%

RM=reference value assigned by CDC reference measurement procedure
CV= coefficient of variation
SD= standard deviation

Reference methods for measuring HDLC and LDL-C are based on separation using ultracentrifugation which is not practical to be used in clinical laboratories (Adult Treatment Panel III (ATP III), 2002; Nauck, Warnick and Rifai, 2002; Rifai, Warnick and Dominiczak, 2001; Warnick, Nauck and Rifai, 2001). This is due to the fact that ultracentrifugation methods are very tedious, time consuming and very difficult to achieve complete and reproducible recovery.

The most common approach to determining LDL-C in the clinical laboratory is using the Friedewald calculation (Eleni, Bairaktari and Seferiadis, 2005; Friedewald, 1972). The principle of the Friedewald calculation is as follows:

1. TC is distributed among the three major lipoprotein classes (HDL, LDL and VLDL).
2. VLDL carries most of the circulating TGs and therefore VLDL-cholesterol (VLDL-C) can be estimated reasonably well from measured total TGs (TG/5 for mg/L or TG/2.2 for mM units).

3. LDL-C is then calculated as: LDL-C=TC-1/2.22 TG (mM).

This is the most commonly used method in the clinical laboratory and in large scale studies. Although the Friedewald method is widely used, the well-known limitation of this method (Bairaktari *et al.,* 2001; Tanno *et al.,* 2010; Tighe *et al.,* 2006; Timón-Zapata, 2011; Turkalp, 2005) increases the interest in improving the accuracy of LDL-C estimated by this equation (Rostami, 2011). The US Centres for Disease Control and Prevention (CDC) uses a reference method based on the Lipid Research Clinic's (LRC) beta-quantification procedure (BQ) for measuring LDL-C (Rifai, Warnick and Dominiczak, 2001; Tremblay *et al.,* 2004). In this method an aliquot of plasma is ultracentrifugated at a density 1.006 kg/L for at least 18 h at 105,000 g to accumulate the VLDL as a floating layer. The amount of LDL-C is then calculated by separating the HDL-C from the lower fraction after ultracentrifugation (Eleni, Bairaktari and Seferiadis, 2005; Nauck, Warnick and Rifai, 2002; Rifai, Warnick and Dominiczak, 2001).

Accuracy in HDL-C measurement has also been important for the calculation of LDL-C using the Friedewald formula and also non-HDL-C. As recommended by NCEP, the CDC method is the current secondary reference method for HDL-C measurement (Warnick *et al.,* 2009; Warnick, 1995). There are three key steps to this method:

1. Ultracentrifugation at a density of 1.006 kg/L to isolate HDL and LDL from other lipoproteins

2. Selective precipitation of LDL with heparin/$MnCl_2$

3. Analysis of cholesterol in the HDL (supernatant) using the Abell-Kendall assay. Abell-Kendall assay is used as a standard assay for TC measurement (Rifai, Warnick and Dominiczak, 2001).

Since there are only a few laboratories capable of performing the ultracentrifugation steps necessary in the CDC method, and due to the high volume (greater than 5.0 mL) of sample required, the Cholesterol Reference Method Laboratory Network (CRMLN) also developed the Designated Comparison Method (DCM) based on a modified dextran sulphate procedure (Kimberly *et al.,* 1999). This method uses 50 kDa dextran sulphate with $MnCl_2$ for the precipitation of non-HDL, followed by measurement of the cholesterol in the supernatant by the CDC reference method.

1.3 Evolution of methods for the measurement HDL-C and LDL-C

Other than reference methods based on ultracentrifugation, laboratory based tests using electrophoresis, chromatography and spectrophotometry have been developed. Due to the differences in the size and charge of various lipoproteins, isolation can be also achieved using electrophoretic techniques, with visualization achieved using lipophilic dyes (Warnick, Nauck and Rifai, 2001). However, due to the fact that the lipophilic dyes are not specific for a class of lipid such as cholesterol, TGs or PLs, these techniques cannot be used for quantitative analysis, but can be used for qualitative analysis of lipoproteins (Contois, 1999; Nauck, 1995; Rifai, Warnick and Dominiczak, 2001). Visual presentation is a distinct advantage of the electrophoretic methods, facilitating observation of atypical lipoproteins. For the routine clinical laboratory, both ultracentrifugation and electrophoresis have disadvantages especially when the workload is high.

A variety of HPLC methods have been used to separate lipoproteins such as HDL, LDL, VLDL and CM, but this has been impeded by the poor stability of the columns used for separation. Even improved HPLC techniques which separate serum lipoproteins based on their size using two connected columns with subsequent determination of cholesterol concentration using an online enzymatic reaction cannot be used in the routine clinical laboratory (Nauck, Warnick and Rifai, 2002; Okazaki, 1997; Okazaki *et al.,* 2008; Osman and Yap, 2006; Usui, 2000; Usui *et al.,* 2000; Usui *et al.,* 2000; Warnick, Nauck and Rifai, 2001).

Many spectrophotometric methods have been developed to measure HDL-C and LDL-C due to its widespread adoption and simple methodology. Initially, cholesterol was measured using non-enzymatic spectrophotometry in the form of the Liebermann-Burchard (L-B) and Killani-Zak assays (Burke, Diamondstone and Velapoldi, 1974). The L-B reaction is performed in an acetic acid-sulphuric acid-acetic anhydride medium based on the fact that cholesterol reacts with various strong acids of the Brønsted and Lewis types to yield coloured products. The Killani-Zak assay was based on direct treatment of the serum with a reagent composed of ferric chloride dissolved in a glacial acetic acid-sulphuric acid mixture (Zlatkis, Zak and Boyle, 1953). However, poor specificity, instability of the colorimetric reagent, and standardization difficulties were some of the disadvantages of this method (Richmond, 1973). The selectivity of spectrophotometric methods was improved significantly by using enzymes such as

cholesterol esterase (ChEs), cholesterol oxidase (ChOx) and horseradish peroxidise (HRP) (Allain *et al.,* 1974). The spectrophotometric measurement of esterified cholesterol requires three enzymatic reaction steps, as shown in Fig. 1.4, where 4AAP is 4-aminoantipyrine and Trinder's dye such as phenol is an enhancer. As can be seen, cholesterol esters released from lipoproteins (in the presence of non-specific surfactants such as Triton X-100) are hydrolysed to non-esterified cholesterol (FC) in the presence of ChEs. In the next step, the –OH group of non-esterified cholesterol produced in the presence of ChOx and oxygen is oxidized to a ketone (cholest-4-en-3-one) and H_2O_2. For a complete reaction with ChEs, each molecule of cholesterol ester present in the lipoprotein will yield one molecule of cholesterol. The cholesterol will then produce one molecule of H_2O_2 in the presence of cholesterol oxidase and one molecule O_2 their concentration being proportional to the concentration of cholesterol ester.

Measuring the amounts of O_2 consumed or the H_2O_2 produced are the preferred methods of quantifying cholesterol spectrophotometrically. Due to the consumption of oxygen by other substances in clinical samples such as ascorbic acid, any method measuring the amount of oxygen consumed in Fig. 1.4 is not accurate (Marazuela *et al.,* 1997). Therefore, measuring the amount of H_2O_2 produced was found to be a more accurate method of quantifying blood TC (Allain *et al.,* 1974; Salè *et al.,* 1984). In the presence of a reducing compound, HRP catalyses the reduction of H_2O_2 and produces a coloured product which can be measured spectrophotometrically. Trinder (1969) developed an assay strategy for H_2O_2 measurement employing HRP catalytic oxidation of phenol in the presence of 4-AAP to form a quinoneimine dye (Fig. 1.4). In the final step, a highly coloured quinoneimine is produced in the presence of 4-AAP, a Trinder's dye such as phenol and HRP, the optical density of which can be measured at 500 nm when phenol is used as the Trinder's dye. (Vojinovi´c *et al.,* 2004; Trinder, 1969). 4-AAP is accepted as a sensitive reagent for detection of phenols (Emerson, 1943). The concentration of cholesterol ester and cholesterol are proportional to the concentration of H_2O_2 formed.

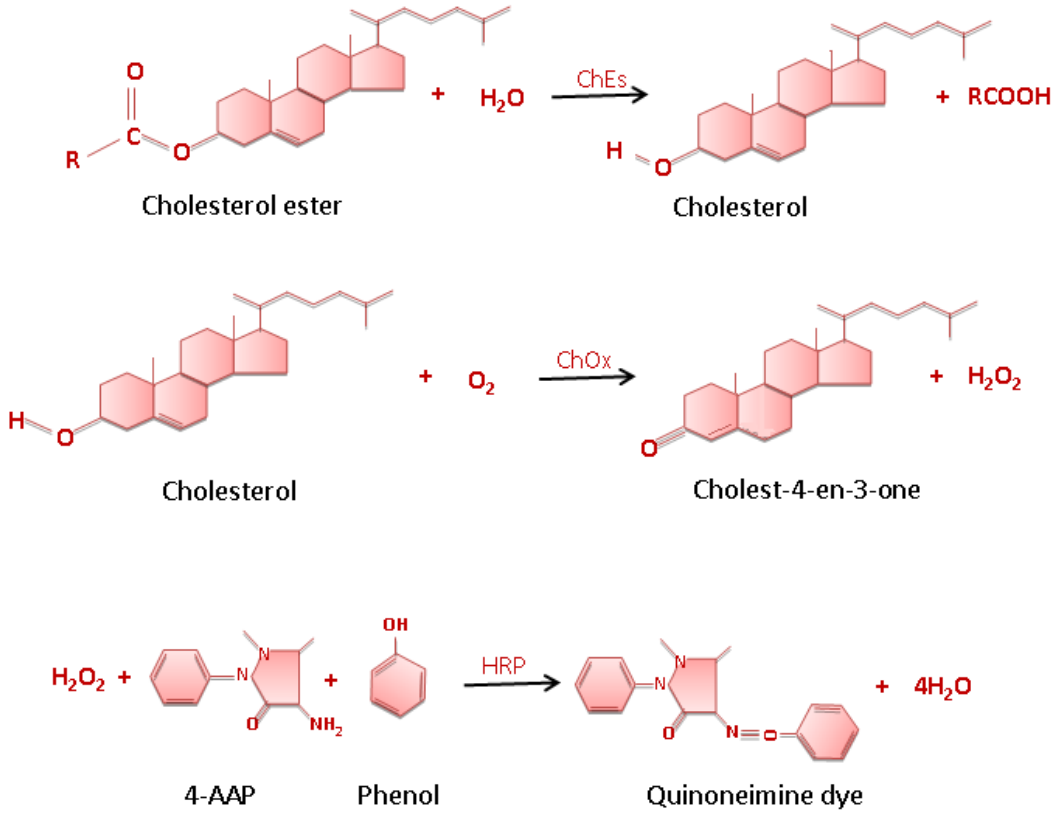

Fig. 1.4. Spectrophotometric measurement of cholesteryl ester and cholesterol using cholesterol esterase (ChEs) and cholesterol oxidase (ChOx). The generation of H_2O_2 is detected using horseradish peroxidase (HRP) in the presence of 4-aminoantipyrine (4-APP) and Trinder's dye (phenol), generating a quinoneimine dye, the optical density of which is measured at 500 nm (Allain *et al.*, 1974).

There are several assay methodologies for measuring cholesterol in specific lipoproteins. All of these are based on the colorimetric measurement of the H_2O_2 produced after the enzymatic reaction of cholesterol when measured spectrophotometrically following the formation of a dye complex. For the selective spectrophotometric measurement of HDL-C, two additional aspects of the assay need to be employed. Firstly, the enzymes must gain effective access to the cholesterol associated with the lipoprotein fraction. Secondly, the enzymes must also only gain access to the cholesterol from the specific lipoprotein fraction to be measured and be prevented from catalysing cholesterol present in other fractions. In the following sections, some of the approaches that have been used for measuring HDL-C and adapted by clinical laboratories will be reviewed.

1.3.1 Chemical precipitation methods for HDL-C measurement

In chemical precipitation methods, lipoproteins other than the target, e.g., HDL-C are aggregated and rendered insoluble using polyanions in combination with divalent cations which can then be sedimented by low-speed centrifugation, while HDL remains soluble (Rifai, Warnick and Dominiczak, 2001). The supernatant containing HDL-C can then be recovered for cholesterol analysis. The larger and lipid-rich lipoprotein such as VLDL and LDL, form insoluble complexes more readily than the smaller and protein-rich HDL. The insoluble complexes may either remain suspended in the solution or float to the surface in the presence of high concentrations of TG-rich lipoproteins. Heparin-Mn^{2+} has been a popular polyanion/divalent ion combination which has been used to assign target values to reference materials (Warnick, 1978). Due to some inconsistency in commercial heparin properties to be used routinely, other polyanion/divalent ion combination such as dextran sulphate-Mg^{2+} (50 kDa) (Warnick, Benderson and Albers, 1982), phosphotungstic acid-Mg^{2+} (Lopes-Virella, 1977) and polyethylene glycol (PEG)(Briggs, 1981) have been used as alternatives.

The actual mechanism of non-HDL-C precipitation in the presence of polyanions and divalent cations such as Mg^{2+} has not yet been fully elucidated. However, based on the fact that that all non-HDLs have one molecule of apo-B100 containing a cluster of positively charged residues (Fernández-Higuero *et al.,* 2014), these may interact with negatively charged groups on the polyanions such as dextran sulphate. Divalent metal cations such as Mg^{2+} may interact with the negatively charged polar phosphate headgroup of phospholipids on the lipoproteins which, in combination with polyanions can facilitate formation of insoluble complexes of non-HDL (Kim and Nishida, 1979; Warnick, Benderson and Albers, 1982).

1.3.2 Homogeneous methods

Homogeneous assays were a major step forward in improving the precision of earlier precipitation methods. Full automation eliminated manual pipetting, off-line pre-treatment, centrifugation and separation steps and improved assay precision, in line with recommended NCEP criteria. The development of such assays has been an area of intense commercial research and development dominated by several Japanese companies including Kyowa Medex, Seikisui Medical (formerly Daiichi Pure Chemicals Company), Deneka Seiken Co., Sysmex International Reagents (formerly International Reagents Corporation, IRC), Wako Chemicals, UMA and

Serotec (Miller *et al.,* 2010). However, the precise mechanisms involved in the interaction between the lipoproteins and the assay reagents used in these assays remain unclear (Kondo, 1999). In their review, Warnick *et al*. (2001) described some of these homogeneous assay methodologies for HDL-C in detail and compared them with conventional assay methods. The authors reported that all five methods demonstrated acceptable accuracy, precision and total error by meeting the NCEP criteria, making them suitable for clinical application. A brief description of the principles of each of the assays is given below.

IRC was the first to publish a report of a HDL-C fully automatable homogeneous assay based on an immunological separation method in 1996. Based on this method, CM, VLDL and LDL were first aggregated using a reagent containing PEG and then protected with antibodies to apo B and apo C. In the next step, unprotected HDL-C underwent enzymatic reaction as described in Fig. 1.4. In the final step, guanidine salts were used to stop the enzymatic reaction and clear the reaction mixture. The final absorbance was measured at 600 and 700 nm (Rifai, Warnick and Dominiczak, 2001; Warnick, Nauck and Rifai, 2001). In spite of the fact that this assay showed reasonable precision, accuracy and specificity, the addition of four different reagents limited its application to a small number of automated analyzers (Kakuyama, Kimura and Hasiguchi, 1994).

In 1995, Kyowa Medex reported a homogeneous assay for HDL-C which its selectivity was based on a combination of PEG-modified enzymes with α-cyclodextrin sulphate in the presence of a small amount of dextran sulphate with no need for precipitation of lipoprotein aggregates. PEG-modified ChEs and ChOx showed selective catalytic activity towards lipoprotein fractions. The reactivity increased in the order LDL < VLDL ≈ CM < HDL (Sugiuchi *et al.,* 1995). Although the mechanism for the selectivity of the modified enzymes towards the lipoprotein fractions is not clear, it is suggested that the modified enzymes may be able to recognize differences in hydrated density, net charge, or size of the various lipoprotein fractions. Size-exclusion chromatography revealed that PEG-modified ChEs breaks up the lipoprotein particles more effectively than the native enzyme, probably because of the amphiphilic properties of the attached PEG molecules (Sugiuchi *et al.,* 1995). Therefore, HDL should be more susceptible to the modified enzyme than non-HDL-C, explaining the observed differences in reactivities of cholesterol moieties of the lipoprotein fractions. H_2O_2 generated from the enzymatic reaction is then measured spectrophotometrically (Arranz-Peña, 1998; Sugiuchi *et*

al., 1995; Warnick, Nauck and Rifai, 2001). This method was evaluated in several studies including comparison with RM and DCM methods, which showed correlations of 0.993 and 0.996, respectively. The dynamic range reported for this method was 30 to 150 mg/L (Warnick, Nauck and Rifai, 2001).

Daiichi developed a homogeneous assay for HDL-C which employed a synthetic polymer together with a polyanion to block the non-HDL lipoproteins. Cholesterol in HDL was then exposed to the enzymes in the presence of a selective detergent which gives specificity for HDL-C. Kondo *et al.* (1999) visualized the formation of HDL-polymer complexes after the addition of polymer and polyanionin (the first reaction) using electron microscopy. This showed that this complex breaks down in the presence of a detergent in the second reaction. It also showed that the polyanion in reagent 1 (phosphotungstate) caused the aggregation of almost all lipoprotein. However, the exact roles of the polyanion and synthetic polymer remain unknown (Kondo, 1999). The detection limit of the method was 0.1–0.1 mM with linearity to at least 5.2 mM (Harris *et al.,* 1997; Kondo, 1999; Lin, 1998; Naucka, März and Wieland, 1998). Commercial reagent sets included two reagent additions; the first with the polyanion and polymer blocking agents and the second with detergent and enzymes. A disposable assay strip for measuring HDL-C was also reported by Daiichi group consisting of a reagent layer which formed on a support made of polyethylene terephthalate (PET). The reagent layer included the sample supply layer containing ChOx and ChEs and specific surfactants and the detecting layer was contained of colorimetric reagent. A polyoxyethylene alylene tribenzyl phenyl ether (Emulgen B-66) that increases the solubility of the HDL-C fraction over other lipoproteins is contained in the lower sample supply layer and a polyoxyethylene-polyoxypropylene condensation product (Pluronic F-88) that inhibits the non-HDL from dissolving was contained in upper sample supply layer. The correlation coefficient between the measured value obtained by the test piece and that obtained by direct Daiichi method and the aggregation method were 0.962 and 0.974, respectively (Tamura *et al.,* 2009).

Fig. 1.5 illustrates a generalised approach to the selective detection of HDL-C in the presence of other lipoproteins using the homogeneous principle based on a blocking reagent which selectively prevents access of a HDL-C selective surfactant to the non-HDL lipoproteins. Addition of the surfactant selectively solubilises the HDL, allowing access to ChEs and ChOx. The generated H_2O_2 can then be measured in the conventional manner as described in Fig. 1.4.

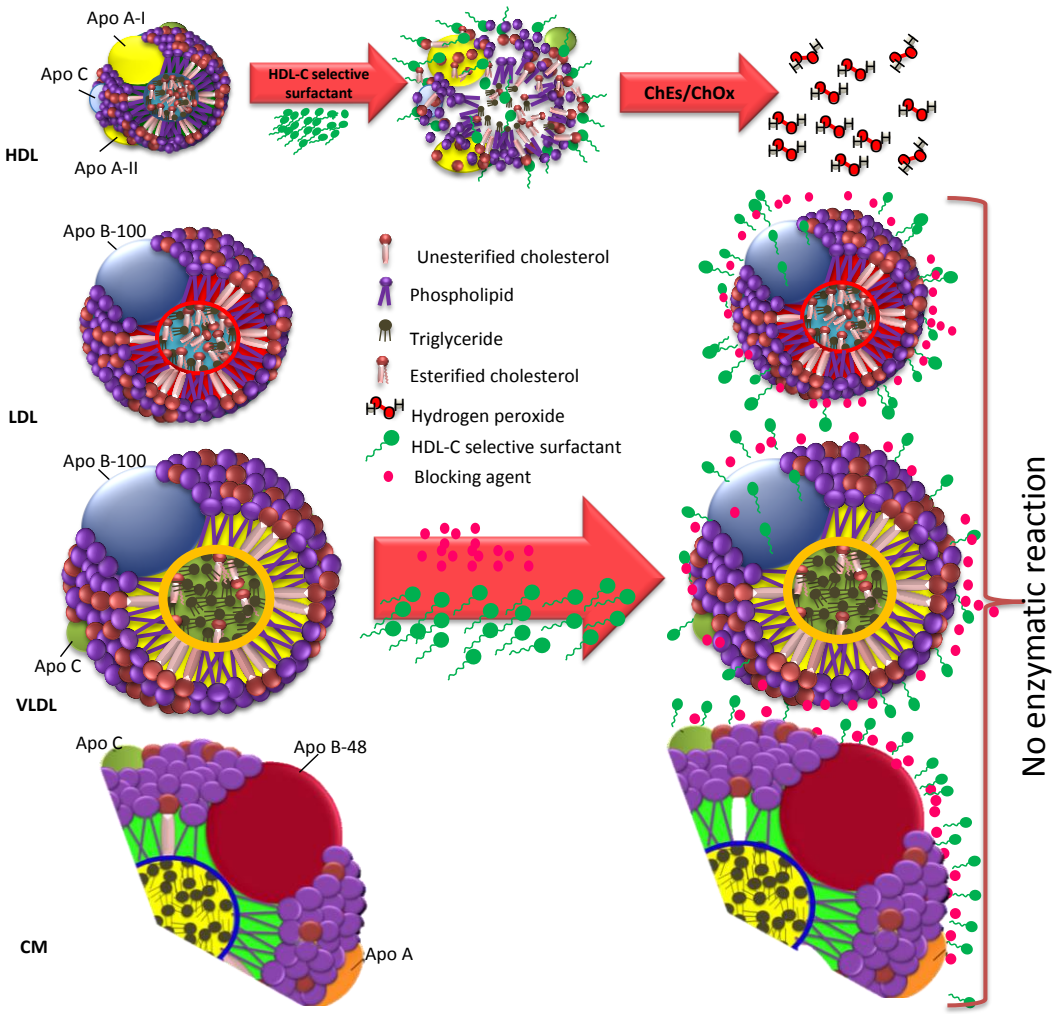

Fig. 1.5. Generalised homogeneous assay methodology for the selective determination of HDL-C. A blocking reagent selectively prevents access of a specific surfactant to the non-HDL lipoproteins. Addition of the surfactant selectively solubilises the HDL, allowing access to ChEs and ChOx. The generated H_2O_2 can then be measured in the conventional manner.

According to the homogeneous method for LDL-C, lipoproteins other than LDL such as VLDL, HDL and CM are removed in the first step using the first reagent described by each method. In the second stage, LDL-cholesterol undergoes an enzymatic reaction to produce H_2O_2which is measured colorimetrically (Bairaktari *et al.,* 2001; Nauck, Warnick and Rifai, 2002). Although the mechanism that confers selectivity to LDL-C from a specific surfactant is also not well understood, the same general mechanism is thought to apply as for HDL-C in that the surfactant may be able to distinguish differences in hydrated density, net charge, or size of the various lipoprotein fractions (Pembouong *et al.,* 2011; Sugiuchi, 1998). Several authors

have reviewed homogeneous assay methodologies for LDL-C measurement and compared them with conventional assay methods (Bayer *et al.,* 2005).

A comparison of homogeneous assay kits for the measurement of HDL-C clinical samples showed a maximum coefficient of variation (CV) of 14.5% for 990 heath check-up samples with almost all of the assay kits except Serotec partially reacting with apo E-rich HDL (Iizuka *et al.,* 2012). The CV of 26.9% was observed for LDL-C measurement using different LDL-C assay kit with IDL ratio and high serum bilirubin levels affecting the final value using all the kits. The study was performed by dividing the patients samples into 6 groups based according to their lipid profile and bilirubin levels. However a good correlation was observed between LDL-C measured using Sekisui assay kit and that calculated using Friedewald equation showing some differences for LDL-C value in the presence of different concentrations of TG (Iizuka *et al.,* 2012).

1.4 Enzyme kinetics

In an enzyme-catalyzed reaction, the enzyme binds to the substrate to form an enzyme-substrate complex transition-state species, which leads to the formation of a product. Michaelis and Menten introduced a useful model for the kinetics of enzyme-catalyzed reactions in 1913 based on a typical reaction of the conversion of a single substrate to a product. As can be seen in Fig. 1.6A, for the measurement of reaction rates, at different substrate concentrations, the initial rate of the reaction is measured. At lower substrate concentrations, the reaction is first order and the rate of the enzymatic reaction is dependent on substrate concentration. At higher substrate concentrations, the rate of the enzymatic reaction reaches a maximum value called V_{max}. At V_{max}, enzymes are completely saturated with substrate. The substrate concentration at one-half of its V_{max} is used as a measure of the enzyme's affinity for the substrate and is known as the Michaelis-Menten constant (K_M). A small K_m is indicative of a higher affinity. Enzymes with multiple substrates such as ChEs and ChOx have more complex kinetic properties and can also vary according to their source (Polleqioni, et al,. 1999; Srisawasdi, Prasertsincharoen and Kroll, 2012). However, these can often be approximated by Michaelis-Menten kinetics.

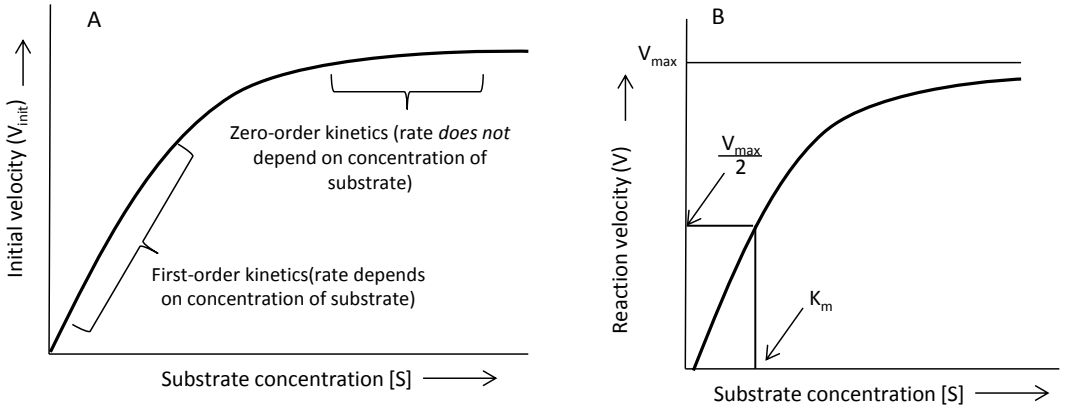

Fig. 1.6. Kinetics of enzyme-catalysed reactions with respect to substrate concentration, A: Relationship between initial reaction rate and substrate concentration. B: Relationship between reaction velocity and substrate concentration. (Adapted from Campbell and Farrell, 2006).

The assay in this study contains the enzymes ChEs and ChOx which are coupled together to produce H_2O_2. Assays using more than one enzyme show unusual features such as having a very long lag phase which may affect the next step of the enzymatic reaction. Depending on the activity of the enzymes (ChEs and ChOx) and the lag phase of the coupled enzymatic reaction, the rate of H_2O_2 production may vary but not exceed the rate of production of esterified cholesterol. Looking at the first and second steps, this shows that cholesterol ester in the presence of ChEs produces non-esterified cholesterol. Non-esterified cholesterol then, in the presence of the second enzyme ChOx produces H_2O_2. In this coupled assay, the concentration of non-esterified cholesterol depends on its rate of formation by ChEs and rate of removal by ChOx. If the concentration of cholesterol ester is greater than its K_M for ChEs, the first reaction may approach zero order. The concentration of cholesterol ester reduces overtime and the concentration of non-esterified cholesterol increases to reach a steady-state concentration which is high enough that the rate of ChOx becomes almost equal to the rate of ChEs. The rate at which non-esterified cholesterol reaches its steady-state concentration very much depends on the K_M for ChOx and the concentration of ChOx present. The time during which the non-esterified cholesterol is building up results in a lag phase in the enzymatic reaction. In this work, it took about a minute until final product forms, which is directly dependent on the time taken for the intermediate product – which is a substrate for the second reaction – to build up. This is the phenomenon of the lag phase which will affect the rate of the formation of the final product, H_2O_2. It is important to optimise the conditions in a way so as to minimise this lag phase. All

the absorbance measurements to quantify substrate concentrations should be performed during the steady state phase (Copeland, 2000, Tipton, 1992).

1.5 Non-HDL-C or LDL-C

The term 'non-HDL-C' is used for cholesterol associated with apo-B-containing lipoproteins which is reported as an alternative for CVD risk score. It has been found that for the majority of patients with TG less than 200 mg/dL it is better to employ a calculation method for LDL-C measurement rather than the homogeneous method (Deventer *et al.,* 2011; Liu *et al.,* 2006). It has also been shown that VLDL which is enriched with cholesterol could be the main source of the bias observed with many homogeneous LDL-C assay kits (Bayer *et al.,* 2005). A comparison between homogeneous LDL-C, calculated LDL-C and non-HDL-C as a coronary vascular disease (CVD) risk factor was performed by Deventer *et al.,* (2011) showing in general better correlation of non-HDL-C as CVD risk predictor for patients with TG between 200 and 400 mg/dL when compared to the CDC reference method, while homogeneous LDL-C showed a better correlation when compared to Friedewald calculation. Compared to the Friedewald calculation, the non-HDL-C result is free from errors related to VLDL estimation as well as errors related to LDL-C specificity (Deventer *et al.,* 2011). In addition, non-HDL-C is calculated from TC - which is normally very precise - and HDL-C measurement, introducing no extra expense and is also capable of assessing risk in patients with elevated TG. Only bias related to HDL-C measurement may impact the clinical accuracy of non-HDL-C (Contois, Warnick and Sniderman, 2011). Compared to LDL-C, non-HDL-C was shown to be a better risk factor for ischaemic stroke among the Chinese population (Wu *et al.,* 2013). Even among statin-treated patients, association between CVD and non-HDL-C was found to be greater than LDL-C and apoB (Boekholdt *et al.,* 2012). Non-HDL-C (apoB) or the TC to HDL-C ratio has also found to be stronger ASCVD risk predictors than LDL-C for men with type 2 diabetes (Pischon *et al.,* 2013). However, apoB was shown to be a better risk predictor for CHD in men than the cholesterol carried by lipoproteins containing apoB proteins (Pischon *et al.,* 2013).

1.6 Point of care testing for HDL-C and TC

In order to remove sample transport requirements, reduce processing and assay times and facilitate near patient testing, point of care devices that measure HDL-C

and LDL-C directly are very attractive in biomedical diagnostics. In general, point of care testing needs relatively small volumes of whole blood directly from a fingerstick and test results are available soon after sampling, which is highly advantageous in self-management of hypercholesterolaemia. There is no requirement for transportation of samples to a central laboratory which helps to reduce result turnaround time and transport costs. Earlier diagnosis and disease management as well as potential for improving patient satisfaction and cost-effectiveness are some other advantages offered by POCT (Gubala *et al.,* 2012; Lee *et al.,* 2011).

A number of both professional use and consumer point of care devices for measurement of cholesterol are commercially available. The CardioCheck PA and Cholestech LDX are two systems that support the UK NHS health check vascular risk assessment. Cardiocheck PA is based on a spectrophotometric method and the measurement of the light reflected off a disposable test strip that has changed colour after applying blood sample. The analyzer converts this reading into an HDL result and displays it (Méndez-González, Bonet-Marqués and Ordóñez-Llanos, 2010; Panz *et al.,* 2005). The Cholestech LDX system combines the enzymatic methodology with 'solid-phase' technology to measure the quantity of TC, HDL-C, TGs, glucose, and others in the blood (capillary or venous), serum or plasma. The sample is applied to a Cholestech LDX cassette. The cassette is then placed into the Cholestech LDX analyzer that can measure the resultant colour by reflectance photometry (The Medical Services Advisory Committee (MSAC), 2001). The LDX uses the Friedewald equation to calculate LDL-C while the Cardiocheck PA measures LDL-C directly. A comparison between Cholestech LDX POC and hospital reference laboratory validates the use of the Cholestech LDX analyser for point of care lipid measurements in clinical practice under the use of a well-trained operator (Carey *et al.,* 2006). A comparison between the performance of these two point-of-care analyzers and clinical diagnostic laboratory methods for the measurement of TC, HDL-C and LDL-C has been reported (Dale, Jensen and Krantz, 2008; Panz *et al.,* 2005). Both of them were found to have acceptable performance, which offers healthcare professionals a rapid POC method for the measurement of cholesterol in specific lipoproteins. Moreover, determination of the accuracy and precision of TC, TG and HDL cholesterol measures by a nurse on capillary blood using the Cardiochek system suggested that this approach was appropriate for predicting CHD risk and provided reliable fractionated lipid

information which was consistent with traditional clinical chemistry platforms (Patel *et al.,* 2011).

1.7 Biosensors

The evolution of point of care tests from central laboratory testing towards low cost, consumer diagnostics based on biosensors has been exemplified by the development of glucose biosensors (Turner, 2013). The progression from optical to electrochemical measurement methods is a natural evolution for many diagnostic devices as they progress from laboratory tests typically based on spectrophotometry, to electrochemical devices which allow lower cost instrumentation and system simplification. Cholesterol testing is also going through this evolution (Belluzo, Ribone and Lagier, 2008; Kimmel *et al.,* 2012). Biosensors not only could revolutionise point-of-care testing but have also been successfully used in the food industry, environmental monitoring and pharmaceutical industries through their ability to convert complex bioanalytical measurements into simple and easy to perform techniques.

A biosensor is an integrated analytical device consisting of a bio-receptor which is a biomolecule capable of recognizing the target analyte and a transducer which converts a biological response into a measurable signal enabling the measurement of target analyte without using any reagent. Therefore, the selection of biomolecules, immobilisation matrix, immobilisation method and transducer are important steps in the fabrication of a biosensor. For electrochemical detection, electrodes such as platinum, gold, carbon based materials such as graphite; carbon black and carbon fiber are suitable (Arya, Datta and Malhotra, 2008). For the purpose of this work, ChOx and ChEs are normally employed as the bio-receptors capable of recognizing esterified and non-esterified cholesterols.

There is a wide variety of deposition and immobilisation techniques as well as transducers that have been applied to the development of cholesterol biosensors (Arya, *et al.,* 2008). The following section is a general review of the techniques most relevant to the current work.

1.7.1 Immobilisation techniques

There is no general method available which is capable of being applied to the immobilisation of all enzymes. However, the most commonly used immobilisation

techniques could be classified into five categories (Hanefeld *et al.,* 2008*)*: non-covalent adsorption and deposition, immobilisation using ionic interactions, covalent attachment, cross-linking of an enzymes and finally entrapment in a polymeric gel or capsule. In general, enzymes can be attached to a support using reversible techniques such as physical adsorption and ionic linkages or using irreversible techniques such as covalent bonds (Brena and Batista-Viera, 2006). Covalent bonding is normally strong and formed between functional groups present on the enzymes surface and reactive groups present on the solid support, while physical bonds such as hydrophobic interactions, van der Waals binding or ionic interactions are generally not very strong and enzymes may leach readily from the carrier (Cantone *et al.,* 2013).

Although covalent binding of enzymes to the carrier has the advantage of the enzyme being attached to the carrier tightly, non-covalent binding was chosen as the method employed in this work. Depending on the deposition technique used, an aqueous solution of enzyme is deposited on a solid support, followed by evaporation of the aqueous phase. This typically requires an optimised enzyme formulation to improve the rate of re-solubilisation as well as enzyme activity following deposition. Immobilisation via ionic interactions is based on the fact that enzymes may possess a net positive or negative charge depending on their isoelectric point and the pH of the solution. For the purpose of ionic interaction, based on the predominant charge on the enzyme, an oppositely charged ion exchanger may be introduced.

The properties of enzymes such as size, conformational flexibility, isoelectric point, surface functional groups/charge density, stability under immobilisation conditions and hydrophilic or hydrophobic regions are important factors when choosing an immobilisation technique. Factors relating to the reaction system itself such as diffusion limitations, enzyme inhibition, viscosity of the mixture, etc., are also important parameters which should be taken into account when considering an immobilisation technique.

Despite the many enzyme immobilisation techniques described in the patent and research literature, only a few techniques have been found to be suitable for mass production and commercialisation (DiCosimo *et al.,* 2013).

Printing technologies such as screen printing and inkjet printing have been found to be amenable towards industrial scale-up of biosensors. Screen printing was first

used in the 1980's for the production of enzyme electrodes and currently half of the disposable glucose electrodes are screen printed using curable polymer inks (Turner, 2013). While screen printing is capable of forming a biosensor with thick films, inkjet printing is capable of using very small quantities of enzyme reagents required for the fabrication of disposable biosensors with minimum waste and high reproducibility which lends itself well attractive in mass production of disposable biosensors (Delaney, Smith and Schubert, 2009). Drop-on-demand which is based on producing droplets when required, and continuous inkjet in which droplets are formed from a continuous stream to the substrate when needed, are two types of inkjet printing techniques. Droplets can be generated using piezoelectric, thermal and electrostatic techniques (Hutchings and Martin, 2013). Among them, drop-on-demand techniques capable of deposition of pico-litre to nano-litre size reagent containing proteins precisely to a designated sensor area with minimum waste were found to be more suitable for fabrication of disposable biosensors and point of care diagnostic products (Delaney, Smith and Schubert, 2009).

1.7.2 Transducer

The final essential component of the biosensor configuration is the transducer which is the component of the biosensor capable of converting the biochemical response into a measurable electrical signal. Electrochemical and optical are the two major modes of transduction which have been mostly applied to cholesterol biosensor fabrication. Electrochemical sensors are devices that provide information about a sample from the measurement of some electrochemical parameter such as potential, current or resistance (Grieshaber, MacKenzie and Reimhul, 2008; Kimmel *et al.,* 2012; Wang *et al.,* 2008). Therefore, different groups of electrochemical sensors such as potentiometric, voltammetric, conductometric etc. have been developed. Among these, voltammetric sensors are arguably the most common, especially in research laboratories. Voltammetric sensors are based on the measurement of current response as a function of applied potential. A special case of voltammetric sensor is the amperometric sensor where a fixed potential is applied and the current response is measured. The current response is directly proportional to the number of electrons transferred, and therefore, to the concentration of the electroactive species (Brett and Brett, 1998):

$$O + ne^- \leftrightarrows R \quad (1)$$

where O (oxidised spices) receives *n* electrons in order to transform into R (reduced species). When certain chemical species are oxidized or reduced (redox reactions) at inert metal electrodes, electrons are transferred between the electroactive species and the working electrode. The direction is based on the properties of the electroactive species and can be controlled by applying a constant potential to the working electrode (Brett and Brett, 1998; Kissinger and Heineman, 1996). The relationship between the moles, *N*, of electroactive species reacted (either oxidised or reduced) and charge passed through the sensor, Q, is known as Faraday's law:

$$N = Q/nF \quad (2)$$

where *F* is the Faraday constant. The application of a defined potential and measurement of the transfer of current is normally measured in an electrochemical cell connected to a potentiostat. Electrochemical cells composed of either two or three electrodes are necessary. These consist of a reference electrode, a working electrode and a counter electrode or combined reference and counter electrode. The working electrode is typically constructed from an inert conductor such as a precious metal (Pt, Au) or carbon. A reference electrode such as Ag/AgCl, provides a fixed potential against which the potential applied to the working electrode is measured and controlled. A third electrode, the counter (or auxiliary) electrode is employed to allow current to flow between the working and auxiliary electrodes while the potential is measured between the working and reference electrodes (Brett and Brett, 1998; Wang *et al.,* 2008).

Cyclic voltammetry (CV) and chronoamperometry are two common electroanalytical techniques used to measure electron transfer processes. CV is useful to obtain information about the redox potential and electrochemical kinetics of these processes. Fig. 1.7 (left) illustrates a potential waveform (plot of potential vs. time) and Fig. 1.7 (right) depicts a typical CV (plot of the current vs. potential) for a fully reversible one electron transfer reaction.

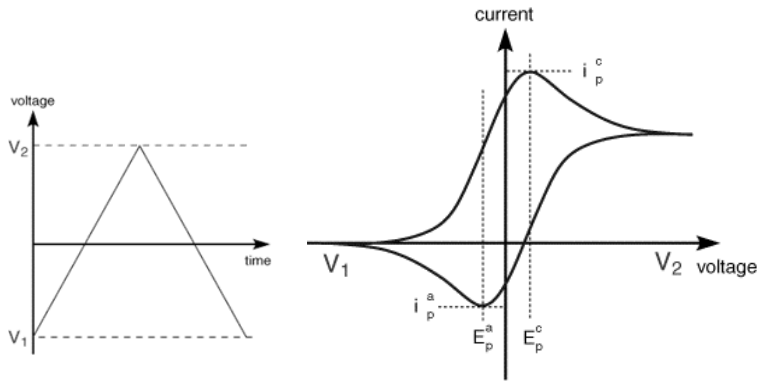

Fig. 1.7: Potential waveform (left) and Cyclic voltammogram (right) for a reversible one electron transfer process.

As can be seen, the voltage is swept between two values (V_1 to V_2) at a fixed rate. When the voltage reaches V_2 the scan is reversed and the voltage is swept back to V_1. According to Fig. 1.7 (right), by scanning the potential negatively, the reduction occurs from V_1 (the initial potential) to V_2 which is the point the potential switches. The resulting current is called the cathodic current (i_c). The peak of which ($I_{p,}^{c}$) occurs at the peak cathodic potential, E_p^c. Initially, only non-Faradic currents flow for potentials $<V_2$, until the potential approaches E_p^c. As E_p^c is approached, the surface concentration of substrate decreases, while the flux to the surface and therefore the current increases. When the potential passes E_p^c, the surface concentration becomes zero and mass transfer of substrate to the surface reaches its maximum rate. Current then decreases due to mass transport limitation (Bard and Faulkner, 2001). Theoretical analysis of the CV leads to the Randles-Sevcik equation for the peak current in linear sweep voltammetry:

$$i_p = 2.69 \times 10^5 n^{3/2} A D^{1/2} R v^{1/2} \quad (3)$$

where the i_p is peak current (in *A*), n is the number of electrons, *A* is the area of the electrode (in cm^2), *R* is the concentration (in mM), *D* is the diffusion coefficient (in cm^2s^{-1}) and v is the scan rate (in Vs^{-1}). The peak separation between the two potentials for a reversible system and the number of electrons involved in the reaction is governed by the Nernst equation:

$$\Delta E_p = E_{pa} - E_{pc} = \frac{59}{n} mV \quad (4)$$

where E_{pa} and E_{pc} are the maximum anodic and cathodic potentials, respectively and *n* is the number of electrons transferred. According to the Nernst equation, a

fast one electron process has ΔE_p of 59 mV and the peak potentials are independent of the scan rate.

As with glucose, cholesterol lends itself effectively to electrochemical measurement as many of the transduction principles employed are transferrable, such as the use of electron transfer mediators or H_2O_2 to measure the oxidation of cholesterol (Aravind *et al.,* 2011; Dey and Raj, 2014; Fang, 2011; Fang, 2011; Huang, Juan and Guilabult, 1977; I. Karube, Hara and Mastsuoka, 1982; Kumar, Pandey and Brantley, 2006; Manjunatha *et al.,* 2012; Pundir, 2008; Ruecha *et al.,* 2014; Shih, Yang and Lin, 2009; Shin and Kameoka, 2012; Shin and Kameoka, 2012; Yang, Shen-Ming Chen and Lin, 2011; Yang, Shen-Ming Chen and Lin, 2011; Özer *et al.,* 2007). However, despite the growing importance of the selective determination of HDL-C for monitoring and managing hypercholesterolaemia, coupled with the suitability of electrochemical assay methodologies to translate well to cholesterol testing, there are only a few reports of the measurement of H_2O_2 generated from the enzymatic reaction of HDL-C using electrochemical methods (Kinoshita *et al.,* 1998; Murphy *et al.,* 2009).

1.8 Electrochemical determination of H_2O_2

H_2O_2 has been widely studied and is readily amenable to measurement using electrochemical methods. As already discussed, there are several optical assay methodologies for measuring cholesterol, and more specifically HDL-C. All of these methods determine the amount of H_2O_2 produced after the enzymatic reaction of cholesterol when measured spectrophotometrically following the formation of a dye complex. Given that electrochemical techniques lend themselves very well to the fabrication of low cost point of care and disposable diagnostic devices, it would seem a common sense approach to develop electrochemical biosensor methodologies that are capable of measuring cholesterol using a similar principle, with the measurement of H_2O_2 performed electrochemically. Most of the H_2O_2 sensing used as a platform for development of electrochemical biosensors for total cholesterol were fabricated by ChEs, ChOx and HRP immobilized onto various membrane or matrix containing a redox mediator such as biosensors based on peroxidase oxidation of potassium ferrocyanide by H_2O_2 (Foster, Cassidy and O'Donoghue, 2000), HRP-hydroxymethyl ferrocene (HMF)-carbon paste electrode (hydroxymethyl ferrocene as mediator) (Charpentier and El Murr, 1995), Chox, ChEs, HRP and potassium ferrocyanide immobilized on the screen-printed carbon

electrodes (Li *et al.,* 2005), Chox, ChEs, HRP co-immobilised on 3-aminopropyl-modified controlled-pore glass (APCEG)/rotating disk using a soluble-redox mediator of 4-tert-butylcatechol (Salinas *et al.,* 2006). Fang, *et al.,* (2011) reported a disposable screen printed TC biosensor using ink formula containing ChEs and cholesterol dehydrogenase as enzymes, a nicotinamide adenine dinucleotide (NAD) as the coenzyme, and a 1,10-phenanthroline-5,6-dione (PD) as the electron mediator. However, some enzyme-less H_2O_2 sensors capable of direct oxidation or reduction of H_2O_2 were fabricated which were employed as a platform for cholesterol measurement. Multiwall carbon nanotubes (MWCNTs) (Yang, Shen-Ming Chen and Lin, 2011), reduced graphene oxide–dendritic Pd nanoparticle (rGO–nPd) hybrid material (Dey and Raj, 2014), a gold (Au) electrode modified with MWCNTs uniformly dispersed in nafion matrix (Urmila *et al.,* 2011), graphene, polyvinylpyrrolidone (PVP) and polyaniline (PANI)-modified electrodes with a 3-fold increase in the current signal compared to an unmodified electrode exhibiting electrocatalytic activity towards the oxidation of H_2O_2 (Ruecha *et al.,* 2014) are some of the examples of the non-enzymatic electrochemical determination of H_2O_2. Due to the biocompatibility of nanoparticles, their ability to provide a large surface area and their excellent conductivities, nanoparticles were employed to improve the sensitivity of H_2O_2 sensors (Lian *et al.,* 2009). Lian, *et al.,* (2009) reported a silver electrode which was roughened by electrochemical oxidation-reduction cycle in a KCl solution with an excellent electrocatalytic activity for the reduction of H_2O_2. Later on, in 2011, a fully printed electrocatalyst for H_2O_2 reduction using a screen-printed silver electrode as a platform was introduced by Killard and coworkers (2012). Screen printed silver paste electrodes dip-coated in solution containing 3.3×10^{-2} M dodecylbenzenesulfonic acid (DBSA) in 0.1 M KCl (DBSA/KCl) solution showed an 80-fold enhancement in electrocatalytic reduction of H_2O_2 at -0.1V vs Ag/AgCl compared to un-modified silver electrodes. This increase in catalysis was reported to be due to the fact that surfactant at lower concentration may form a spheroidal micelles aggregate in aqueous solutions which can convert to lamellar structures on the surface of the electrode. The presence of chloride salt such as NaCl was also found to be important in catalysis as the modification was significant above certain concentrations of salt. This aggregation may have occurred due to hydrophobic interactions between the long hydrocarbon chains which is not a favour interaction with water and the hydrophilic head group with a favour interaction with water (Gonzalez-Macia, Smyth and Killard, 2011). The electrocatalytic layer was also inkjet printed (Gonzalez-Macia, Smyth and Killard,

2012b) and used as a platform for glucose sensing (Gonzalez-Macia, Smyth and Killard, 2012a). The following is a possible electrocatalytic mechanism for H_2O_2 reduction at a silver electrode as reported by Honda *et al.,* (1983 & 1986) and adapted by Welch *et al* (2005):

$$H_2O_2 + e^- \rightleftharpoons OH_{(ads)} + OH^- \quad (5)$$

$$OH_{(ads)} + e^- \rightleftharpoons OH^- \quad (6)$$

$$2OH^- + 2H^+ \rightleftharpoons 2H_2O \quad (7)$$

Since Eq. (5) is the rate-determining step, it is believed that lamellar structures formed in the presence of the surfactant/salt may stabilize OH_{ads} which favours this reaction.

1.9 Conclusion

While near patient testing and point of care testing are becoming very important, a major challenge continues to be the development of sensitive and accurate methods capable of performing rapid analysis by untrained users. Biosensors based on electrochemical techniques are ideally suited to this due to their high sensitivity and selectivity, rapid response time and low-cost (Grieshaber, MacKenzie and Reimhul, 2008; Kimmel *et al.,* 2012; Wang *et al.,* 2008).

These days, the movement of many routine blood tests away from the central laboratory, to the point-of-care is a major trend in healthcare provision. This is also the case for cholesterol. Moving away from free cholesterol testing and focusing on measuring HDL-C, non-HDL-C and HDL-C/TC ratios as major risk factors for heart disease has been recommended by the NCEP. Homogeneous methods which can be fully automated are found to be a good answer to the need of clinical laboratories to cope with increasing workload. Using biosensors and point of care devices could be even more effective for the monitoring patient health. Given the effective development of homogeneous methodologies based on spectrophotometric measurement, it seems inevitable that such methodologies will soon migrate onto electrochemical biosensor platforms suitable for consumer use. Electrochemical sensors based on the electrocatalysis of H_2O_2 are particularly suited to the development of cholesterol biosensors.

Screen printing and, more recently inkjet-printing have been applied to the fabrication of sensors and biosensors. Combining the benefits of both techniques for the fabrication of distinct components of the biosensor which are most suited to the particular printing method may provide a viable means of low cost, high throughput mass production of such sensors.

1.10 Aims and objectives

The purpose of this research was to develop low cost electrochemical biosensors for the direct, selective measurement of HDL-C and TC using screen printed silver electrodes with enhanced electrocatalytic reduction of H_2O_2 at a silver paste electrode. This was achieved through the following objectives:

- ***Development of single step assay methodologies for the selective measurement of HDL-C and TC using the homogeneous enzyme assay principle (Chapter 3):*** A room temperature spectrophotometric assay with minimum sample dilution factor based on an assay methodology using a HDL-C or TC selective surfactants designed to assist the development and optimization of the assay properties required for the development of the HDL-C and TC biosensors (e.g., buffers, surfactants, enzymes, concentrations and incubation times)

- ***Adaptation of the assay reagent formulation to the screen printed silver paste H_2O_2 electrocatalyst (Chapter 4):*** Investigation of the effects of assay reagents such as buffer, surfactant, samples, delipidated serum and enzymes on electrochemical behaviour of H_2O_2 at DBSA/KCl modified SPEs and establishment of an optimised HDL-C assay formulation to measure H_2O_2 in delipidated serum.

- ***Evaluate the ability of the sensors to measure cholesterol in clinical samples (Chapter 5):*** Development of HDL-C and TC biosensors by employing external addition of assay reagents using the DBSA/KCl modified electrodes and also exploring the potential for using surfactant/salt combinations used in HDL-C and TC assays.

- ***Integrate the assay reagent formulation into the sensor using inkjet printing (Chapter 6):*** Formulation and analytical characterisation of an assay reagent ink suitable for inkjet-printing designed to improve sensor stability, limit of detection, analytical range and reproducibility of the assay.

References

Adult Treatment Panel III (ATP III) (2002) Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. 2012 (2/23/2012).

Allain, C.C., Poon, L.S., Chan, C.S.G., Richmond, W. and Fu, P.C. (1974) Enzymatic determination of serum total cholesterol. *Clinical Chemistry*. 20 (4), pp. 470-475.

Aravind, S.S.J., Baby, T.T., Arockiadoss, T., Rakhi, R. and Ramaprabhu, S. (2011) A cholesterol biosensor based on gold nanoparticles decorated functionalized graphene nanoplatelets. *Thin Solid Films*. 519 (16), pp. 5667-5672.

Ariga, K., Ji, Q., Mori, T., Naito, M., Yamauchi, Y., Abe, H. and Hill, J.P. (2013) Enzyme nanoarchitectonics: organisation and device application. *Chemical Society Review*. 42, pp. 6322-6345.

Arranz-Peña, M.L. (1998) Comparison of two homogeneous assays with a precipitation method and an ultracentrifugation method for the measurement of HDL-cholesterol. *Clinical Chemistry*. 44 (12), pp. 2499.

Arya, S.K., Datta, M. and Malhotra, B.D. (2008) Recent advances in cholesterol biosensor. *Biosensors and Bioelectronics*. 23 (7), pp.1083-1100.

Ashmaig, M.E., Gupta, S., McConnell, J.P. and Warnick, G.R. (2013) Validation of a novel homogeneous assay for of HDL3-C measurement. *Clinica Chimica Acta*. 425 (0), pp .37-41.

ATP III, Adult treatment panel III (2001) Executive summary of the third report of the national cholesterol education program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adult (Adult treatemnt panel III). *The Journal of the American Medical Association*. 285, pp. 2486-2497.

Bachorik, P.S. (1995) National Cholesterol Education Program recommendations for measurement of low-density lipoprotein cholesterol: executive summary. The National Cholesterol Education Program Working Group on Lipoprotein Measurement. *Clinical Chemistry*. 41 (10), PP. 1414-20.

Bairaktari, E., Elisaf, M., Tzallas, C., Karabina, S.A., Tselepis, A.D., Siamopoulos, K.C. and Tsolas, O. (2001) Evaluation of five methods for determining low-density lipoprotein cholesterol (LDL-C) in hemodialysis patients. *Clinical Biochemistry*. 34 (8), PP. 593-602.

Bard, A.J., Faulkner, L.R. (2001) *Electrochemical methods: Fundamentals and Applications.* John Wiley & Sons, inc. 2nd Edition.

Batki, A.D., Nayyar, P. and Thomason, H.L., (2009) *Buyers' Guide: Point-of-Care Testing for Cholesterol Measurement*. Report number: CE09020.University of Birmangham: The centre for evidence-based purchasing (CEP).

Bayer, P., Veinberg, F., Couderc, R., Cherfils, C., Cambillau, M., Cosson, C., Fradin, S., Gillery, P., Steinmetz, J., Legrand, A., Egloff, M. and Beucler, I. (2005)

Multicenter evaluation of four homogenous LDL-cholesterol assays. *Ann Biol Clin*. 63 (1), PP. 27-41.

Belluzo, M.S., Ribone, M.E. and Lagier, C.M. (2008) Assembling amperometric biosensors for clinical diagnostics. *Sensors*. 8, pp. 1366-1399.

Bethesda, M.D. (1995) National Cholesterol Education Program. Working Group on Lipoprotein Measurement. Recommendations on lipoprotein measurement. *NIH Publication no. 95-3044; National Heart, Lung, and Blood Institute*. 41, pp. 1414-1420.

Boekholdt, S.M., Arsenault, B.J., Mora, S., Pedersen, T.R., LaRosa, J.C., Nestel, P.J., Simes, R.J., Durrington, P., Hitman, G.A., Welch, K.M., DeMicco, D.A., Zwinderman, A.H., Clearfield, M.B., Downs, J.R., Tonkin, A.M., Colhoun, H.M., Gotto, J.r., Ridker, P.M. and Kastelein, J.J. (2012) Association of LDL cholesterol, non-HDL cholesterol, and apolipoprotein B levels with risk of cardiovascular events among patients treated with statins: a meta-analysis. *JAMA*. 307, pp. 1302-1309.

Brena, B.M. and Batista-Viera, F. (2006) Immobilization of enzymes. In: Guisan, J.M., ed. (2006) *Immobilization of Enzymes and Cells*. Springer, pp. 15-30.

Brett, C.M.A. and Brett, A.M.O. (1998) *Electroanalysis*. Oxford Science Publication.

Briggs, C.J. (1981) Evaluation of the polyethylene glycol precipitation method for the estimation of high-density lipoprotein cholesterol. *Annals of Clinical Biochemistry*. 18, pp. 177-81.

Burke, R.W., Diamondstone, B.I. and Velapoldi, R.A. (1974) Mechanisms of the Libermann-Burchard and Zak color reactions for cholesterol. *Clinical Chemistry*. 20 (7), pp. 794-801.

Campbell, M.C., Farrell, S.O. (2006) *Biochemistry*, Fifth Ed. Thamson Brook Cole.

Cantone, C., Ferrario, V., Corici, L., Ebert, C., Fattor, D., Spizzo, P. and Gardossi, L. (2013) Efficient immobilisation of industrial biocatalysts:criteria and constraints for the selection of organic polymeric carriers and immobilisation methods. *Chemical Society Review*. 42, pp. 6262-6276.

Carey, M., Markham, C., Gaffney, P., Boran, G. and Maher, V. (2006) Validation of a point of care lipid analyser using a hospital based reference laboratory. *Irish Journal of Medical Science*. 175 (4), PP. 30-35.

Caudill, S., Cooper, G., Smith, S. and Myers, G. (1998) Assessment of current National Cholesterol Education Program guidelines for total cholesterol, triglyceride, HDL-cholesterol, and LDL-cholesterol measurements. *Clinical Chemistry*. 44 (8), pp. 1650-1658.

Chapman, M.J., Assmann, G., Fruchart, J.C., Shepherd, J. and Sirtori, C. (2004) Raising high-density lipoprotein cholesterol with reduction of cardiovascular risk: the role of nicotinic acid - a position paper developed by the European Consensus Panel on HDL-C. *Current Medical Research and Opinion*. 20 (8), pp. 1253-1268.

Charpentier, L. and El Murr, N. (1995) Amperometric determination of cholesterol in serum with use of a renewable surface peroxidase electrode. *Analytica Chimica Acta*. 318 (1), pp. 89-93.

Christie, W.W. (2012) *Plasma Lipoproteins: Composition, Structure and Biochemsitry.* Available from: http://lipidlibrary.aocs.org/lipids/lipoprot/index.htm [Accessed 31 July 2012].

Contois, J.H., Warnick, G.R. and Sniderman, A.D. (2011) Reliability of low-density lipoprotein cholesterol, non-high-density lipoprotein cholesterol, and apolipoprotein B measurement. *Journal of Clinical Lipidology*. 5 (4), pp. 264-272.

Contois, J.H. (1999) Quantitative determination of cholesterol in lipoprotein fractions by electrophoresis. *Clin Chim Acta*. 282 (1-2), pp. 1.

Cooper, G.R. (1986) Interlaboratory testing of the transferability of a candidate reference method for total cholesterol in serum. *Clinical Chemistry*. 32 (6), pp. 921.

Dale, R.A., Jensen, L.H. and Krantz, M.J. (2008) DYSLIPIDEMIA: Comparison of Two Point-of-Care Lipid Analyzers for Use in Global Cardiovascular Risk Assessments. *Ann Pharmacother*. 42, pp. 633-639.

Datta, S., Christena, L.R. and Rajaram, Y.R.S. (2013) Enzyme immobilisation: an overview on techniques and support materials. *3 Biotechnology*. 3, pp. 1-9.

Delaney, J.T., Smith, P.J. and Schubert, U.S. (2009) Inkjet printing of proteins. *Soft Matter*. 5, pp. 4866-4877.

Deventer, H.E., Miller, W.G., Myers, G.L., Sakurabayashi, I., Bachmann, L.M., Caudill, S.P., Dziekonski, A., Edwards, S., Kimberly, M.M., Nakajima, K., Nakamura, M., Nilsson, G., Shamburek, R.D. and Vetrovec, G.W. (2011) Non–HDL Cholesterol Shows Improved Accuracy for Cardiovascular Risk Score Classification Compared to Direct or Calculated LDL Cholesterol in a Dyslipidemic Population. *Clinical Chemistry*. 57 (3), pp. 490-501.

Dey, R.S. and Raj, C.R. (2014) Enzyme-integrated cholesterol biosensing scaffold based on in situ synthesized reduced graphene oxide and dendritic Pd nanostructure. *Biosensors and Bioelectronics*. 62 (0), pp. 357-364.

DiCosimo, R., McAuliffe, J., Poulose, A.J. and Bohlmann, G. (2013) Industerial use of immobilized enzymes. *Chemical Society Review*. 42, pp. 6437-6474.

Eleni, T., Bairaktari, K. and Seferiadis, I. (2005) Evaluation of Methods for the Measurement of Low-Density Lipoprotein Cholesterol. *Journal of Cardiovascular Pharmacology and Therapeutics*. 10, pp. 45-54.

Emerson, E., (1943) The Condensation of Aminoantipyrine II; A New Color Test for Phenolic Compounds". *Organic Chemistry*. 8, 417.

Fang, C. (2011) A disposable amperometric biosensor for determining total cholesterol in whole blood. *Sensors and Actuators B: Chemical*. 155 (2), pp. 545-550.

Fernández-Higuero, J.A., Salvador,A.M., Martín, C., Carlos, J., Milicua, G. and Arrondo, J.L.R., (2014) Human LDL Structural Diversity Studied by IR Spectroscopy. *PLoS One.* 9(3): e92426

Foster, R., Cassidy, J. and O'Donoghue, E. (2000) Electrochemical Diagnostic Strip Device for Total Cholesterol and Its Subfractions. *Electroanalysis*. 12 (9), pp. 716-721.

Friedewald, W.T. (1972) Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clinical Chemistry*. 18 (6), pp. 499-502.

Garrett, R.H. and Grisham, C.M. (1995) *Biochemistry* . 2nd ed. Orlando, FL.: Saunders College.

Gonzalez-Macia, L., Smyth, M.R. and Killard, A.J. (2011) Enhanced electrochemical reduction of hydrogen peroxide at metallic electrodes modified with surfactant and salt. *Electrochimica Acta*. 58, pp. 562-570.

Gonzalez-Macia, L., Smyth, M.R. and Killard, A.J. (2012a) Evaluation of a silver-based electrocatalyst for the determination of hydrogen peroxide formed via enzymatic oxidation. *Talanta*. 90, pp. 989-996.

Gonzalez-Macia, L., Smyth, M.R. and Killard, A.J. (2012b) A Printed Electrocatalyst for Hydrogen Peroxide Reduction. *Electroanalysis*. 24 (3), pp. 609-614.

Grieshaber, D., MacKenzie, R. and Reimhul, E. (2008) Electrochemical Biosensors - Sensor Principles and Architectures. *Sensors*. 8, pp. 1400-1458.

Gubala, V., Harris, L.F., Ricco, A.J. and Tan MX, W., D.E. (2012) Point of care diagnostics: status and future. *Anal. Chem.* 84, pp. 487-515.

Hanefeld, U., Cao, L.Q. and Magner, E. (2013) Enzyme immobilisation: fundamental and application. *Chemical Society Review*. 42, pp. 6211-6212.

Harris, N., Galpchian, V., Thomas, J., Iannotti, E., Law, T. and Rifai, N. (1997) Three generations of high-density lipoprotein cholesterol assays compared with ultracentrifugation/dextran sulfate-Mg2+ method. *Clinical Chemistry*. 43 (5), pp. 816-823.

Honda, M., Koderaa, T. and Kita, H. (1983) On the electrochemical behavior of H2O2 AT Ag in alkaline solution. *Electrochimica Acta,* 28 (5), pp. 727-733.

Honda, M., Koderaa, T. and Kitab, H. (1986) Electrochemical behavior of H_2O_2 at Ag in HClO4 aqueous solution, *Electrochimica Acta*. 31 (3), pp. 377–383

Huang, H.S., Juan, S.S. and Guilabult, G.G. (1977) Amperometric determination of total cholesterol in serum, with use of immobilized cholesterol ester hydrolase and cholesterol oxidase. *Clinical Chemistry* 23, pp. 671-676.

Hutchings, I.M. and Martin, G.D. (2013) *Inkjet Technology for Digital Fabrication*. 1st ed. UK: John Wiley & sons Ltd.

Karube, K., Hara, H. and Mastsuoka, S. (1982) Amperometric determination of total cholesterol in serum with use of immobilized cholesterol esterase and cholesterol oxidase. *Anal. Chim. Acta*. 139, pp. 127-132.

Iizuka, Y., Ishijima, M., Kato, T., Nanmoku, T., Kuwa, K., Kawakami, Y. and Isobe, K. (2012) Clinical sample comparison of eight homogeneous assay kits for high density lipoprotein cholesterol and low density lipoprotein cholesterol. *J Anal Bio-Science*. 35, pp. 168-178.

Ito, Y., Satoh, N., Ishii, T., Kumakura, J. and Hirano, T. (2014) Development of a homogeneous assay for measurement of high-density lipoprotein-subclass cholesterol. *Clinica Chimica Acta*. 427 (0), pp. 86-93.

Kakuyama, T., Kimura, S. and Hasiguchi, Y. (1994) Fully automated determination of HDL-cholesterol from human serum with Hitachi 911. *Clinical Chemistry*. 40, pp. A1104.

Kim, Y.C. and Nishida, T. (1979) Nature of the interaction of dextran sulfate with high and low density lipoproteins in the presence of Ca2+. *Journal of Biological Chemistry*. 254, pp. 9621-9626.

Kimberly, M.M., Leary, E.T., Cole, T.G. and Waymack, P.P. (1999) Selection, Validation, Standardization, and Performance of a Designated Comparison Method for HDL-Cholesterol for Use in the Cholesterol Reference Method Laboratory Network. *Clinical Chemistry*. 45 (10), pp. 1803-1812.

Kimmel, D.W., LeBlanc, G., Meschievitz, M.E. and Cliffel, D.E. (2012) Electrochemical Sensors and Biosensors. *Analytical Chemistry, 84 (2), pp. 685–707*.

Kinoshita, H., Torimura, M., Kano, K. and Ikeda, T. (1998) Amperometric determination of high-density lipoprotein cholesterol using polyethylene glycol-modified enzymes and a peroxidase-entrapped electrode. *Annals of Clinical Biochemistry*. 35, pp. 739-744.

Kissinger, P.T. and Heineman, W.R. (1996) *Laboratory Techniques in Electroanalytical Chemistry*. Second ed. Marcel Dekker, Inc.

Kondo, A. (1999) Dynamic reaction in a homogeneous HDL-cholesterol assay visualized by electron microscopy. *Clinical Chemistry*. 45 (11), pp. 1974.

Kumar, A., Pandey, R.R. and Brantley, B. (2006) Tetraethylorthosilicate film modified with protein to fabricate cholesterol biosensor. *Talanta*. 69 (3), pp. 700-705.

Lee, E.J., Shin, S.D., Song, K.J., Kim, S.C., Cho, J.S., Lee, S.C., Park, J.O. and Cha, W.C. (2011) A point-of-care chemistry test for reduction of turnaround and clinical decision time. *American Journal of Emergency Medicine*. 29, pp. 489-495.

Li, G., Liao, J.M., Hu, G.Q., Ma, N.Z. and Wu, P.J. (2005) Study of carbon nanotube modified biosensor for monitoring total cholesterol in blood. *Biosensors and Bioelectronics*. 20 (10), pp. 2140-2144.

Lian, W., Wang, L., Song, Y., Yuan, H., Zhao, S., Li, P. and Chen, L. (2009) A hydrogen peroxide sensor based on electrochemically roughened silver electrodes. *Electrochimica Acta*. 54 (18), pp. 4334-4339.

Lin, M.J. (1998) Evaluation of homogeneous high-density lipoprotein cholesterol assay on a BM/Hitachi 747-200 analyzer. *Clinical Chemistry*. 44 (5), pp. 1050.

Liu, J., Sempos, C.T., Donahue, R.P., Dorn, J., Trevisan, M. and Grundy, S.M. (2006) Non-high-density lipoprotein and very-low-density lipoprotein cholesterol and their risk predictive values in coronary heart disease. *American Journal of Cardiology*. 98, pp. 1363-1368.

Lopes-Virella, M. (1977) Cholesterol determination in high-density lipoproteins separated by three different methods. *Clinical Chemistry*. 23 (5), pp. 882.

Manjunatha, R., Shivappa Suresh, G., Savio Melo, J., D'Souza, S.F. and Venkatarangaiah Venkatesha, T. (2012) An amperometric bienzymatic cholesterol biosensor based on functionalized graphene modified electrode and its electrocatalytic activity towards total cholesterol determination. *Talanta*. 99 (0), pp. 302-309.

Marazuela, M.D., Cuesta, B., Moreno-Bondi, M.C. and Quejido, A. (1997) Free cholesterol fiber-optic biosensor for serum samples with simplex optimization. *Biosensors and Bioelectronics*. 12 (3), pp. 233-240.

Méndez-González, J., Bonet-Marqués, R. and Ordóñez-Llanos, J. (2010) Lipid Profile in Ambulatory Subjects Using 3 Point-of-Care Devices and Comparison With Reference Methods. *The Journal of Near-Patient Testing & Technology*. 9 (2), pp. 102-107.

Miller, W.G., Myers, G.L., Sakurabayashi, I., Bachmann, L.M., Caudill, S.P., Dziekonski, A., Selvin, E., Kimberly, M., Korzun, W.J., Leary, E.T., Nakajima, K., Nakamura, M., Nilsson, G., Shamburek, R.D., Vetrovec, G.W., Warnick, G.R. and Remaley, A.T. (2010) Seven Direct Methods for Measuring HDL and LDL Cholesterol Compared with Ultracentrifugation Reference Measurement Procedures. *Clinical Chemistry*. 56 (6), pp. 977-986.

Murphy, L., Burrows, C., Bayly, S., Wilkinson, K. and Harris, J. (2009) *HDL Cholesterol Sensor using Selective Surfactant* Anon. US20090130696 A1.

Nauck, M. (1995) Quantitative determination of high-, low-, and very-low-density lipoproteins and lipoprotein(a) by agarose gel electrophoresis and enzymatic cholesterol staining. *Clinical Chemistry*. 41 (12), pp. 1761-7.

Nauck, M., Warnick, G.R. and Rifai, N. (2002) Methods for Measurement of LDL-Cholesterol: A Critical Assessment of Direct Measurement by Homogeneous Assays versus Calculation. *Clinical Chemistry*. 45 (2), pp. 236-254.

Naucka, M., März, W. and Wieland, H. (1998) New immunoseparation-based homogeneous assay for HDL-cholesterol compared with three homogeneous and two heterogeneous methods for HDL-cholesterol. *Clinical Chemistry*. 44, pp. 1443-1451.

Okada, M., Matsui, H., Ito, Y. and Fujiwara, A. (2001) Direct measurement of HDL cholesterol:Method eliminating apolipoprotein E-rich particles. *Journal of Clinical Laboratory Analysis*. 15 (4), pp. 223-229.

Okazaki, M. (1997) Evaluation of precipitation and direct methods for HDL-cholesterol assay by HPLC. *Clinical Chemistry*. 43 (10), pp. 1885-1890.

Okazaki, M., Usui, S., Nakamura, M. and Yamashita, S. (2008) Evaluation of an HPLC method for LDL-cholesterol determination in patients with various lipoprotein abnormalities in comparison with beta-quantification. *Clinica Chimica Acta*. 395 (1-2), pp. 62-67.

Osman, H. and Yap, K.C. (2006) Comparative sensitivities of cholesterol analysis using GC, HPLC and spectrophotometric methods. *The Malaysian Journal of Analytical Sciences*. 10 (2), pp. 205-210.

Özer, B.C., Özyörük, H., Çelebi, S.S. and Yıldız, A. (2007) Amperometric enzyme electrode for free cholesterol determination prepared with cholesterol oxidase immobilized in poly(vinylferrocenium) film. *Enzyme and Microbial Technology*. 40 (2), pp. 262-265.

Panz, V.R., Raal, F.J., Paiker, J., Immelman, R. and Miles, H. (2005) Performance of the CardioChek PA and Cholestech LDX point-of-care analysers compared to clinical diagnostic laboratory methods for the measurement of lipids. *Cardiovascular Journal of South Africa*. 16 ,. 112-117.

Patel, J.V., Thorpe, G.H., Springer, L. and Hughes, E.A. (2011) Accuracy and precision of point-of-care testing for serum cholesterol, triglycerides and HDL cholesterol. *Atherosclerosis*. 218 (2), pp. e8-e9.

Pembouong, G., Morellet, N., Kral, T., Hof, M., Scherman, D., Bureau, M. and Mignet, N. (2011) A comprehensive study in triblock copolymer membrane interaction. *Journal of Controlled Release*. 151 (1), pp. 57-64.

Pischon, T., Girman, C.J., Sack, F.M., Rifai, N., Stampfer, M.J. and Rimm, E.B. (2013) Non-high-density lipoprotein cholesterol and Apolipoprotein B in the prediction of cornary heart disease in men. *Circulation*. 112, pp. 3375-3383.

Pollegioni, L., Wels, G., Pilone, M.S., Ghisla, S. (1999) Kinetic mechanisms of cholesterol oxidase from Streptomyces hygroscopicus and Brevibacterium sterolicum. *European Journal of Biochemistry*. 264 (1), pp. 140-51.

Pundir, C.S. (2008) Fabrication of Pt based amperometric cholesterol biosensor using Cellulose acetate membrane. *Journal of Scientific & Industrial Research*. 67, pp. 299-306.

Richmond, W. (1973) Preparation and properties of a cholesterol oxidase from nocardia sp. and its application to the enzymatic assay of total cholesterol in serum. *Clinical Chemistry*. 19 (12), pp. 1350-1356.

Rifai, N., Warnick, R. and Dominiczak, M.H., eds. (2001) *Handbook of Lipoprotein Testing*. Washington: AACC Press.

Rostami, M. (2011) Comparison of Friedwald formula with direct measurement of LDL-cholesterol. *Clinical Biochemistry*. 44 (13), pp. S147.

Ruecha, N., Rangkupan, R., Rodthongkum, N. and Chailapakul, O. (2014) Novel paper-based cholesterol biosensor using graphene/polyvinylpyrrolidone/polyaniline nanocomposite. *Biosensors and Bioelectronics*. 52 (0), pp. 13-19.

Salè, F.O., Marchesini, S., Fishman, P.H. and Berra, B. (1984) A sensitive enzymatic assay for determination of cholesterol in lipid extracts. *Analytical Biochemistry*. 142 (2), pp. 347-350.

Salinas, E., Rivero, V., Torriero, A.A.J., Benuzzi, D., Sanz, M.I. and Raba, J. (2006) Multienzymatic-rotating biosensor for total cholesterol determination in a FIA system. *Talanta*. 70 (2), pp. 244-250.

Segrest, J.P., Jones, M.K., Loof, H.D., Brouillette, C.G. and Anantharamaiah, G.M. (1992) The amphipathic helix in exchangeable apolipoproteins: a review of secondary structure and function. *Journal of Lipid Research*. 33, pp. 141-166.

Segrest, J.P., Jones, M.K., Loof, H.D. and Dashti, N. (2001) Structure of apolipoprotein B-100 in low density lipoproteins. *Journal of Lipid Research*. 42, pp. 1346-1367.

Shih, W., Yang, M. and Lin, M.S. (2009) Development of disposable lipid biosensor for the determination of total cholesterol. *Biosensors and Bioelectronics*. 24 (6), pp. 1679-1684.

Shin, Y.J. and Kameoka, J. (2012) Amperometric cholesterol biosensor using layer-by-layer adsorption technique onto electrospun polyaniline nanofibers. *Journal of Industrial and Engineering Chemistry*. 18 (1), pp. 193-197.

Stone, N.J., Robinson, J.G., Lichtenstein, A.H., Bairey Merz, C.N., Blum, C.B., Eckel, R.H., Goldberg, A.C., Gordon, D., Levy, D., Lloyd-Jones, D.M., McBride, P., Schwartz, J.S., Shero, S.T., Smith Jr., S.C., Watson, K. and Wilson, P.W.F. (2014) 2013 ACC/AHA Guideline on the Treatment of Blood Cholesterol to Reduce Atherosclerotic Cardiovascular Risk in Adults: A Report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. *Journal of the American College of Cardiology*. 63 (25, Part B), pp. 2889-2934.

Srisawasdi, P., Prasertsincharoen, N., Kroll, M.H. (2012) Porcine pancreas: a superior source of cholesterol esterase for total serum cholesterol assay by the enzymatic kinetic method. *Journal of Clinical Laboratory Analysis.* 26(6), pp.420-30.

Sugiuchi, H., Uji, Y., Okabe, H., Irie, T., Uekama, K., Kayahara, N. and Miyauchi, K. (1995) Direct measurement of high-density lipoprotein cholesterol in serum with

polyethylene glycol-modified enzymes and sulfated alpha-cyclodextrin. *Clinical Chemistry*. 41 (5), pp. 717-723.

Sugiuchi, H. (1998) Homogeneous assay for measuring low-density lipoprotein cholesterol in serum with triblock copolymer and alpha-cyclodextrin sulfate. *Clinical Chemistry*. 44 (3), pp. 522-531.

Talwalkar, P.G., Sreenivas, C.G., Gulati, A. and Baxi, H. (2013) Journey in guidelines for lipid management: From adult treatment panel (ATP)-I to ATP-III and what to expect in ATP-IV. *Indian J Endocrinol Metab.* 17, pp. 628-635.

Tamura, H., Nishino, S., Yamaguchi, T. and Hino, K. (2009) *Test Piece for Measureing HDL- Cholesterol,*Daiichi Chemical Co., L. US7575884.

Tanno, K., Okamura, T., Ohsawa, M., Onoda, T., Itai, K., Sakata, K., Nakamura, M., Ogawa, A., Kawamura, K. and Okayama, A. (2010) Comparison of low-density lipoprotein cholesterol concentrations measured by a direct homogeneous assay and by the Friedewald formula in a large community population. *Clinica Chimica Acta*. 411 (21-22), pp. 1774-1780.

The Medical Services Advisory Committee (MSAC) (2001) *Near Patient Testing using Cholestech LDX.* Available from: http://www.msac.gov.au [Accessed 12/11/2012].

Tighe, D.A., Ockene, I.S., Reed, G. and Nicolosi, R. (2006) Calculated low density lipoprotein cholesterol levels frequently underestimate directly measured low density lipoprotein cholesterol determinations in patients with serum triglyceride levels ≤ 4.52 mmol/l: An analysis comparing the LipiDirect® magnetic LDL assay with the Friedewald calculation. *Clinica Chimica Acta*. 365 (1-2), pp. 236-242.

Trinder, P., (1969) Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. *Annals of Clinical Biochemistry*.6,24.

Timón-Zapata, J. (2011) Extreme concentrations of high density lipoprotein cholesterol affect the calculation of low density lipoprotein cholesterol in the Friedewald formula and other proposed formulas. *Clin Biochem*. 44, pp. 1451-1456.

Torabi, s.f., Khajeh, K., Ghasempur, S., Ghaemi, N. and Siadat, S.O.R. (2007) Covalent attachment of cholesterol oxidase and horseradish peroxidase on perlite through silanization: Activity, stability and co-immobilization. *Journal of Biotechnology*. 131 (2), pp. 111-120.

Tremblay, A.J., Morrissette, H., Gagné, ,J., Bergeron, J., Gagné, C. and Couture, P. (2004) Validation of the Friedewald formula for the determination of low-density lipoprotein cholesterol compared with β-quantification in a large population. *Clinical Biochemistry*. 37 (9), pp. 785-790.

Turkalp, I. (2005) Analytical performance of a direct assay for LDL-cholesterol: a comparative assessment versus Friedewald's formula. *Anadolu Kardiyoloji Dergisi: AKD = the Anatolian Journal of Cardiology*. 5 (1), pp. 13-17.

Turner, A.P.F. (2013) Biosensors: sense and sensibility. *Chemical Society Review*. 42, pp. 3184-3196.

Urmila, S., Madhuri, D., Barbora, A., Borthakur, L., Verma, M., Bora, A. and Goswami, U. (2011) Multiwalled carbon nanotube-based bi-enzyme electrode for total cholesterol estimation in human serum *Journal of Experimental Nanoscience,* 6 (1), pp. 84-95.

Usui, S. (2000) Assessment of between-instrument variations in a HPLC method for serum lipoproteins and its traceability to reference methods for total cholesterol and HDL-cholesterol. *Clinical Chemistry*. 46 (1), pp. 63-72.

Usui, S., Nakamura, M., Jitsukata, K. and Nara, M. (2000) Assessment of between-instrument variations in a HPLC method for serum lipoproteins and its traceability to reference methods for total cholesterol and HDL-cholesterol. *Clinical Chemistry*. 46 (1), pp. 63-72.

Vojinovi´c, V., Azevedo, A.M., Martins, V.C.B., Cabral, J.M.S., Gibson, T.D., Fonseca, L.B., (2004) Assay of H2O2 by HRP catalysed co-oxidation of phenol-4-sulphonicacid and 4-aminoantipyrine: characterisation and optimisation. *Journal of Molecular Catalysis B: Enzymatic.* 28, 129–135.

Wang, U., Xu, H., Zhang, J. and Li, W. (2008) Electrochemical Sensors for Clinic Analysis. *Sensors*. 8, pp. 2043-2081.

Warnick, G.R., Benderson, J. and Albers, J.J. (1982) Dextran sulfate-Mg2+ precipitation procedure for quantitation of high density-lipoprotein cholesterol. *Clinical Chemistry*. 28 (6), pp. 1379-1388.

Warnick, G.R., Kimberly, M.M., Waymack, P.P., Leary, E.T. and Myers, G.L. (2009) Standardization of measurements for cholesterol, triglycerides, and major lipoproteins. *Labmedicine*. 39, pp. 481-490.

Warnick, G.R., Nauck, M. and Rifai, N. (2001) Evolution of Methods for Measurement of HDL-Cholesterol: From Ultracentrifugation to Homogeneous Assays. *Clinical Chemistry*. 47 (9), pp. 1579-1596.

Warnick, G.R. (1995) National Cholesterol Education Program recommendations for measurement of high-density lipoprotein cholesterol: executive summary. The National Cholesterol Education Program Working Group on Lipoprotein Measurement. *Clinical Chemistry*. 41 (10), pp. 1427.

Warnick, G.R. (1978) A comprehensive evaluation of the heparin-manganese precipitation procedure for estimating high density lipoprotein cholesterol. *Journal of Lipid Research*. 19 (1), pp. 65-76.

Welch, C.M., Banks, C.E., Simm, A.O. and Compton, R.G. (2005) Silver nanoparticle assemblies supported on glassy-carbon electrodes for the electro-analytical detection of hydrogen peroxide. *Analytical and Bioanalytical Chemistry*, 382, pp. 12-21

Wu, J., Chen, S., Liu, L., Gao, X., Zhou, Y., Wang, C., Zhang, Q., Wang, A., Hussain, M., Sun, B., Wu, S. and Zhao, X. (2013) Non-high-density lipoprotein cholesterol vs low-density lipoprotein cholesterol as a risk factor for ischemic stroke: a result from the Kailuan study. *Neurol Res.* Epub ahead of print .

Yang, J.U., Shen-Ming Chen, Y.L. and Lin, K.C. (2011) Fabrication of a Cholesterol Biosensor Based on Cholesterol Oxidase and Multiwall Carbon Nanotube Hybrid Composites. *International Journal of Electrochemical Science*. 6 pp. 2223-2234.

Zlatkis, A., Zak, B. and Boyle, A.J. (1953) A new method for the direct determination of serum cholesterol. *Journal of Laboratory and Clinical Medicin*. 41, pp. 486-492.

Chapter 2

Materials and methods

2.1 Materials

Dodecylbenzenesulphonic acid (DBSA-D0989) was purchased from TCI Europe. Polyoxyethylene octyl phenyl ether (Triton X-100), sodium chloride (KCl), potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4), disodium hydrogen phosphate (Na_2HPO), N,N-dimethyl-m-toluidine, 3-(N-morpholino) propanesulfonic acid (MOPS), Tris-HCl, NaOH, $MgCl_2$, Poly(diallyldimethylammonium chloride) solution (PDA) 20% (w/v) in H_2O were purchased from Sigma-Aldrich (UK). 4-Aminoantipyrine HCl was from BDH (Dorset, UK). Cholesterol oxidase (ChOx, CO5F; 19.4 U/mg), Cholesterol esterase (ChEs, CE4F; 130.5 U/mg) and Horseradish peroxidise (HRP, HRP4C; 295 U/mg) were purchased from BBI Enzymes (Gwent, UK). LDL-C (P232-8), HDL-C (P233-8) isolated from human serum and delipidated serum (S139) all from human plasma were purchased from Scipac Ltd (Kent, UK). Clinical serum samples were kindly donated by Alere (San Diego).

Other materials were obtained as follows: 30% (v/v) hydrogen peroxide solution (Merck, UK), Polyoxyethylene derivatives (Emulgen B-66, Kao Corporation (Japan), HDL and LDL/VLDL cholesterol assay kit (Abcam, UK). Silver (PF-410), carbon (Electrodag 6017SS), and Ag/AgCl (Electrodag 6038SS) screen printing inks were from Henkel (Netherlands). 16 nozzles print cartridge (Fujifilm, USA), Costar 96-Well microplates (VWR, UK), Pluronic F-68 (POE-POP) (Sigma, UK) and Polyvinylpyrrolidone K30 (PVP) from Fluka. Poly (ethylene) terephthalate (PET) substrates (film type: SU320/175 mic) obtained from HiFi Industrial Film Ltd. (Stevenage/UK)) and 25 µm polyester pressure sensitive adhesive (PSA) spacer layer and hydrophilic cyclic polyolefin (Zeonor) lid.

2.2 Buffers and solutions

2.2.1 Phosphate buffered saline solution (PBS)

Unless otherwise stated, all electrochemical measurements were carried out in phosphate buffered saline solution (PBS) which contained 0.1 M phosphate, 137 mM NaCl and 2.7 mM KCl. This was prepared by mixing solution containing 0.1 M Na_2HPO4, 137 mM NaCl and 2.7 mM KCl with a solution containing 0.1 M KH_2PO_4, 137 mM NaCl and 2.7 mM KCl to a pH of 6.8.

2.2.2 MOPS buffer

50 mM MOPS was prepared in deionised water and pH adjusted to 7.3 using 1 M NaOH.

2.2.3 Tris-HCl buffer

0.1 M Tris-HCl was prepared in dionised water and pH was adjusted with 1 M HCl.

2.2.4 DBSA/KCl modification solution

A solution of 33 mM DBSA/0.1 M KCl was prepared by mixing 2.35 mL water, 1.25 mL 0.1 M DBSA and 0.15 mL 2.5 mM KCl (Gonzalez-Macia, Smyth and Killard, 2012).

2.2.6 Cholesteryl acetate stock solution

Cholesteryl acetate (50 mg) was dissolved on 1.0 mL of Triton X-100 by slowly heating and stirring until solution was clear. 0.1 M PBS (pH 6.8) was added to get a final concentration of 500 mg/dL. The solution was stored at 4°C.

2.3 Instrumentation

Screen-printing was performed using an automated DEK 248 machine (Weymouth, UK). Scanning electron microscopy (SEM) using secondary electron (SE) detection was carried out with a Philips XLS0 ESEM with acceleration voltage of 20 keV. Both Gaseous Secondary Electron (GSE) and Back-scattered Secondary Electron (BSE) were used. A GRAPHTEC Craft Robo ProS (Model No. CE50000-40-CRP) cutting plotter and a Robo Master-Pro software were used to prepare the PSA and Zeonor patterns for encapsulation of the biosensor to provide a thin layer cell. Electrode patterns were drawn using AutoCAD and uploaded into the Robo Master software. All spectrophotometric measurements were performed using 96 well microplates with a FLUOstar Optima plate reader (BMG Labtech, UK) with Optima software (version: 2.1). Inkjet-printing was performed using a Dimatix Materials Printer DMP-2831 with Dimatix Drop manager DMP-2800 series software (Fujifilm Dimatix, Inc., US). The MEMS-based Dimatix cartridge with 16 nozzles (20 µm diameter) spaced at 254 µm was used. Most of the electrochemical measurements were carried out using a PGSTAT128N potentiostat with NOVA 1.6 software

(Metrohm, UK) and some of them performed using SP-200 potentiostat with EC-lb V10.19 software. Unless otherwise stated, all measurements were performed at a room temperature of 25±3°C. All potentials were referred to the Ag/AgCl reference electrode unless otherwise stated.

2.4 Methods

2.4.1 Screen printing of electrodes and silver modification

Screen-printed electrodes (SPEs) (Ag working, Ag/AgCl reference and carbon counter with silver connector) were fabricated using an automated DEK 248 machine (Weymouth, UK) according to the design shown in Fig. 2.1. Briefly, a layer of silver paste was deposited onto PET substrate and cured in a convection oven at 120°C for 5 minutes. A single layer of Ag/AgCl was then screen printed and cured in a convection oven at the same temperature for 5 min, followed by two layers of carbon over-printed on silver and cured for 5 min at 120°C.

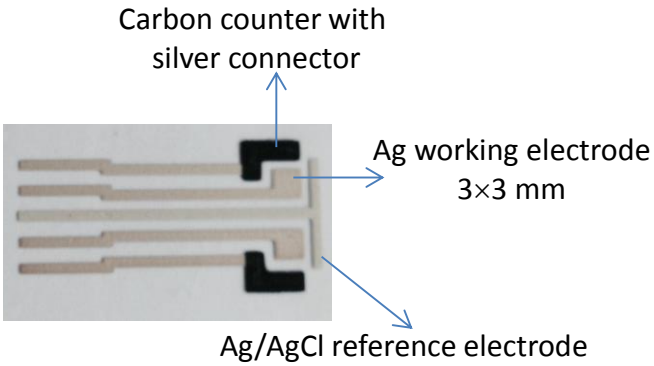

Fig. 2.1. Image of the dual electrode system fabricated using screen printing.

In the three-electrode system, a 3 ×3 mm Ag screen printed electrode (PF-410) and an Ag/AgCl screen printed electrode (6038SS) were used as a working electrode and reference electrode, respectively. The counter electrode was composed of a screen printed silver conductor (PF-410) overprinted with carbon (Electrodag 6017SS) to ensure minimal resistance and electrochemical interference. The area of each working electrode was 9 mm^2. Electrodes were either used in single or dual electrode mode, either in open batch experiments, or following encapsulation with a 25 µm pressure sensitive adhesive (PSA) spacer layer and hydrophilic cyclic polyolefin (Zeonor) lid, yielding a sample volume of 8 µL (Fig. 2.2).

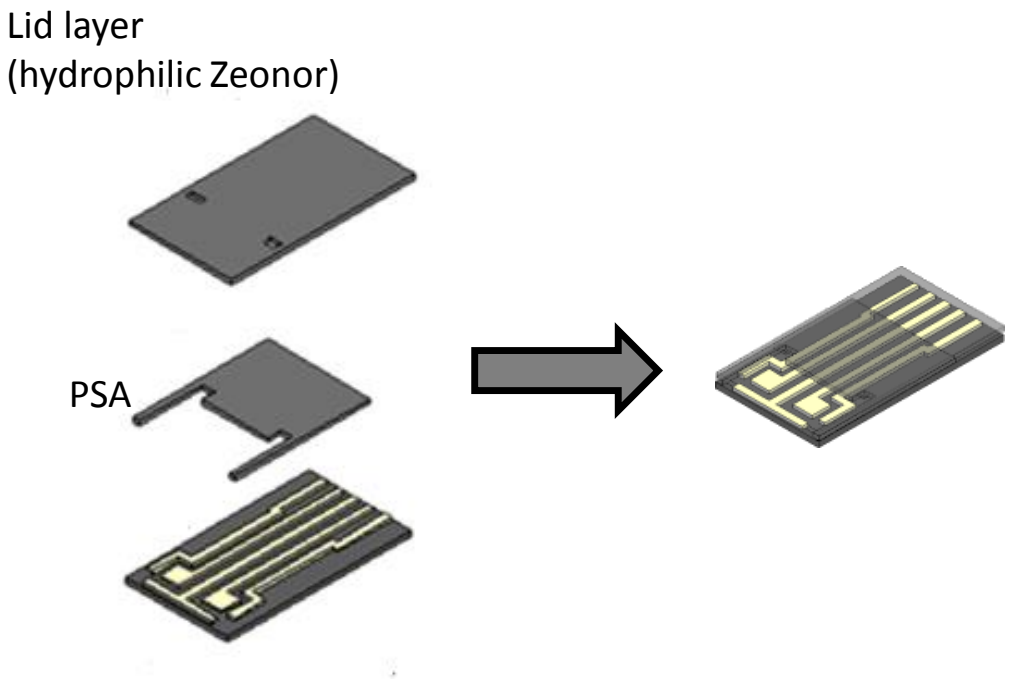

Fig. 2.2. Design layout of the encapsulated electrodes

Different techniques such as dip-coating, drop-coating and inkjet-printing were employed to modify silver screen printed electrodes. For dip-coating, the entire electrode was dipped in modification solution, while for drop-coating, defined volumes of modification solution were pipetted onto the silver electrode surface and left for a minimum 10 min. Where appropriate, modification solutions of DBSA/KCl, 6% (w/v) Emulgen B-66/PBS or 0.5% Triton X-100/PBS were inkjet-printed at a nozzle voltage of 26 V using a 16-nozzle head cartridge without filtration on silver screen printed electrodes with 20 µm dot spacing. Modification was typically performed with 5 to 15 layers of modification solution. Electrodes were then left to dry for at least 10 min and then rinsed using deionised water to remove the excess of modification solution that did not interact with the electrode surface just before use.

2.4.2 Spectrophotometric assay development

Spectrophotometric measurement of HDL-C was performed according to the literature as summarised in Table 2.1 (Nakamura *et al.,* 2009, Hino *et al.,* 1998, Tamura *et al.,* 2009), and was used as the initial platform for assay development. 300 µL of a reagent 1 was mixed with 3 µL of serum sample and heated at 37 °C for 5 min. the absorbance was measured at 545 nM. The 100µL of reagent 2 was

added to the mixture and the difference between absorbance was measured at 545 nM at 37 °C.

Table 2.1. Spectrophotometric methodology for HDL-C measurement at 37°C according to the literature.

Reagent 1	Reagent 2	Buffer
ChOx (1-5 U/mL) HRP (5 U/mL) 4-AAP (0.05 % (w/v))	Emulgen B-66 (0.1%w/v) ChEs (1.2 U/mL) DMT (0.02-0.04% (w/v))	Tris-HCl (0.1 M, pH 7.0) MOPS (50 mM, pH 7.3)

Fig. 2.3 shows a flow diagram for spectrophotometric assay methodology developed at room temperature for this work using 96 well microtitre plates. The final volume of reaction solution in each well was 100 µL. A final concentration of each assay component was: 6.1% (w/v) Emulgen B-66/PBS, 0.075 % (w/v) 4-AAP, 14 U/mL HRP and 0.06 % (v/v) DMT, 23 U/mL ChOx and 39 U/mL ChEs. Unless otherwise stated, incubation was at 25 ±3°C (Hino, Nakamura and Manabe, 1998; Nakamura *et al.,* 2009; Tamura *et al.,* 2009; Yamamoto, Yamamoto and Nakanishi, 2011).

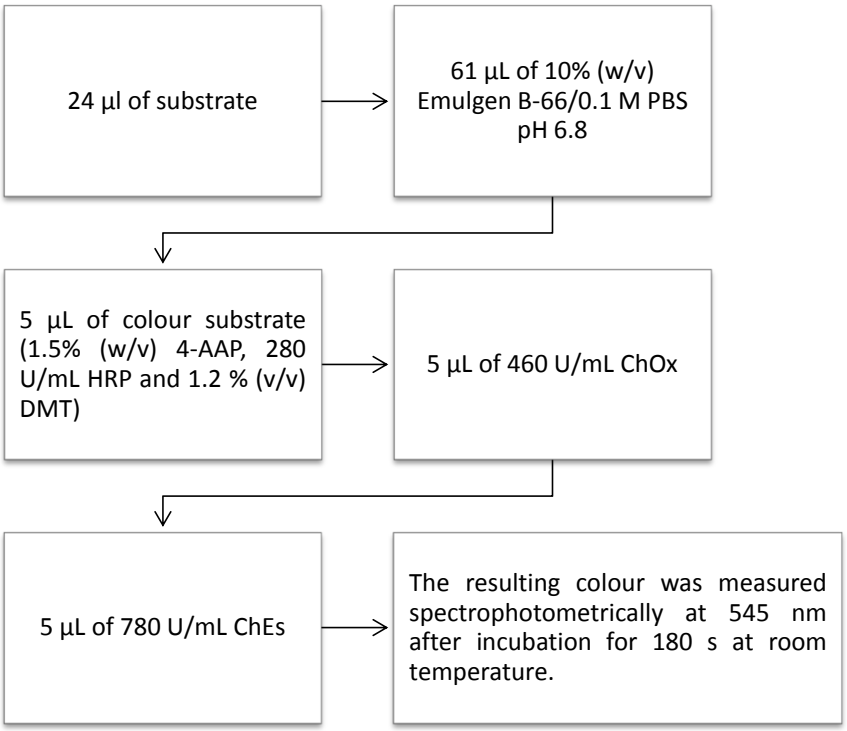

Fig. 2.3. Flow diagram for spectrophotometric assay methodology developed at room temperature for this work using 96 well microtitre plates

2.4.3 Electrochemical measurements using the three electrode system

All electrochemical measurements were carried out on the Ag SPEs in either an open stirred batch system or an enclosed (encapsulated) electrode sample chamber with a volume of 8 µL. In the stirred batch system, all the measurements were performed in 4 mL 0.1M PBS pH 6.8 unless otherwise stated. In the case of the encapsulated sample chamber, the modified SPEs were enclosed within a chamber of volume encapsulated using PSA and Zenor (Fig. 2.1 and Fig. 2.2). Encapsulation was performed by enclosing each electrode using a 25 µm pressure sensitive adhesive (PSA) spacer layer and hydrophilic cyclic polyolefin (Zeonor) lid to yield a sample volume of 8 µL. 20 µL of the reaction solution were placed on a PET sheet and the sample chamber was filled by capillary action.

Cyclic voltammetric measurement was perfumed using a narrow potential window of -0.25 to 0 V (vs Ag/AgCl) where the scan started and finished at -0.25 V vs. Ag/AgCl, with a scan rate of 100 mV/s. Chronoamperometric measurement was performed either in stirred batch solution using three electrode system at 150 rpm using stirring plate and at -0.1 V vs (Ag/AgCl) when the steady state current obtained or using encapsulated electrodes at -0.1 V vs Ag/AgCl with the current response measured at 420 s following 240 s incubation.

2.4.4 Measurement of HDL and TC using biosensors with externally added assay reagents

The general approach to the measurement of HDL-C and TC using the developed biosensors is shown in Fig. 2.4. Typically in the case of the measurement of HDL-C, a HDL-C selective surfactant, or in the case of TC a non-selective surfactant was mixed with ChEs and ChOx and allowed to incubate for a period of time before application to the electrode. In the next step, cholesterol esters released from HDL were hydrolyzed to FC in the presence of ChEs. The FC produced was oxidized by ChOx to cholest-4-en-3-one. ChEs was reduced to the active oxidised state by transferring electrons to molecular oxygen, resulting in the production of H_2O_2.

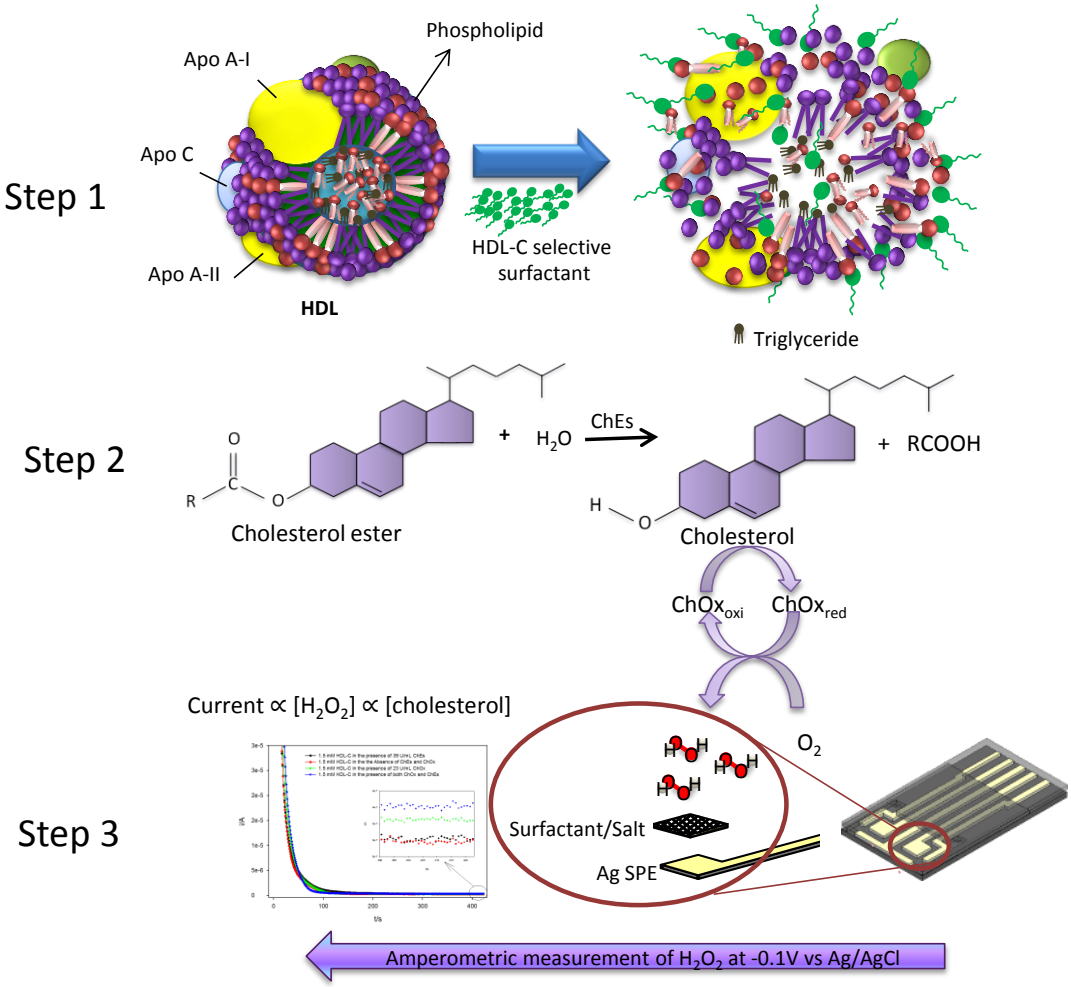

Fig. 2.4. General approach to the amperometric measurement of HDL-C using externally added reagents. Step 1 and 2: externally addition of assay reagent containing HDL-C selective surfactant solubilises the HDL which allows access to cholesterol esterase (ChEs) and cholesterol oxidase (ChOx) presents in the reagent at room temperature. Step 3: The generated H_2O_2 can then be measured using modified electrodes at -0.1 V vs. Ag/AgCl which is measured at 420 s.

Assay reagents were externally added to known concentrations of substrate as shown in Fig. 2.5. The amperometric measurement of H_2O_2 released after enzymatic reaction was then performed at -0.1 V vs Ag/AgCl and the current was typically measured at 420 s. Assay reagent parameters were studied including surfactants (Triton X-100 and Emulgen B-66), buffers (PBS, MOPS or Tris-HCl), enzyme concentrations (20 to 156 U/mL) and incubation times (180 and 240 s). The optimised HDL-C biosensor was based on a final concentration of 6% (w/v) Emulgen B-66/PBS, 39 U/mL ChEs and 23 U/mL ChOx while the optimised TC biosensor was based on 0.5% (v/v) Triton X-100/PBS, 156 U/mL ChEs and 60 U/mL ChOx.

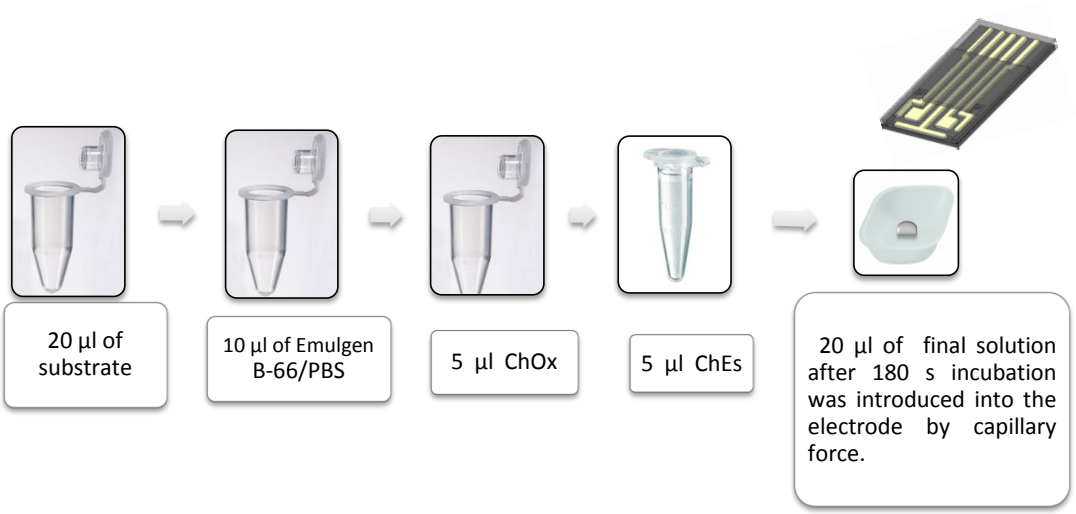

Fig. 2.5. Schematic of assay reagent addition for amperometric measurement of HDL-C using external addition of reagents.

2.4.5 Analysis of clinical samples

Two sets of samples were provided by Alere and were stored at -80°C. One set was spiked human serum samples and the other set was composed of donor samples which were un-manipulated 'off the clot' serum samples from a donor population in San Diego, USA (Table 2.2).

Table 2.2. Composition of the clinical serum samples as per certificate of analysis provided by Alere.

Samples ID	TG (mg/dL)	TC (mM)	GLU (mg/dL)	HDL-C(mM)
1	39.5	1.47	26	0.3
2	138	3.57	48	0.72
3	163.5	4.23	70	0.95
4	183.5	4.93	92	1.2
5	127.5	4.89	113	1.39
6	127	4.17	136	1.7
7	126.5	3.63	183	2.175
8	160.5	5.56	231	0.699
9	211	6.34	267	1.24
10	174	7.5	316	0.44
11	205	8.96	360	0.62
1085S	104.1	5.5	73	2.33
1087S	94.1	4.11	128.8	1.2
1106s	289.9	3.7	120.7	0.74

Each of the samples was analysed using the Abcam cholesterol assay kit for HDL-C and FC while TC was taken as reported in the certificate of analysis. Some of the spiked human serum samples were used as calibrators and the rest were used as clinical samples for HDL-C and TC measurement. For analysis of the clinical samples using the developed biosensors, optimised assay reagent was externally added to the samples and amperometric measurement was performed after 240 s incubation at room temperature using modified electrodes.

2.4.6 Measurement of HDL and LDL/VLDL using the Abcam cholesterol assay kit

Measurement of FC and HDL-C present in clinical samples was performed using the Abcam cholesterol assay kit (ab65390), according to manufacturer's instructions. 100 µL of serum samples were mixed with 100 µL of 2X precipitation buffer in microcentrifuge tubes and incubated for 10 min at room temperature, followed by centrifugation at 5,000 rpm for 10 min. The supernatant containing the HDL-C fraction was incubated for 60 min at 37 °C in the presence of the reaction mix containing 44 µL cholesterol assay buffer, 2 µL cholesterol probe, 2 µL cholesterol enzyme and 2 µL ChEs. For FC measurement, 2 µL cholesterol assay buffer was added instead of ChEs (ab65390 assay protocol). The absorbance of the final solution was then measured at 570 nm. A calibration curve was prepared by measuring the absorbance of cholesterol standards (0 to 5 µg/well).

2.4.7 Development of integrated HDL-C and TC biosensors

Integrated HDL-C and TC biosensors were fabricated using either modified or unmodified electrodes followed by the deposition of assay reagent formulations directly onto the electrode surface using inkjet printing. The average deposited droplet volume of 9.2×10^{-6} µL which was experimentally obtained using the drop volume calibration feature. Parameters such as the number of printed layers and the print design were evaluated. Each printed layer was either on a single silver electrode (Fig. 2.6A), or both silver electrodes (Fig. 2.6B) using a 3.2 $\times$ 3.2 mm pattern each with 25,921 droplets, equivalent to a total volume of 0.238 µL per layer, or on the whole cells area including working, reference and counter electrodes (Fig. 2.6C) of 15 $\times$ 8 mm at a final total volume of 2.77 µL and a drop count of 301,151.

The printed assay reagent formulation was optimised in terms of enzyme concentrations, number of printed layers, incubation time and stabilisers such as PVP and PDA. The final optimised integrated HDL-C biosensor employed four printed layers of assay reagent, of which each layer was composed of a 2.77 µL reagent solution containing ChOx (218 U/mL) and ChEs (633 U/mL) in 6% (w/v) Emulgen B-66/PBS in the presence of 0.2% (w/v) PVP and PDA deposited onto unmodified electrodes on an area of 15 × 8 mm. Optimised TC biosensors were based on ChEs (1044 U/mL) and ChOx (388 U/mL) in 0.25% (v/v) Triton X-100/PBS in the presence of 0.2% (w/v) PVP and PDA . Sensors were then encapsulated as described in Section 2.4.1.

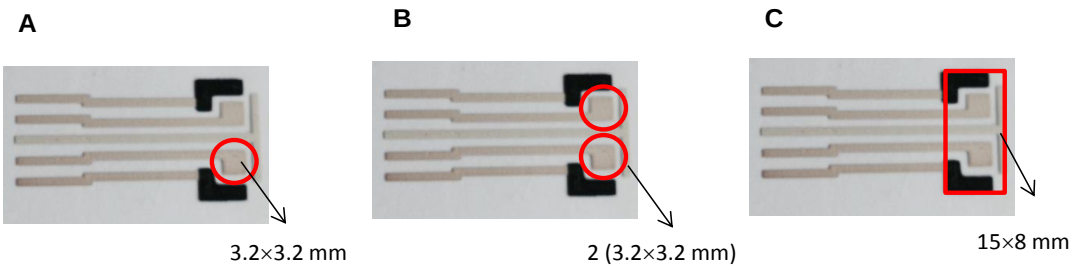

Fig. 2.6. Schematic showing patterns of printing used for the deposition of assay reagent formulation. A: 3.2 × 3.2 mm single silver working electrode, B: 3.2 × 3.2 mm on two working electrodes and C: 15 × 8 mm across dual three-electrode cells.

Fig. 2.7 illustrates the general approach for amperometric measurement of cholesterol using the integrated biosensors. Application of both modified and un-modified silver electrodes were assessed for the development of the integrated biosensors. Typically, 20 µL of sample was directly placed on a clean, flat hydrophobic surface and introduced to the integrated biosensor by capillary filling. The deposited assay reagent containing surfactant and enzymes on the electrode surface was then re-solubilised upon contact to the sample. Some of the main challenges in development of the integrated biosensor are discussed in Chapter 6.

The sensor was allowed to incubate for 240 s with cholesterol sample in order to release H_2O_2 following enzymatic reaction and the current was measured using chronapmerometry at -0.1 V vs. Ag/AgCl after 420 s.

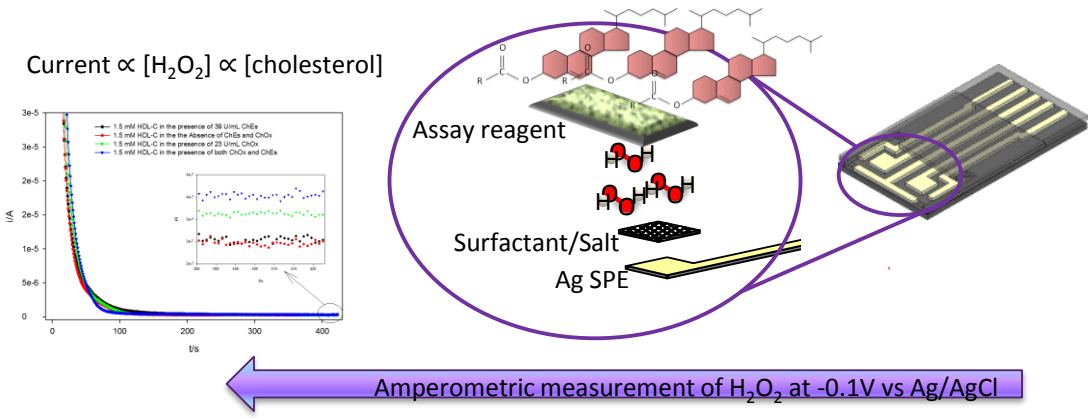

Fig. 2.7. General approach to the amperometric measurement of cholesterol in delipidated serum using integrated biosensors. Application of the sample to the sensor solubilises the reagent layer. H_2O_2 is produced following the reaction of surfactants with lipoproteins and enzymes with cholesterols at room temperature for 240 s. Electrodes, either modified or unmodified catalyse the reduction of H_2O_2 at the silver electrode surface at -0.1 V vs. AgCl which is measured at 420 s.

2.5 Research Governance

All of the research was carried out in accordance with UWE's Code of Good Research Conduct (http://www1.uwe.ac.uk/research/researchgovernance). All relevant ethical and health and safety requirements were adhered to.

References

Gonzalez-Macia, L., Smyth, M.R. and Killard, A.J. (2012) A Printed Electrocatalyst for Hydrogen Peroxide Reduction. *Electroanalysis*. 24 (3), pp. 609-614.

Hino, K., Nakamura, M. and Manabe, M. (1998) *Method for Quantitatively Determining Cholesterol* Daiichi Pure Chemicals Co., Ltd. 5773304.

Nakamura, M., Taniguchi, Y., Yamamoto, M. and Manabe, M. (2009) *Method of Pretreatment of Sample for Quantitating Cholesterol and Method for Quantitating Cholesterol in Specific Lipoproteins.*Daiichi Pure Chemicals Co., Ltd. 20090226945.

Tamura, H., Nishino, S., Yamaguchi, T. and Hino, K. (2009) *Test Piece for Measureing HDL- Cholesterol,*Daiichi Chemical Co., L. US7575884.

Yamamoto, S., Yamamoto, M. and Nakanishi, K. (2011) *Method of Measuring Lipid in Specific Lipoprotein.*Daiichi Chemical Co., L. US 2011/0091917.

Chapter 3

Development and optimisation of assays for the measurement of TC and HDL-C

3.1 Introduction

As already discussed in Chapter 1, there are several assay methodologies for measuring cholesterol, and more specifically HDL-C. All of these methods determine the amount of H_2O_2 produced after the enzymatic reaction of cholesterol when measured spectrophotometrically following the formation of a dye complex. Although the homogeneous assay methodologies allow direct and selective analysis of HDL-C using automated devices, development of a spectrophotometric assay capable of measuring HDL-C at room temperature with minimum dilution factor is critical as a foundation for the development of an analogous electrochemical biosensor and so it is necessary to understand the mechanisms and kinetics of the enzymatic reaction under conditions suitable for development of the electrochemical biosensor.

In this chapter, a spectrophotometric assay with minimum dilution factor was developed for HDL-C and TC measurement. In order to assist the development and optimization of the assay properties, a spectrophotometric assay was designed by considering several assay methodologies reported in literatures and patents (Hino, Nakamura and Manabe, 1998; Nakanishi, Nakamura and Hino, 1998; Tamura *et al.,* 2009). An assay employing 39 U/mL ChEs, 23 U/mL ChOx, 1% (w/v) Emulgen B-66, 0.05% (w/v) 4-AAP, 0.04% (v/v) DMT and 5 U/mL HRP was used as the starting point for assay development.

Optimisation studies were performed to assess the most suitable enzyme concentrations capable of measuring HDL-C in its clinically relevant range at room temperature which would be more suitable for subsequent room temperature operation of the biosensor. To ensure that the assay was optimised for the quantitative measurement of HDL-C, each step of the assay was studied and optimised to ensure that it did not constitute a rate limiting step in the assay. Enzyme assays can be performed either when the reaction has gone to completion, or kinetically, by looking at the rates of reaction during the earlier phase of the assay (Copeland, 2000; Tipton, 1992).

The effect of different surfactants such as Emulgen B-66, Triton X-100, and sodium cholate on the selective measurement of HDL-C over LDL-C was evaluated. Triton X-100, and sodium cholate were used as non-selective surfactants for the measurement of TC present in HDL-C and LDL-C samples for comparison with the TC measured in the presence of HDL-C selective surfactant. Optimisation was then

performed to evaluate the most suitable surfactant concentrations for spectrophotometric measurement of HDL-C and TC. The final optimised formulation was applied for the selective measurement of HDL-C in mixtures of HDL-C and LDL-C prepared in delipidated serum.

3.2 Development of a spectrophotometric assay for the measurement of cholesterol

In this section, the operation of an assay methodology employing 39 U/mL ChEs, 23 U/mL ChOx, 1% (w/v) Emulgen B-66, 0.05% (w/v) 4-AAP, 0.04% (v/v) DMT and 5 U/mL HRP for HDL-C measurement in its clinically relevant range was monitored spectrophotometrically for 1800 s at room temperature (RT). This was performed to evaluate the possibility of occuring the enzymatic reaction at room temperature while using 1 in 4 dilution of the sample (24 µL sample in a 100 µL total reaction volume).

Fig. 3.1 shows the effect of the HDL-C concentration on the reaction kinetics of the assay. Under these experimental conditions, initial reaction rates were similar for all concentrations of HDL-C, suggesting that the reaction rate was not limited by the substrate concentration. The responses typically reached a plateau sooner for lower concentrations of HDL-C. Most reactions had reached completion by approx. 800 s. The inset shows a plot of absorbance vs. H_2O_2 concentration at 400 and 800 s (6.6 and 13.3 min) showing linearity only up to approx. 1.2 or 1.6 mM. This could be due to enzyme limitation due to the reduced rate of reaction for these coupled enzyme reactions at RT in which a lag phase can result. This lag phase can result from a delay in the formation of the final product which occurs as a result of the build up in concentration of the intermediate product which is a substrate for the second reaction (Fig. 3.2) (Copeland, 2000). Alternatively, the delay could also be due to the time required for the dissolution of HDL-C to make the esterified cholesterol available for enzymatic catalysis.

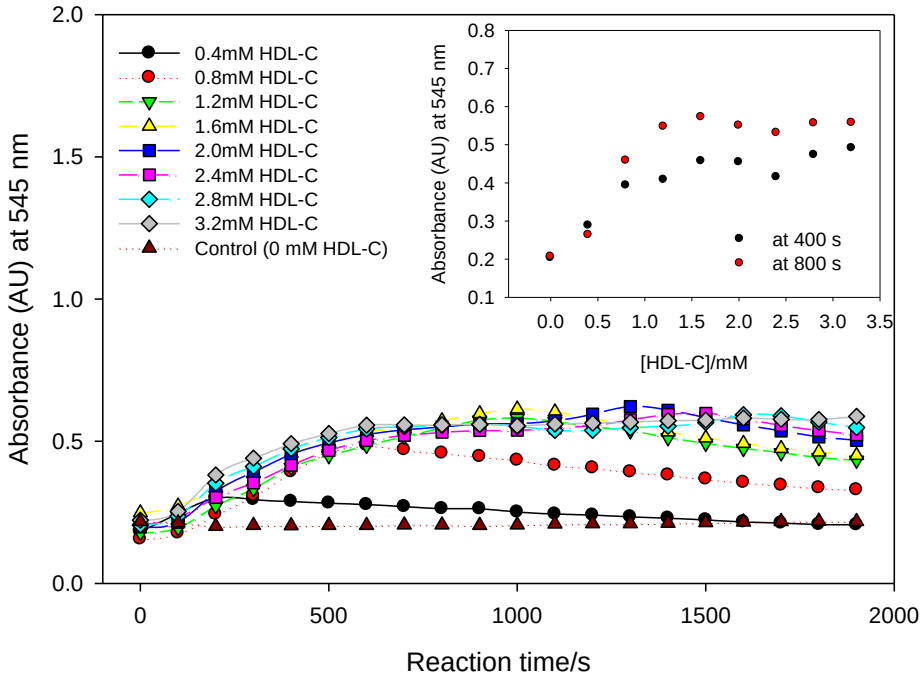

Fig. 3.1. The effect of HDL-C concentration on the kinetics of the spectrophotometric assay. Inset: Plot of absorbance vs. HDL-C concentration (t=400 and 800 s).

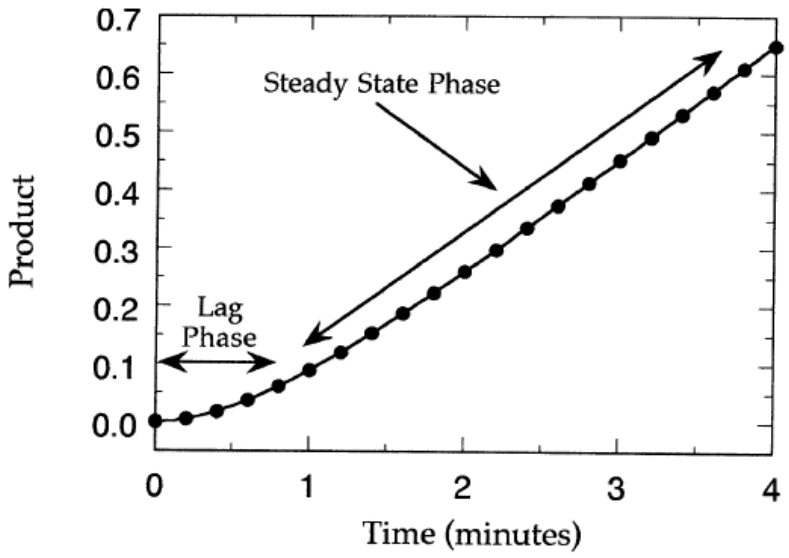

Fig. 3.2. Kinetic response of a coupled enzyme reaction showing the lag phase and steady state phase [Used with permission of the publisher (Copeland, 2000)].

Therefore, the use of HDL-C as a substrate adds an additional kinetic step in the reaction of HDL-C. To reduce the complication introduced by the use of HDL-C in

this optimization study, cholesteryl acetate was then employed. Cholesteryl acetate has been shown to be a suitable substrate for ChEs in the electrochemical determination of blood TC (Fang, 2011; Hooda *et al.,* 2009). It is soluble in water and easier to work with than HDL-C. Control experiments were performed with 3 mM cholesteryl acetate as substrate at RT (Fig. 3.3).

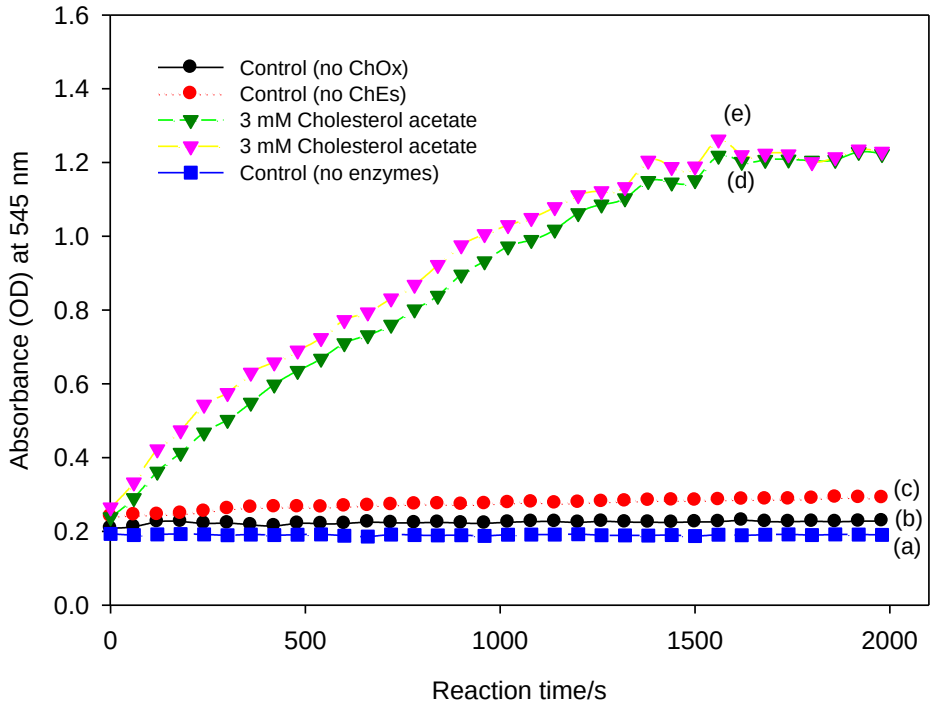

Fig. 3.3. Use of 3 mM cholesteryl acetate as substrate for assay optimisation. a) No ChEs or ChOx; b) no ChOx; c) no ChEs; d and e) repeats with all assay components.

These confirmed that catalysis of the cholesteryl acetate using this assay protocol was very effective, with no observable lag phase and reaching a final absorbance reading of 1.2 AU after approximately 1500 s. This demonstrated that cholesteryl acetate would act as an effective substrate with which to optimise the enzymatic steps of the assay. However, assay optimisation must be performed at a concentration which is within the linear range of the spectrophotometric assay. Assays employing this protocol typically use an effective 1 in 4 dilution of the sample (24 µL sample in a 100 µL total reaction volume). Given that the method showed linearity up to 1.2 mM HDL-C concentration, further optimisation was performed using 1.2 mM cholesteryl acetate concentrations. Consequently, the optimization of each step was carried out using cholesteryl acetate as substrate,

commencing with the final step. When the optimum concentration of an assay component was ascertained, it was fixed at that concentration for subsequent optimisation of the previous assay step.

Since the colorimetric measurement of H_2O_2 is the final step in the enzymatic reaction of cholesteryl acetate, it is very important that this step is not rate limiting. Therefore, for the optimization of the colorimetric substrate formulation, the formulation developed by Hino *et al.* (1998) and Tamura *et al.* (2009) and adapted here was termed the 'initial formulation' (Table 3.1).

To see if any increase in concentrations of this formulation would have an effect on the rate of the reaction, two additional colorimetric reagent formulations with reagents at 1.5 times and 3 times that of the initial formulation were prepared (Table 3.1). Concentrations of ChEs (107 U/mL) and ChOx (72 U/mL) which were approx. three times greater than those reported by Tamura *et al.* (2009) were used as start points in this optimisation to ensure that neither one would be rate limiting. In the electrochemical sensor for cholesterol, this step will be replaced by the measurement of the reduction of H_2O_2 at the electrode. However, for assay development using the spectrophotometric assay, it was important to ensure that this assay step did not limit the performance of the assay when performed at RT.

Table 3.1. Colorimetric reagent formulations employed for assay optimisation.

	Initial formulation	**1.5 x concentration**	**3 x concentration**
4-AAP (% w/v)	0.05	0.075	0.15
DMT (% v/v)	0.04	0.06	0.12
HRP (U/mL)	9	14	28

Fig. 3.4 illustrates the effect of the three different concentrations of colorimetric reagent on the kinetics of the assay at RT in 1.2 mM cholesteryl acetate. All curves in Fig. 3.4 showed a typical substrate utilization profile where the final steady state response was related to the total concentration of substrate. No lag phase was observed at the beginning of the reaction. The reaction rates for 1.5x and 3x were more rapid than for the initial formulation, which suggested some rate limitation from the colorimetric reagent in the initial formulation. However, the similarity of 1.5x and 3x suggests no rate limitation at these concentrations. The 1.5x and 3x mixtures also reached saturation more rapidly at approx. 200 s, as opposed to 400

s for the initial formulation. Further assay optimization was performed based on a colorimetric reagent formula at 1.5x the initial formula concentrations.

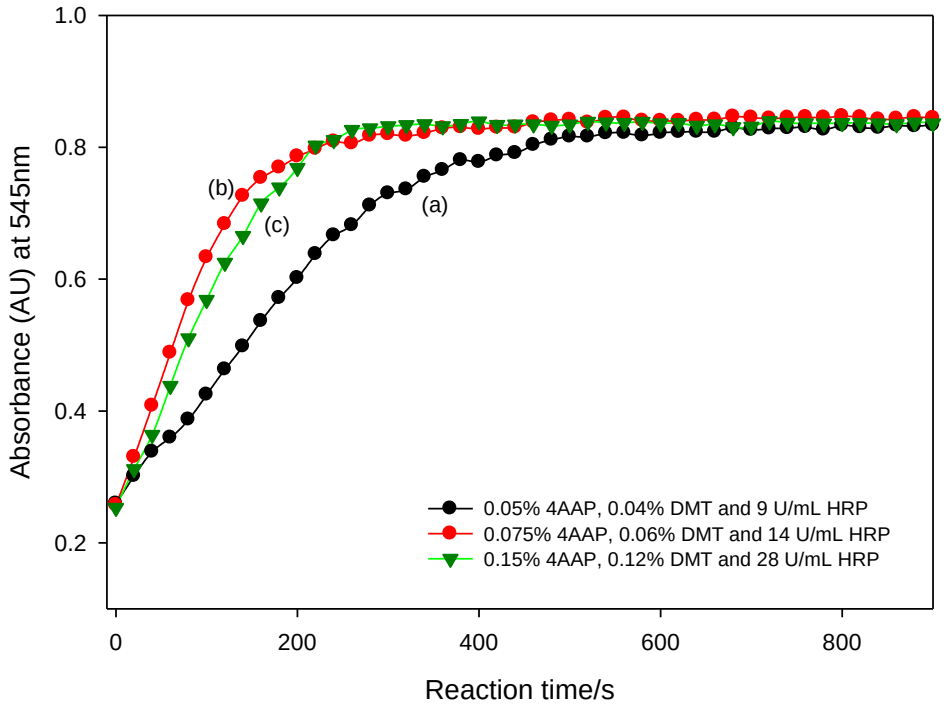

Fig. 3.4. Effect of colorimetric reagent formulation concentration on assay reaction kinetics in 1.2 mM cholesteryl acetate at RT using a) Initial formulation; b) 1.5x and; c) 3x reagent concentrations.

Following optimisation of the colorimetric reagent, optimisation of ChEs was performed using concentrations of 39, 79 and 107 U/mL, again using an elevated concentration of ChOx of 72 U/mL in an attempt to ensure no kinetic limitation in this step. Fig. 3.5 shows the effect of ChEs concentration on the overall reaction kinetics of the enzyme assay. As can be seen, the final absorbance value for all three concentrations was approx. 0.9 AU, and all had similar initial reaction rate kinetics. This shows that increasing the concentration of ChEs from 39 U/mL to 78 and 107 U/mL had no effect on the rate of the process, which suggests that the ChEs concentration was not rate limiting at these concentrations. Therefore, 39 U/mL ChEs was selected for further assay optimisation.

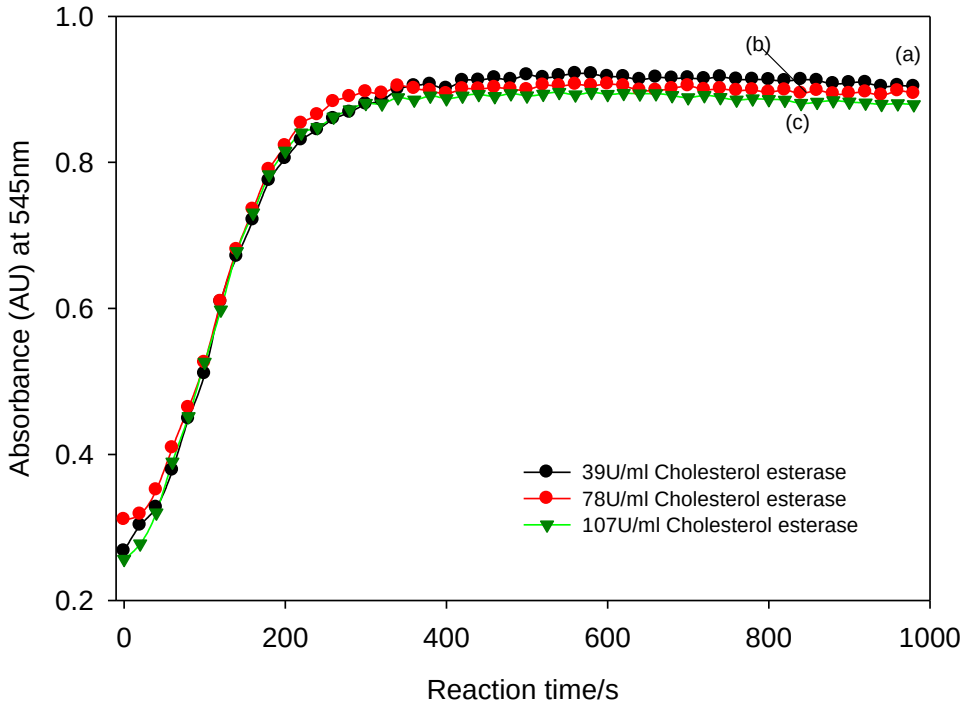

Fig. 3.5. Effect of ChEs concentration on assay reaction kinetics in 1.2 mM cholesteryl acetate at RT using a) Initial formula; b) 2x and; c) 3x reagent concentrations.

Based on the optimised conditions for HRP and ChEs, ChOx concentration was next optimised using concentrations of ChOx of 23, 46 and 72 U/mL. 23 U/mL was the initial concentration and 46 and 72 U/mL represented concentrations that were approx. 2 and 3 times more than the initial formulation concentrations. Fig. 3.6 illustrates the plot of absorbance vs. reaction time of 1.2 mM cholesteryl acetate in the presence of 39 U/mL ChEs, 14 U/mL HRP and different concentrations of ChOx. As can be seen, the final absorbance for all three concentrations of ChOx was approx. 0.9 AU. The initial reaction rate kinetics was also similar for all concentrations showing that increasing the concentration of ChOx from 23 U/mL to 46 and 72 had no effect on the reaction rate of the process. This suggests that the ChEs concentration used in this experiment was not rate limiting and therefore 23 U/mL ChOx was selected for future assay optimisation.

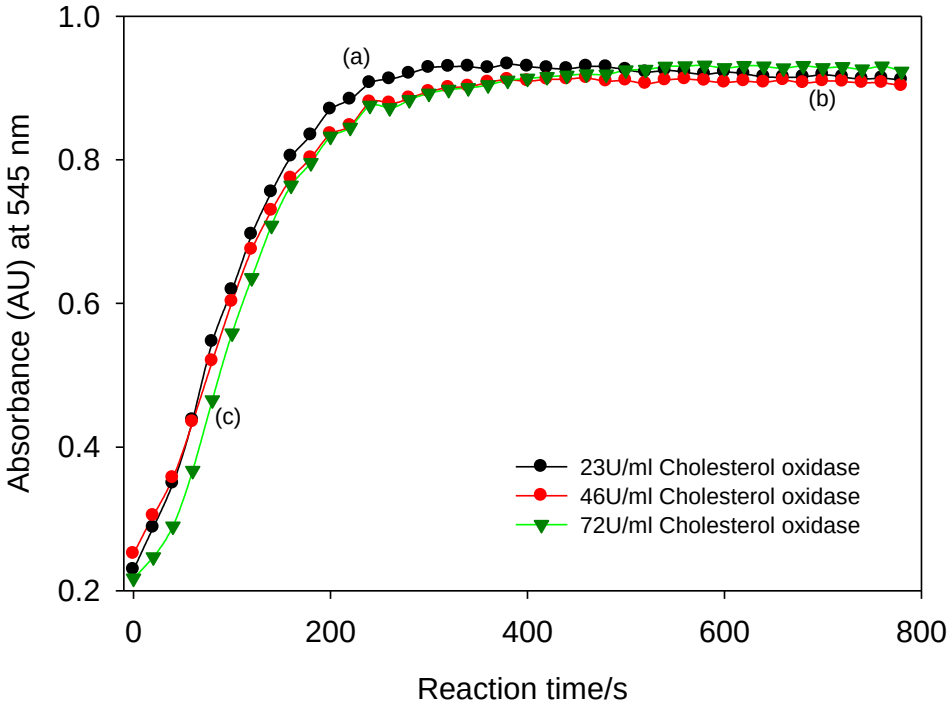

Fig. 3.6. Effect of ChOx concentration on assay reaction kinetics in 1.2 mM cholesteryl acetate and RT using a) Initial formula; b) 2x and; c) 3x reagent concentrations.

A more detailed kinetic study was then performed with the re-optimised assay formulation detailed in Table 3.2. Cholesteryl acetate was then assayed in the range of 0 to 4.0 mM which is in the clinically relevant range. Fig. 3.7 shows the kinetics of the assay at RT.

Table 3.2. Optimised spectrophotometric assay formulation.

Ingredient	Concentration
Cholesterol esterase	39 U/mL
Cholesterol oxidase	23 U/mL
Horseradish peroxidase	14 U/mL
4-AAP	0.075% (w/v)
DMT	0.06% (v/v)

Under these assay conditions, the initial reaction rates were related to the concentration of cholesteryl acetate, with rates being greater for higher concentrations, indicating partial, if not complete substrate (cholesteryl acetate) limitation at RT. The inset in Fig. 3.7 shows the relationship between the cholesteryl acetate concentration and absorbance at 545 nm at 180 and 420 s. As shown, the reaction completion time was increased from 180 to 400 s at substrate concentrations above 2.0 mM. The steady state absorbance value also appeared to

be reasonably stable over the duration of the assay (7 min). The assay showed an acceptable linearity at 420 s from 0 to 4.0 mM cholesteryl acetate (slope=1.54, r^2=0.99). Since most point of care assays must be completed within a matter of minutes (Lee *et al.,* 2011), therefore, a target assay time of 10 min or less for final amperometric measurement of cholesterol was defined.

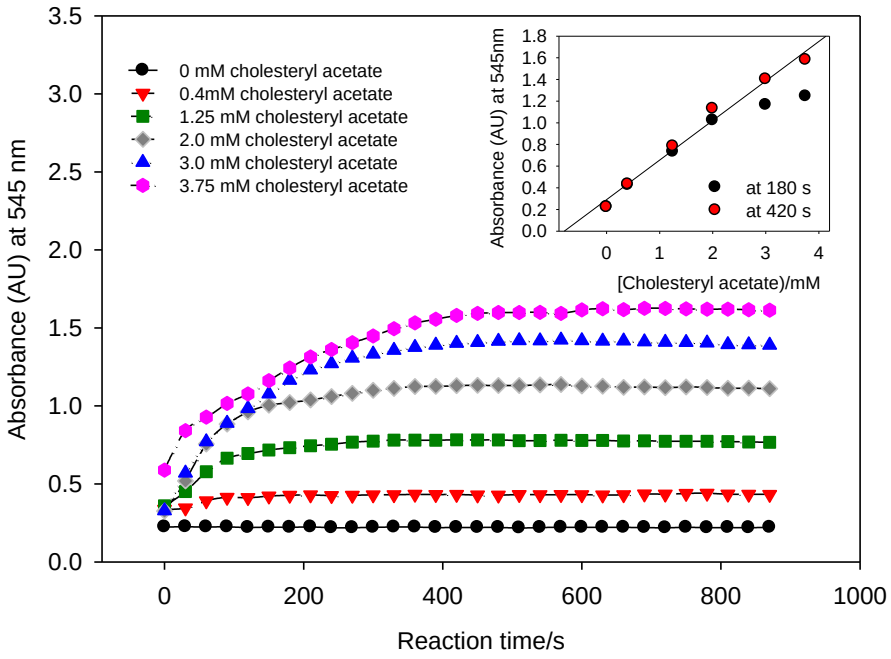

Fig. 3.7. Reaction kinetics of the optimised spectrophotometric assay in cholesteryl acetate at RT. Inset: Plot of absorbance vs. concentration of cholesteryl acetate at t=180 and 420 s.

To sum up, a spectrophotometric method was established at RT for the measurement of cholesteryl acetate following optimisation of the assay reagent concentrations. The method showed reasonable linearity between 0 to 4 mM with an assay time of 420 s. The next step was to apply this assay methodology to the measurement of esterified cholesterols in HDL following solubilisation using selective surfactants, and in TC measurement using non-selective surfactants. To achieve this, optimisation of the surfactants to be used was necessary.

3.3 Evaluation of different surfactants on the selective measurement of HDL-cholesterol and total cholesterol

The spectrophotometric formulation developed in section 3.2 showed an acceptable linearity at 420 s from 0 to 4.0 mM cholesteryl acetate (slope=1.54, r^2=0.99). Since most point of care assays must be completed within a matter of

minutes (Lee *et al.,* 2011)., a target assay time of 10 min or less for final amperometric measurement of cholesterol was defined.

As discussed in the introduction, cholesterol is present in both esterified and non-esterified forms, either free or in association with various lipoproteins. Thus, for the measurement of TC, all of this must be made accessible to enzymatic catalysis by ChEs and ChOx. The surfactant Triton X-100 (polyethylene glycol alkyl aryl ether) is a widely used non-ionic surfactant which has been found to be suitable for the total dissolution of cholesterol in blood (Allain *et al.,* 1974; Brahim, Narinesingh and Guiseppi-Elie, 2001; Richmond, 1973; Richmond, 1976; Shih, Yang and Lin, 2009; Zoppi and Fenili, 1976). It has been shown to disrupt all types of lipoprotein structures and is also able to maintain the released cholesterol in micellar suspension. The cholesterol suspension has also been shown to be amenable to enzymatic reaction.

Sodium cholate is another surfactant which has also been used for the total solubilisation of all blood cholesterol (TC) (Akimova and Melgunov, 1984; Allain *et al.,* 1974; Mitra and Dungan, 2001; Richmond, 1976). It has been reported that surfactant molecules with a hydrophobic portion containing fused-ring structures such as sodium cholate, solubilised cholesterol significantly better than surfactants containing a linear hydrocarbon chain such as Triton X-100 (Mitra and Dungan, 2001). However, sodium cholate is an ionic surfactant (Lin *et al.,* 2007) which may have some impact on final amperometric measurement of H_2O_2 using modified silver electrodes.

In contrast, for the selective measurement of HDL-C, a surfactant has been identified which is capable of only solubilising HDL particles, making this fraction selectively available for enzymatic degradation. This surfactant, Emulgen B-66, is a polyoxyethylene derivative with a hydrophile-lipophile balance of 13.2 which enables the selective breakdown of HDL particles, as opposed to other lipoproteins such as LDL, VLDL and CM (Nakamura *et al.,* 2009; Okada *et al.,* 2001; Tamura *et al.,* 2009). Although the exact mechanism of HDL solubilisation remains unclear, it is believed that this effect might be related to the presence of different apoprotein types in HDL and non-HDL. Apoprotein A-I and II are the major apoproteins in HDL, while non-HDLs such as LDL, CM and VLDL all have just one molecule of apoprotein B-100. A surfactant which is selective for HDL-C can distinguish between these proteins and may solubilise the polar lipids via a specific interaction

with this apolipoprotein. This is believed to be related to the surfactant's hydrophilic-lipophilic balance (HLB) (Kane, 1983; Kondo, 1999; Kurosaki and Ogawa, 2009; Okada *et al.,* 2001; Okada, 2001; Segrest *et al.,* 1992; Segrest *et al.,* 2001).

The biological effects of surfactants depend on the concentration and structure of the chosen surfactants resulting from penetration of the membrane, its fluidization and solubilisation. It has also been found that there is parabolic relationship between membrane activity and lipophilicity of non-ionic surfactant which varies in lower and higher concentrations of surfactant (Florence, Tucker and Walters, 1984; Tucker and Florence, 1983). Therefore, these surfactants were studied for their use in the measurement of HDL-C and TC in the assays developed here.

Before the surfactants associated with HDL-C (Emulgen B-66) and TC (Triton X-100 and sodium cholate) measurement could be employed, they were first evaluated for any impact they might have on the behaviour of the optimised enzyme assay. To assess this, the assay was performed using 2.0 mM cholesteryl acetate as substrate in the presence of these surfactants in PBS. Previous studies have indicated the suitability of Emulgen B-66 at 1% (w/v) for measurement of HDL-C (Nakamura *et al.,* 2009; Okada *et al.,* 2001) and 0.5% (v/v) Triton X-100 and 8 mM sodium cholate (Allain *et al.,* 1974; Richmond, 1976) for TC measurement. So to assess the effect on the enzyme assay, these concentrations were employed (Fig. 3.8). The assay control in the absence of ChEs and ChOx gave an absorbance of 0.23 AU. The assay response in PBS was 1.38 AU, while in 1% (w/v) Emulgen B-66, 0.5% (v/v) Triton X-100, and 8 mM sodium cholate the responses were 1.35, 1.39 and 1.35, respectively, showing little, if any significant difference in absorbance between the PBS control and the surfactants, and confirming that the surfactants used had no impact on the enzymatic assay. The next step was to then evaluate these surfactants as the basis of assays for HDL-C and TC.

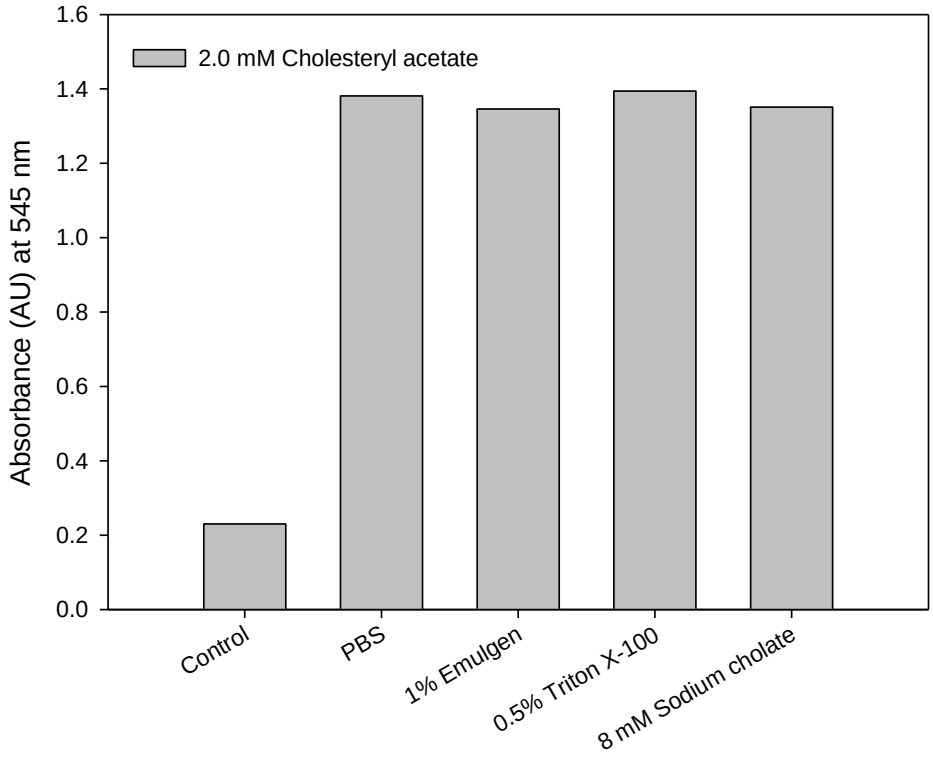

Fig. 3.8. Spectrophotometric enzyme assay of 2.0 mM cholesteryl acetate in the presence of surfactants. Control contained no enzymes.

3.3. 1 Evaluation of the efficiency and selectivity of different surfactants in the measurement of HDL-C and TC

As was mentioned before, lipoproteins are a complex of lipids and apolipoproteins. Apolipoproteins have an amphipathic property. They surround lipids and enable them to be transported within blood stream. The solubility of these lipoproteins in the presence of surfactants might be due to the partial unfolding of their protein and interaction of surfactant with the lipoprotein surface layer. Higher concentrations of ionic and non-ionic surfactants with HLB values less than 14.6 were found to decrease the Stokes' radius of proteins, which might be due to delipidation of the lipoproteins. In the absence of surfactant, protein adsorbs at the water/fluid layer in a diffusion controlled manner which may result in very slow enzymatic reactions with ChEs and ChOx (Miller *et al.,* 2000; Tucker and Florence, 1983).

In the presence of surfactant, there are different types of proteins/surfactant interaction which lead to protein/surfactant complexes with different surface activities compared to pure protein. Ionic surfactants bond to proteins via electrostatic interactions, leading to higher surface activity, while non-ionic

surfactants adsorb competitively with the protein with a hydrophobic interaction which is less surface active than the pure proteins (Miller *et al.,* 2000).

Having established that the identified surfactants had no impact on the behaviour of the enzyme assay, the effect of these surfactants and their concentrations on the measurement of TC and non-esterified cholesterol within HDL-C was assessed. The absorbance value of non-esterified cholesterol was considered as the control in the presence of LDL-C due to the presence of significant levels of non-esterified cholesterol in LDL stock solutions. To do this, 2.0 mM HDL-C (prepared in PBS 0.1 M pH 6.8) was used as a substrate in the presence and absence of the selected surfactants. Since Emulgen B-66 is known to be capable of the solubilisation of HDL-C and Triton X-100 and sodium cholate are known to be capable of the solubilisation of all lipoproteins, all surfactants should be comparable in their ability to solubilise HDL-C. The enzyme assay was used to determine the amount of measurable cholesterol from the HDL sample (2.0 mM) following its solubilisation with the various surfactants. In addition, however, measurement with ChOx alone was used to determine the fraction of measurable non-esterified cholesterol, while measurement with ChEs and ChOx allowed the determination of both esterified and non-esterified forms, as some free cholesterol is always present in the sample. The kinetic assay responses are shown in Fig. 3.9, while the absorbances at 600 s are compiled in the inset and in Table 3.3.

The amount of non-esterified cholesterol measured following HDL-C solubilisation by surfactants was only fractionally greater than that measured in PBS alone, while all surfactants showed similar values. This indicated that most of the non-esterified cholesterol was available without solubilisation. The amount of both esterified and non-esterified cholesterol measured was greater than the amount of non-esterified cholesterol in PBS, which suggested a fraction of esterified cholesterol was available without solubilisation of lipoprotein. However, significantly more esterified cholesterol could be measured in the presence of surfactants. In addition, all surfactants appeared to be comparably effective at solubilisation of the esterified cholesterol fraction present in HDL, and that HDL-C can be effectively solubilised in the presence of 1% (w/v) Emulgen B-66.

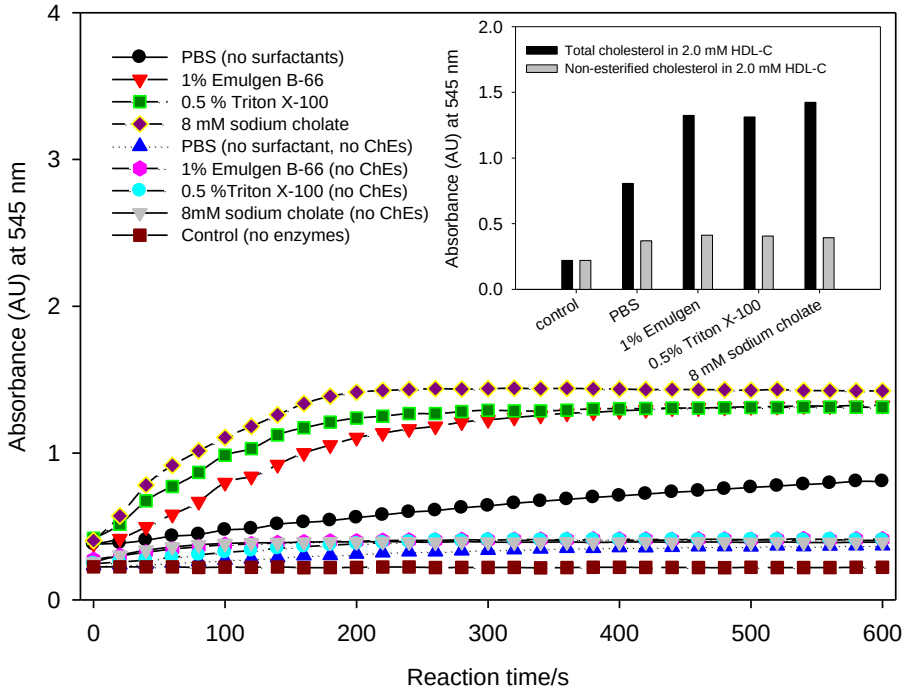

Fig. 3.9. Enzyme assay responses in 2.0 mM HDL-C at RT at 545 nm in different surfactants. Control (reagent blank) contains assay reagent without enzymes. Non-esterified cholesterol was determined in the absence of ChEs Inset: Absorbance at 600 s.

Table 3.3. Measurement of total cholesterol and non-esterified cholesterol from 2.0 mM HDL-C in the presence of specific and non-specific surfactants (Absorbance was measured at 545 nm).

Type of surfactant	Cholesterol fraction measured	
	Esterified and non-esterified cholesterol (ChEs + ChOx) (Absorbance)	Non-esterified cholesterol (ChOx) (Absorbance)
Substrate blank (no enzyme)	0.222	0.222
PBS (no surfactant)	0.807	0.370
1% (w/v) Emulgen B-66	1.32	0.413
0.5% (v/v) Triton X-100	1.31	0.406
8 mM sodium cholate	1.42	0.392

After assessing the effect of individual surfactants on the measurement of cholesterol from HDL-C, the next step was to assess the specificity of Emulgen B-66 for HDL-C. This meant determining its ability (or rather inability) to solubilise non-HDL lipoproteins. Among the non-HDLs (that is LDL, VLDL and CM), LDL-C carries most of the cholesterol in the body. Since the surfactant Emulgen B-66 is believed to exhibit the same kind of effect on all lipoproteins containing apo-B, and since all the non-HDLs possess a single apo-B lipoprotein (Murphy *et al.,* 2009),

LDL-C was used as the representative for all non-HDLs in assessing surfactant selectivity. Enzyme assays were again performed, this time with 2.0 mM LDL-C, either with ChOx or ChEs and ChOx to measure levels of non-esterified or esterified cholesterol, respectively (Fig. 3.10, Table 3.4).

By comparing the results for 2.0 mM HDL-C and 2.0 mM LDL-C, the absorbances observed for 2.0 mM LDL-C were 1.35 and 1.44 in the presence of 0.5% (v/v) Triton X-100 and 8 mM sodium cholate, respectively which were almost the same intensity as the absorbance observed for 2.0 mM HDL-C in the presence of these two surfactants (1.31 and 1.42, respectively). This suggests that both of these surfactants could be used for TC measurement of the samples as both of them were able to disrupt both types of lipoproteins.

The absorbance for measurement of esterified and non-esterified cholesterol from 2.0 mM LDL-C in the presence of 1% (w/v) Emulgen B-66 was 0.65 compared to 1.32 for 2.0 mM HDL-C, which constitutes a reduction of almost half. For a surfactant to selectively measure HDL-C, the differentiation between HDL-C and LDL-C is required to be around 50 to 90% (Murphy *et al.,* 2009). The differentiation between HDL-C and LDL-C is calculated according to the following formula:

$$\text{Differentiation (\%)} = (G_{HDL}-G_{LDL})*100/G_{HDL}$$

where G is the gradient of the measured response vs. known concentrations of the sample. The percentage differentiation between HDL-C and LDL-C using this formula, and after removing background absorbance from the absorbance value observed for each of them in the presence of 1% (w/v) Emulgen B-66, was 60% in this case. This was within the percentage range defined by Murphy *et al*. (2009).

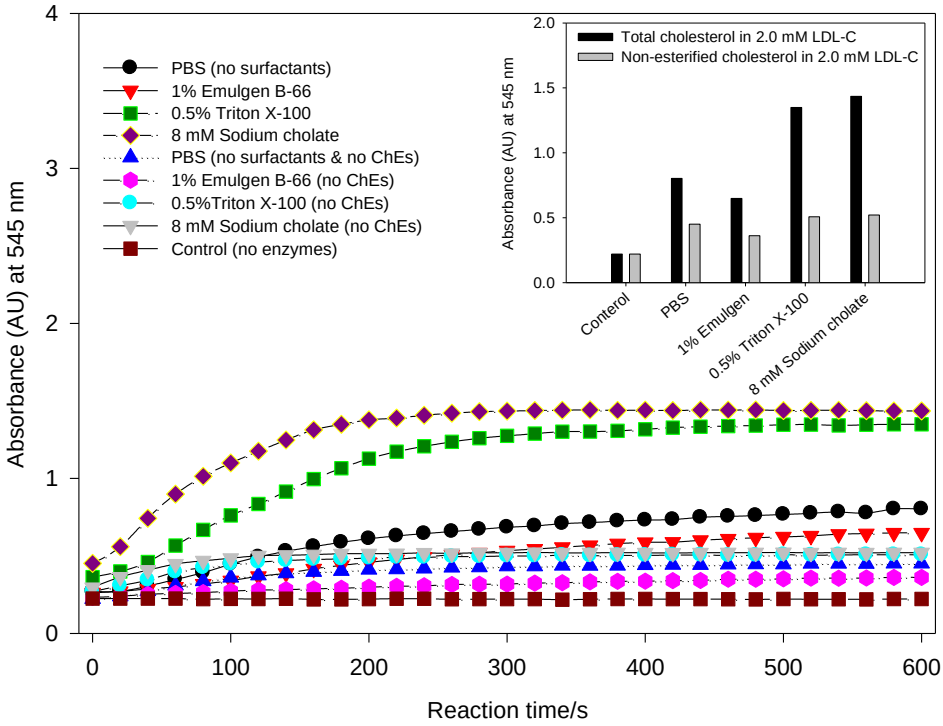

Fig. 3.10. Enzyme assay response in 2.0 mM LDL-C at RT at 545 nm in different surfactants. Control (reagent blank) contains assay reagent without enzymes. Non-esterified cholesterol was determined in the absence of ChEs Inset: Absorbance at 600 s.

Table 3.4. Total cholesterol and non-esterified cholesterol measurement of 2.0 mM LDL-C in the presence of HDL-C specific and non-specific surfactants (Absorbance was measured at 545 nm).

Type of surfactant	Cholesterol fraction measured	
	Esterified and non-esterified cholesterol (ChEs + ChOx) (Absorbance)	Non-esterified cholesterol (ChOx) (Absorbance)
Substrate blank (no enzyme)	0.222	0.222
PBS (no surfactant)	0.803	0.451
1% (w/v) Emulgen B-66	0.649	0.361
0.5% (v/v) Triton X-100	1.35	0.507
8 mM sodium cholate	1.44	0.521

The percentage recovery of TC and cholesterol ester present in 2.0 mM LDL-C was also calculated. This was achieved by assigning the measured concentration of cholesterol from LDL-C in the presence of 0.5% (v/v) Triton X-100 as 100%. The absorbance value of 0.22 AU was used as a background absorbance for the calculation of TC recovery of 2.0 mM LDL-C. For calculating the percentage recovery of cholesterol ester, the absorbance value of non-esterified cholesterol in

the absence of surfactant (0.45 AU) was subtracted from the value of total cholesterol measurement in the presence of surfactants. The percentage recovery of total cholesterol and cholesterol ester from 2.0 mM LDL-C in the presence of 1% (w/v) Emulgen B-66 were found to be approx. 38% and 21.3%, respectively, which demonstrated that further optimisation was required if adequate efficiency was to be achieved.

Assigning 100% to the absorbance value obtained for the recovery of cholesterol from 2.0 mM LDL-C in the presence of 8 mM sodium cholate, this gave percentage recoveries of 35 and 19.4% for TC and esterified cholesterol measurement in 1% (w/v) Emulgen B-66, respectively. This suggests that both Triton X-100 and sodium cholate can be used for total cholesterol measurement. However, Triton X-100 is a non-ionic surfactant and may have less impact on the final amperometric measurement method than cholate. Therefore, Triton X-100 was used for future developments of TC assays.

Given that the selectivity of 1% (w/v) Emulgen B-66 was relatively poor, alternative strategies were investigated to see if this could be improved. Others have used alternative surfactants such as Emulgen A90 (Yamamoto, Yamamoto and Nakanishi, 2011) and Pluronic F-68 (Tamura *et al.,* 2009) to prevent the non-HDL lipoprotein from dissolving. However, it was found that Emulgen A90 demonstrated a decrease in selectivity towards both HDL and LDL cholesterol, while Pluronic F-68 showed no significant improvement in bioassay selectivity towards HDL-C (data not shown). These methods were not studied further.

Others have shown that the addition of di-cations such as $MgCl_2$ improves selectivity (Okada *et al.,* 2001; Okada, 1998). Therefore, the ability of Emulgen B-66 in association with $MgCl_2$ to solubilise LDL-C was evaluated, alongside Triton X-100 which was used to evaluate TC (Fig. 3.11). The best percentage recovery was achieved in the presence of 1% (w/v) Emulgen B-66 and 20 mM $MgCl_2$ which was 40.6 and 17.7% for total cholesterol and esterified cholesterol in 2.0 mM LDL-C, respectively. Therefore, little improvement was seen across the concentration range studied and this approach was not considered further.

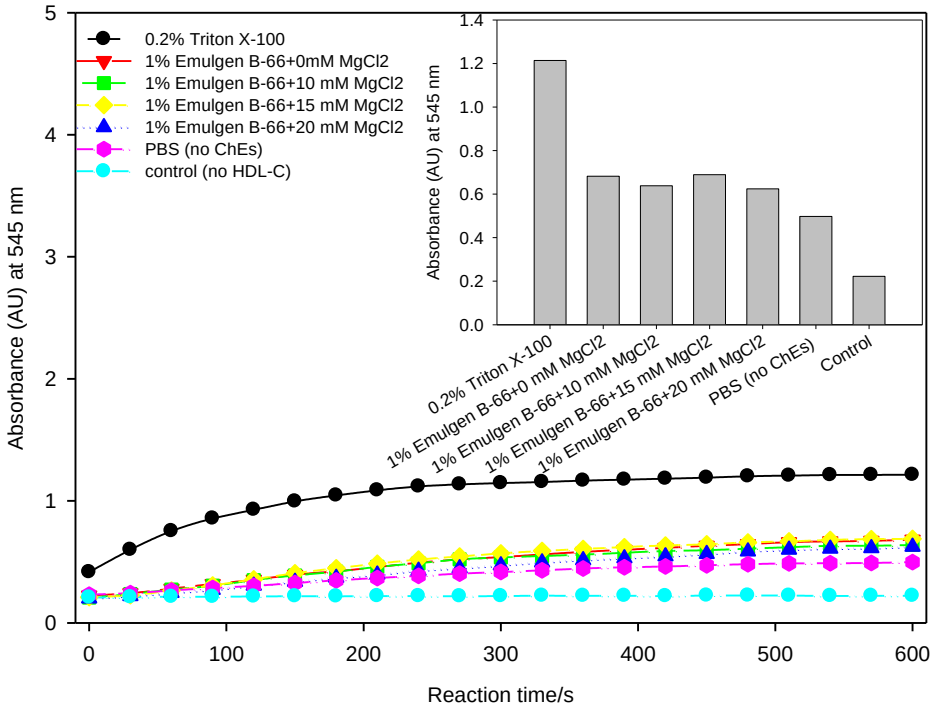

Fig. 3.11. Effect of supplementation of Emulgen B-66 with $MgCl_2$ on selectivity to LDL-C. Enzyme assays in 2.0 mM LDL-C at RT. Inset: Absorbance at 600 s.

In summary, Triton X-100 was selected for further optimisation of TC measurement, while Emulgen B-66 was selected for use in selective HDL-C assays. However, both surfactants required further individual and joint optimisation to achieve the most efficient measurement of both TC, HDL-C, and by subtraction, non-HDL-C. This optimisation is dealt with in the following sections.

3.3. 2 Optimisation of the measurement of total cholesterol

As was discussed earlier, Triton X-100 was able to solubilise both HDL and LDL cholesterols, making their cholesterol esters available for enzymatic catalysis. However, Triton X-100 had only been evaluated at a single concentration and so it was necessary to investigate the optimal conditions for recovery of TC from both HDL and LDL. Others have shown that higher concentrations of Triton X-100 may inhibit the enzymes' activity (Shih, Yang and Lin, 2009; Tan *et al.,* 2005; Vidal, García and Castillo, 1999).

To establish the best concentration of Triton X-100 for TC measurement, the effect of its concentration on the enzymatic determination of 2.0 mM LDL-C was assessed (Fig. 3.12).

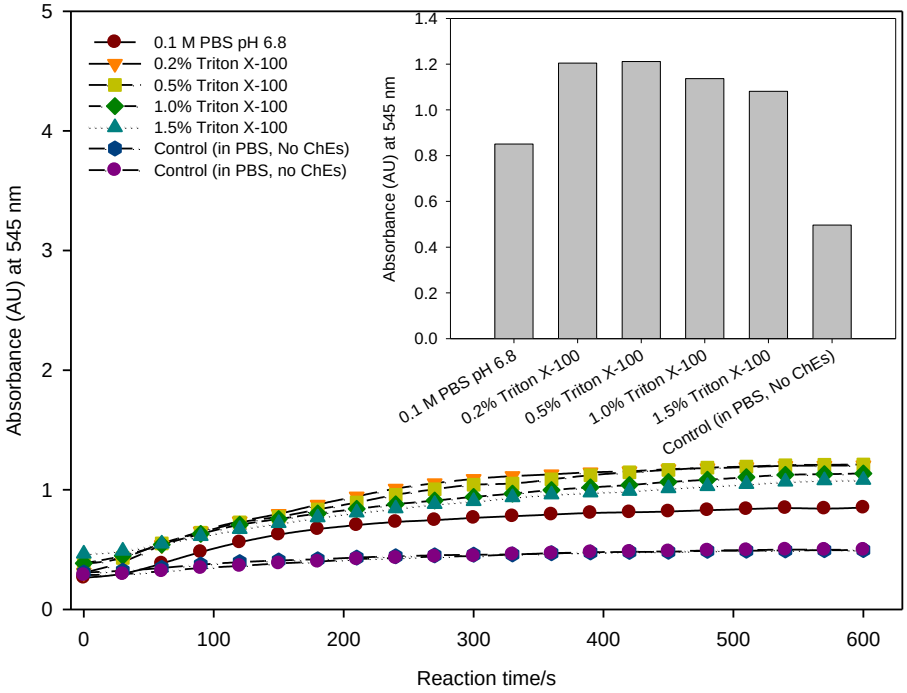

Fig. 3.12. Effect of Triton X-100 concentration on the enzymatic determination of cholesterol in 2.0 mM LDL. Inset: Absorbance at 600 s.

As shown, almost the same absorbance values of 1.20 and 1.21 AU were observed in the presence of 0.2 and 0.5 % (v/v) Triton X-100. This was 1.4 fold higher than that observed in its absence which was 0.85 AU. By increasing the concentration of Triton X-100 from 0.5 to 1.0 and 1.5 % (v/v), the absorbance decreased from 1.21 to 1.14 and 1.08 AU, respectively. As mentioned, this may be due the inhibition of enzyme activity. Nishiya *et al*. (1998) reported that by increasing the concentration of Triton X-100, the reaction rate of ChOx decreased. Non-ionic surfactants such as Triton X-100 form micelles around cholesterol. Enzymes must remove the cholesterol from its micellar form to expedite the enzymatic reaction. Therefore the enzyme's affinity for surfactant is critical in determining the rate of the enzymatic reaction. Higher concentrations of Triton X-100 may make very dense micelles around the cholesterol molecules, which might then interfere with the diffusion of cholesterol to the enzyme (Vidal, García and Castillo, 1999). Further studies of Triton X-100 were performed at 0.5% (v/v).

Reproducibility of the measurements in Triton X-100 was assessed by measuring TC and non-esterified cholesterol levels from HDL-C and LDL-C (2.0 mM) in the presence of 0.5% (v/v) Triton X-100. The measurement of non-esterified cholesterol (with ChOx) was again used to account for the free cholesterol present

in the sample, while TC was then measured in the presence of ChEs and ChOx and Triton X-100 (n=3) (Fig. 3.13). While the kinetics of the reaction in HDL-C appeared to be moderately faster than for LDL-C, both assays had very comparable kinetics and final absorbance values.

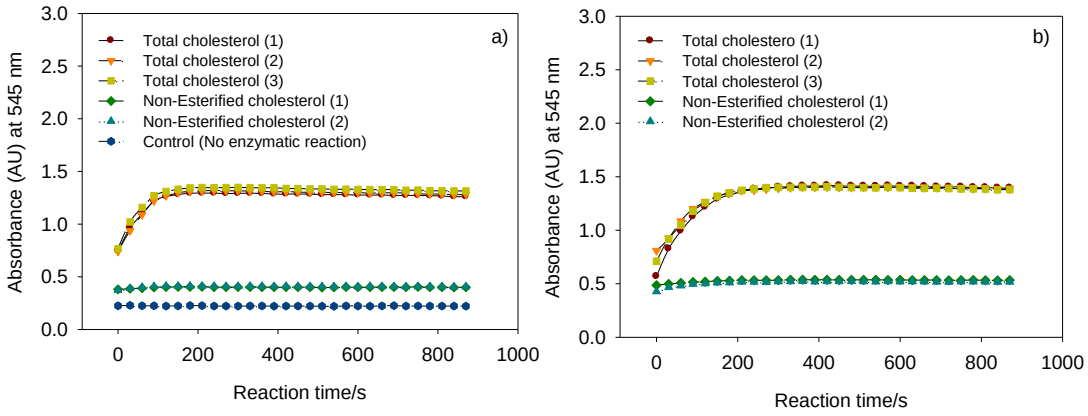

Fig. 3.13. Enzyme assay of non-esterified (ChOx only) and total cholesterol (ChEs and ChOx) in 0.5% (v/v) Triton X-100 in a) 2.0 mM HDL-C and; b) 2.0 mM LDL-C at RT.

The average absorbance and standard deviation of each measurement are shown in Fig. 3.14. The average absorbance value for TC measurement of 2.0 mM HDL and LDL cholesterol were 1.29 ±0.02 AU (RSD=1.6%) and 1.39 ±0.01 AU (rsd=0.7%), respectively. Measurement of non-esterified cholesterol from 2.0 mM HDL and LDL yielded absorbance values of 0.403 ±0.003 (RSD= 0.7%) and 0.53 ±0.01 AU (rsd=2.0%), respectively. Therefore, under the developed assay conditions, the use of 0.2 or 0.5% (v/v) Triton X-100 was found to be an efficient and reproducible means of measuring TC in HDL and LDL using the enzyme assay and was used as the basis of further developments.

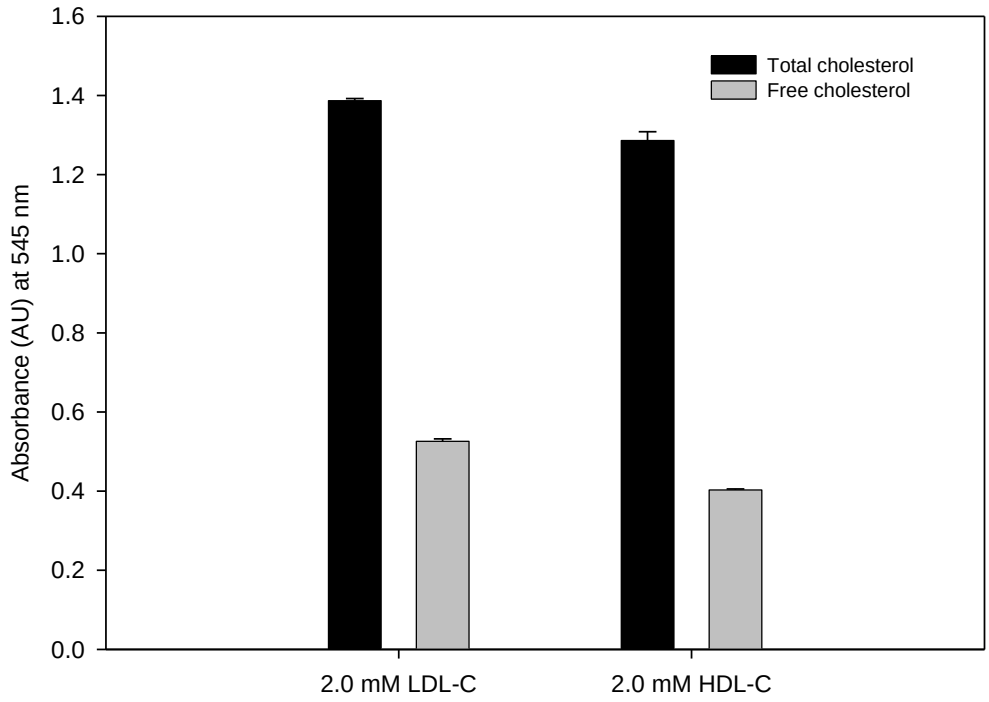

Fig. 3.14. Reproducibility study of enzyme assay measurements of total cholesterol and non-esterified cholesterol from 2.0 mM HDL-C and LDL-C in 0.5% (v/v) Triton X-100 (absorbance at 600 s).

3.3. 3 Optimisation of the selective measurement of HDL-C

As was mentioned earlier (see section 3.3.1), selectivity of the spectrophotometric assay towards HDL-C was achieved by introducing a non-ionic surfactant such as Emulgen B-66 to the assay reagents detailed in Table 3.2. This surfactant was found to be highly selective to HDL-C over LDL-C, by making mainly the cholesterol bound to HDL-C available for enzymatic reaction. To further improve assay selectivity towards HDL-C, the effect of different concentrations of Emulgen B-66 on the recovery of cholesterol from 2.0 mM LDL-C in PBS was assessed. The recovery of cholesterol from LDL-C using 0.2% (v/v) Triton X-100 was taken as being 100%. The kinetics of the enzyme assay was studied in the usual way over 600 s at RT (Fig. 3.15).

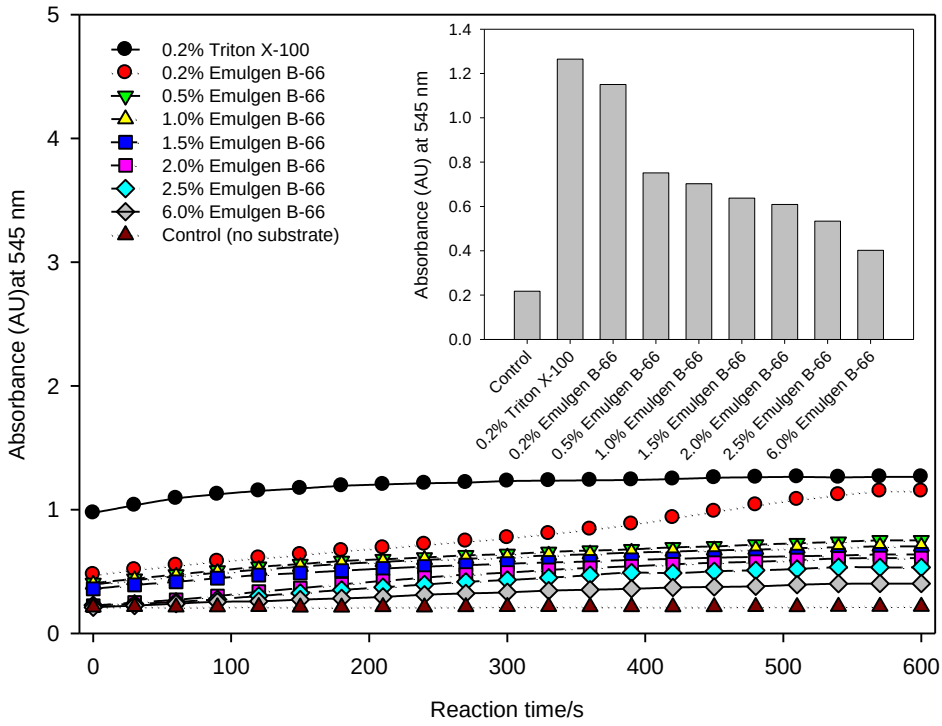

Fig. 3.15. Effect of Emulgen B-66 concentration on recovery of cholesterol from 2.0 mM LDL-C. 0.2 % (v/v) Triton X-100 taken as 100%. Inset: Absorbance values measured at 600s.

As can be seen, for 0.2% (w/v) Emulgen B-66, the absorbance value observed at 200 s was approx. 0.66 AU, compared to 1.19 AU for 0.2% (v/v) Triton X-100. However, the absorbance value increased and reached 1.15 AU at 600 s. Therefore, 0.2% (w/v) Emulgen B-66 was able to inhibit the enzymatic reaction of 2.0 mM LDL-C for just the first 200s. This might be due the inability of lower concentrations of surfactant to form a stable protein-surfactant complex by affecting the adsorption dynamics of apo-lipoprotein B and their surface activity. Hydrophobic interactions between protein and surfactant increase with increasing surfactant concentration, leading to less protein adsorption (Florence, Tucker and Walters, 1984; Miller *et al.,* 2000).

Florence *et al.* also reported different behaviours of non-ionic surfactant at lower and higher surfactant concentrations. Depending on the HLB of the surfactant, at very low surfactant concentrations, the activity of the hydrophobic region of the surfactant may be limited by micellisation, leading to better affinity of the hydrophilic region for the membrane, causing disruption and solubilisation. The slow action might be due to the slow saturation of its bilayers with surfactant and therefore

slower enzymatic reaction (Florence, Tucker and Walters, 1984; Tucker and Florence, 1983).

By increasing the concentration of Emulgen B-66 from 0.2% to 6% (w/v), the percentage recovery of cholesterol from 2.0 mM LDL-C decreased from 89.1% to 17.6% at 600 s. This confirmed that the protein/surfactant interaction was altered by changing the concentration of surfactant, showing an inhibition at higher concentration of Emulgen B-66 which might be due to increased hydrophobic interaction of apolipoprotein B with non-ionic surfactant by causing an increase in the Stokes radius of the LDL particles. This might be due to unfolding of apolipoprotein B molecules and therefore penetration of the phospholipid surface layer (Florence, Tucker and Walters, 1984; Miller *et al.,* 2000).

The percentage recovery and the absorbance values are summarised in Table 3.1. As was shown, the lowest percentage recovery was observed in the presence of 6.0% (w/v) Emulgen B-66 which was 17.6%, including non-esterified cholesterol present in the stock sample.

Table 3.5. Summary of effect of Emulgen B-66 concentration on the recovery of cholesterol from 2.0 mM LDL-C. Recovery in 0.2% (v/v) Triton X-100 taken as 100%. (t=600 s and absorbance was measured at 545 nm).

Type of surfactant	Absorbance (AU)	Percentage recovery (%)
Control	0.217	0.0
0.2% (v/v) Triton X-100	1.27	100.0
0.2% (w/v) Emulgen B-66	1.15	89.1
0.5% (w/v) Emulgen B-66	0.751	51.0
1.0% (w/v) Emulgen B-66	0.703	46.3
1.5% (w/v) Emulgen B-66	0.638	40.1
2.0% Emulgen B-66	0.609	37.4
2.5% Emulgen B-66	0.534	30.2
6.0% Emulgen B-66	0.409	17.6

In a further experiment, Emulgen B-66 at 1%, 2.5% and 6% (w/v) was used to measure esterified cholesterol present in 2.0 mM LDL-C, this time in delipidated serum rather than in PBS to enhance the authenticity of the matrix, and taking 0.5% (v/v) Triton X-100 as 100% cholesterol recovery (Fig. 3.16).

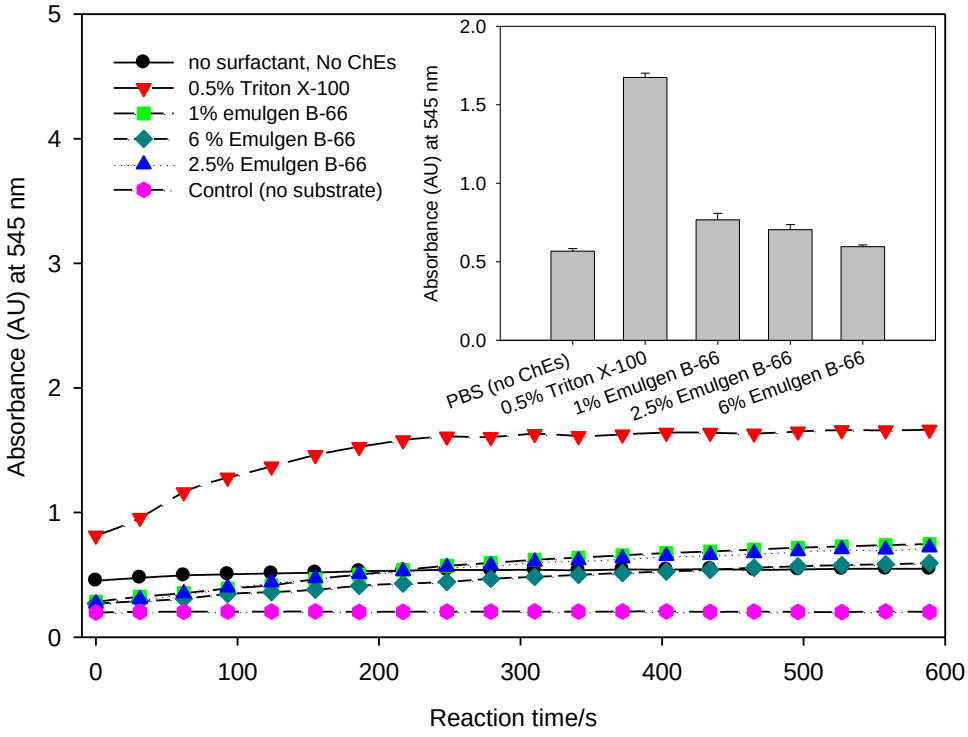

Fig. 3.16. Effect of Emulgen B-66 concentration on the recovery of cholesterol from 2.0 mM LDL-C in delipidated serum. 0.5% (v/v) Triton X-100 taken as 100% recovery. Absorbance in 0.1 M PBS and in the absence of ChEs and surfactant used as control. Inset: Average absorbance of three repeats at 600 s.

The absorbance in the absence of ChEs and surfactant was used as a control. As can be seen, in the presence of Emulgen B-66, the absorbance increased up to approx. 0.77 (±0.04), 0.70 (±0.03) and 0.60 (±0.01) AU for 1%, 2% and 6%, respectively, which were very similar to that observed for non-esterified cholesterol measurement (0.57 (±0.02) AU). This might be due to the presence of high concentrations of non-esterified cholesterol in the stock sample. The absorbance value observed in the presence of 0.5% (v/v) Triton X-100 was 1.67 (±0.03) AU which was more than twice the absorbance observed in the presence of Emulgen B-66. By considering the absorbance in the absence of ChEs and surfactant as a control and the absorbance in the presence of 0.5% (v/v) Triton X-100 as 100%, the percentage recoveries of esterified cholesterol in 2.0 mM LDL-C were 18.0, 12.4 and 2.5% for 1%, 2.5%, and 6% (w/v) Emulgen B-66, respectively. Therefore 6% (w/v) Emulgen B-66 was found to be the most suitable concentration to achieve approx. 97.5% rejection of LDL-C. However, it was now necessary to assess the recovery of cholesterol from HDL-C using this optimised concentration.

To evaluate the effect of 6% (w/v) Emulgen B-66 on the recovery of cholesterol from HDL-C, a similar experiment was performed employing 2.0 mM HDL-C in delipidated serum, again with 0.5% (v/v) Triton X-100 taken as 100% recovery (Fig. 3.17). As shown, the absorbance at 600 s for the measurement of 2.0 mM HDL-C in the presence of Emulgen B-66 and Triton X-100 were 1.16 (±0.03) AU and 1.22 (±0.03), respectively, yielding a percentage recovery in Emulgen B-66 of approx. 95%.

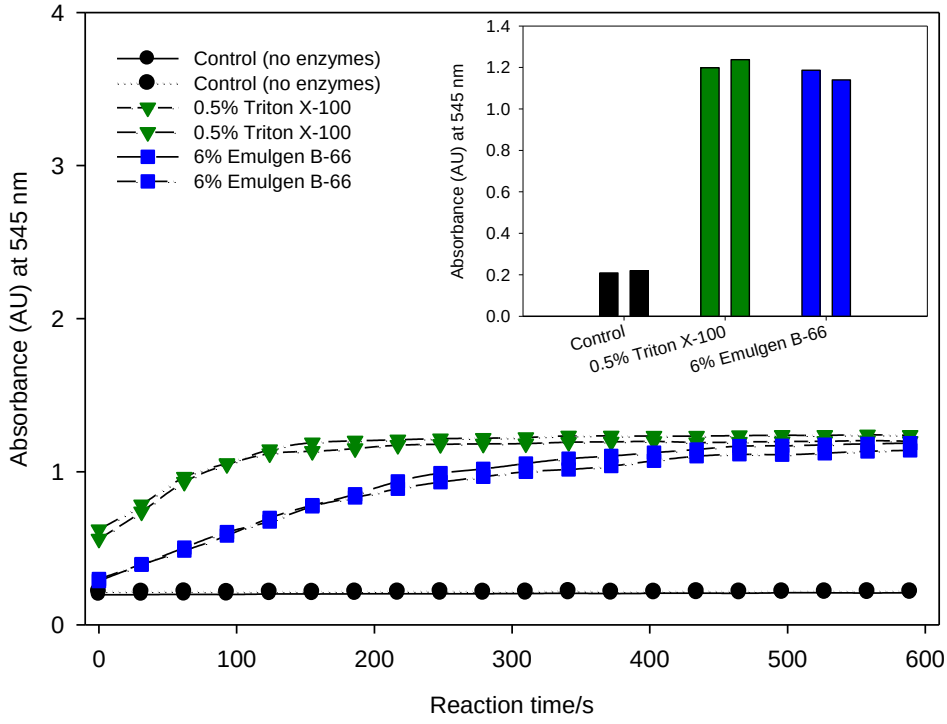

Fig. 3.17. Effect of 6% (w/v) Emulgen B-66 on the recovery of cholesterol from 2.0 mM HDL-C in delipidated serum. 0.5% (v/v) Triton X-100 taken as 100% recovery. Absorbance in the absence of enzymes and surfactant was taken as control. Inset: Absorbance values of two repeats at 600 s.

To sum up, for 6% (w/v) Emulgen B-66, the recovery of cholesterol from LDL-C relative to 0.5% (v/v) Triton X-100 was 2.5%, whereas with HDL-C, this was 95% with inaccuracy of minus 5%. Previously, 0.9% (w/v) Emulgen B-66 in combination with 15 mM $MgCl_2$ was reported by Okada *et al.* (2001) as the best surfactant among four HDL-C selective surfactants employed in their experiment which was capable of measuring 95% of cholesterol content in HDL_3 following 76% in HDL_2 and 10% in LDL-C (Okada *et al.,* 2001). Later on, in 2008, Matsui and Ohta reported that Emulgen 911, Emulgen B-66 and the combination of Emulgen B-66 and Emulgen A-90 with concentrations ranging from 0 to 3 %(w/v) were suitable as

surfactants which specifically act on HDL-C (Matsui and Ohta, 2008). Recently, Ito *et al.* (2014) developed a method for measurement of HDL subclasses, again by employing a surfactant from the polyoxyethyene derivatives with an HLB of 13.6 which was capable of the selective decomposition of HDL_3 (Ito *et al.,* 2014). According to their report, polyoxyethylene benzylphenyl ether derivatives can be used for total HDL-C assay. The maximum concentration of surfactant employed was 3%. However as was shown above, even up to a concentration of 6% (w/v) Emulgen B-66, 95% of cholesterol in HDL-C was recovered. Therefore, 6% (w/v) Emulgen B-66 was used as a HDL-C selective surfactant for further work. However, thus far, recovery had only been evaluated using a single concentration of either HDL-C or LDL-C alone. As both of these will vary across a typical range of 1 to 4 mM in real serum samples, the accuracy of the method should be assessed across a range of values and the assay optimised further if necessary.

3.4 Determination of HDL-C in mixtures of HDLC and LDL-C

The optimised concentrations of enzymes and surfactant were used to measure the cholesterol in 2.0 mM HDL-C while in the presence of 0, 1.0, 2.0, 3.1 and 4.2 mM LDL-C in delipidated serum. 6% (w/v) Emulgen B-66 was used to determine HDL-C levels while non-esterified cholesterol present in the samples was measured by omitting ChEs from the reaction mixtures, the resulting absorbance from which was taken as 0% cholesterol recovery, according to Nakamura *et al.* (2009). As can be seen in Fig. 3.18, increasing the concentration of LDL-C led to an increase in the level of cholesterol measured in the presence of Emulgen B-66. However, when the level of freely available non-esterified cholesterol (which is not made selectively available by the Emulgen B-66) is subtracted, the estimated value for the measured HDL-C could be obtained. Table 3.6 gives a summary of these recoveries, which demonstrates that the assay had a recovery of cholesterol from 2.0 mM HDL-C between 86 and 128%. As can be seen, all the absorbance values for TC measurement in the presence of Emulgen B-66 were above 1.0 AU which lies at the upper end of the linear absorbance range for spectrophotometry (Day and Underwood, 1974). This was observed particularly at higher concentrations of LDL-C, which results in higher concentrations of free cholesterol and subsequently higher TC concentrations of the sample. Others have not mentioned this problem as almost all of the spectrophotometric measurement of HDL-C are based on the use of approx. 4 µL of sample in a total test volume of 100 µL resulting in a maximum concentration of 0.04 mM in the reaction mixture (Warnick, Nauck and

Rifai, 2001). However, one of the objectives of this project was to establish a method with a minimum dilution factor which would be capable of being employed for the direct amperometric measurement of HDL-C. For the method to be considered validated, the accuracy expressed as percent recovery, must be within 80-120% unless otherwise specified (Food and drug administrator, FDA, 2014). As can be seen in Table 3.6, most of the individual recovery results fell within this limit with the exception of two measurements. This was due to the limitations of the method and the inability to run the experiment more than twice within a day to minimise the systematic error. For this reason and considering the total RSD of 15% for measurement of esterified cholesterol in 2 mM HDL-C in the presence of different concentrations of LDL-C which is well within the specified FDA limits, 6% (w/v) Emulgen B-66 deemed to be effective for the selective measurement of HDL-C over LDL-C.

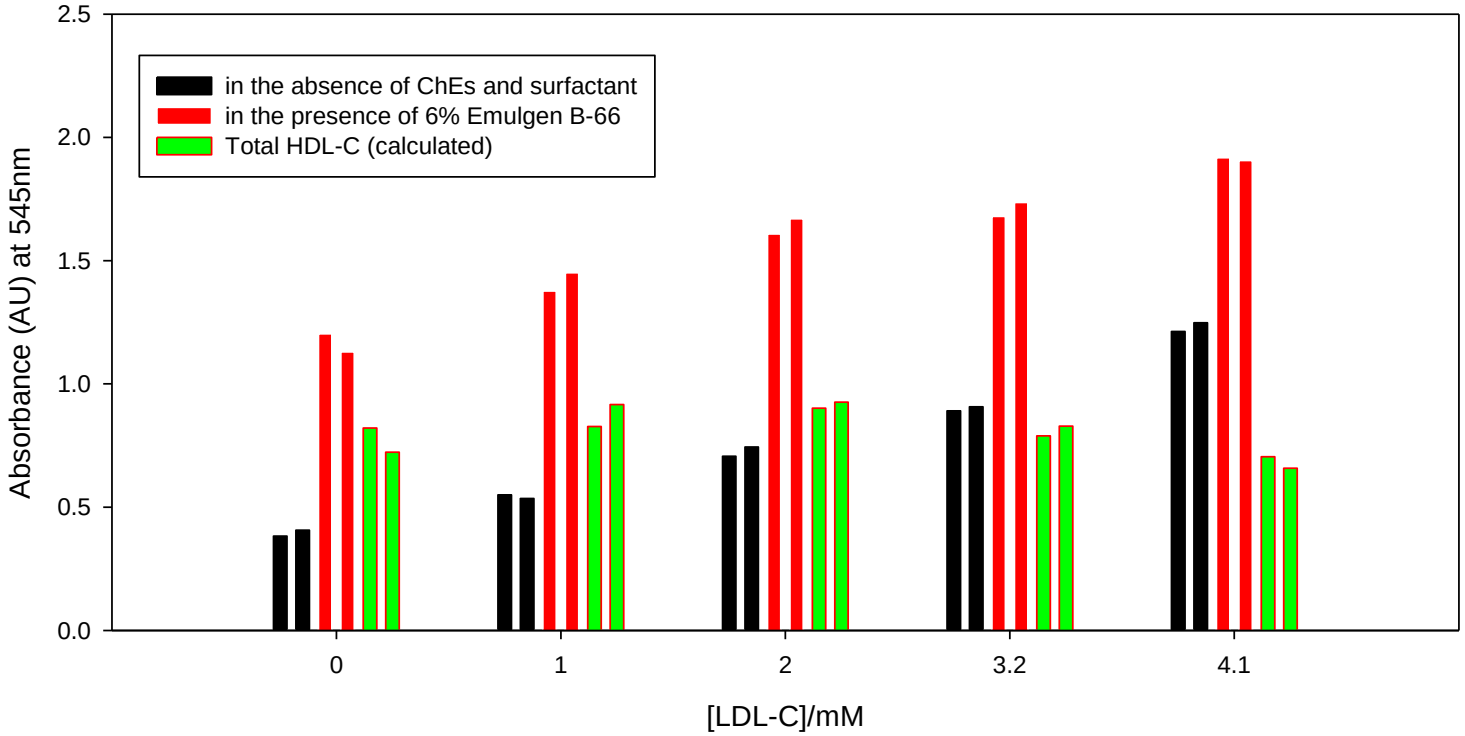

Fig. 3.18. Effect of LDL-C concentration on the measured recovery of cholesterol from 2.0 mM HDL-C: absence of ChEs (free non-esterified cholesterol)(●); 6% (w/v) Emulgen B-66 (●); estimated HDL-C (●), n=2

Table 3.6. Effect of LDL-C concentration on the measured recovery of HDL-C (n=2).

HDL-C (mM)	LDL-C (mM)	Control in the absence of surfactant and ChEs (n=2)		Emulgen B-66 (6%) (absorbance) (n=2)		Cholesterol ester (absorbance) (n=2)		% HDL-C Recovery (n=2)	
		Replicate 1	Replicate 2	Replicate 1	Replicate 2	Replicate 1	Replicate 2	Replicate 1	Replicate 2
2	0	0.383	0.407	1.20	1.23	0.821	0.723	100	100
2	1	0.550	0.535	1.38	1.45	0.828	0.916	101	127
2	2	0.707	0.744	1.61	1.67	0.902	0.926	110	128
2	3.2	0.891	0.907	1.68	1.74	0.789	0.829	96	115
2	4.1	1.21	1.25	1.92	1.91	0.705	0.658	86	91
				Average cholesterol ester (AU)		0.81			
				SD		0.12			
				RSD (%)		15			

3.5 Conclusion

A final formulation (Table 3.2) for spectrophotometric measurement of 0 to 4 mM cholesteryl acetate was established. This assay methodology showed acceptable linearity across this range confirming that none of the enzymes were limiting the enzymatic reaction. The assay time was found to be within 7 min at room temperature and so was suitable for application to an electrochemical biosensor.

Effect of Triton X-100 and sodium cholate as non-specific surfactant and Emulgen B-66 as HDL-C specific surfactant on spectrophotometric measurement of 2.0 mM HDL-C and LDL-C were assessed. Both Triton X-100 and sodium cholate were found to be suitable surfactants in spectrophotometric measurement of total cholesterol in both HDL and LDL cholesterol. Considering that Triton X-100 is a non-ionic surfactant which may have less impact on its future application for amperometric measurement of H_2O_2 using silver modified electrodes, optimisation study was performed using different concentrations of Triton X-100 TC measurement. Therefore, 0.2 to 0.5% (v/v) Triton X-100 was found to be suitable for measuring TC in HDL and LDL using the enzyme assay and was used as the basis of any future experiments. The next step was to find the optimum concentration of HDL-C specific surfactant (Emulgen B-66) capable of reasonable differentiation between HDL-C and LDL-C. Consequently, 6% emulgen B-66 was found to have a good selectivity towards HDL-C over LDL-C. The final formulation was used to measure 2.0 mM HDL-C in the presence of 0 to 4 mM LDL-C. Almost the same signal was observed after subtracting the free cholesterol results across the range.

To sum up, assay reagent summarised in Table 3.2 showed a reasonable linearity for the measurement of 0 to 4 mM Cholesteryl acetate and 6% Emulgen B-66 and 0.2 to 0.5% Triton X-100 were found to be suitable for the measurement of HDL-C and TC respectively. As the aim of this project was to replace the final step of this assay with amperometric measurement using modified silver electrodes, the effect of individual assay components such as ChOx, ChEs, surfactants and samples on modified silver electrodes behaviour will be assessed first and subsequently modified silver electrodes will be employed for the measurement of 0 to 4 mM H_2O_2.

References

Akimova, E.I. and Melgunov, V.I. (1984) Apolipoprotein B: removal of lipids by sodium cholate and reassociation of a lipid-free apoprotein with dipalmitoyl phosphatidylcholine. *Biochemistry International.* 9, pp. 463-743.

Allain, C.C., Poon, L.S., Chan, C.S.G., Richmond, W. and Fu, P.C. (1974) Enzymatic determination of serum total cholesterol. *Clinical Chemistry*. 20 (4), pp. 470-475.

Brahim, S., Narinesingh, D. and Guiseppi-Elie, A. (2001) Amperometric determination of cholesterol in serum using a biosensor of cholesterol oxidase contained within a polypyrrole–hydrogel membrane. *Analytica Chimica Acta*. 448 (1–2), pp. 27-36.

Copeland, R.A. (2000) *ENZYMES: A Practical Introduction to Structure, Mechanism and Data Analysis*. Second edition, New York: Wiley-VCH, Inc.

Day, R.A. and Underwood, A.L. (1974) *Quantitative Analysis. 3rd Edition. Prentice Hall. Inc.,* New Jersy: Prentice Hall Inc.

Fang, C. (2011) A disposable amperometric biosensor for determining total cholesterol in whole blood. *Sensors and Actuators B: Chemical*. 155 (2), pp. 545-550.

Florence, A.T., Tucker, I.G. and Walters, K.A. (1984) Interaction of non-ionic polyoxyethylene alkyl and aryl ethers with membranes and other biological system. In: Anon.(1984) *Structure/performance Relationships in Surfactants*. American Chemical Society, pp. 189-207.

Food and drug administrator, FDA (2014) *Volume II - Methods, Method Verification and Validation ORA-LAB.5.4.5.* Available from: http://www.fda.gov/ScienceResearch/Field-Science/ucm171877.htm.

Hino, K., Nakamura, M. and Manabe, M. (1998) *Method for Quantitatively Determining Cholesterol* Daiichi Pure Chemicals Co., Ltd. 5773304.

Hooda, V., Gahlaut, A., Kumar, H. and Pundir, C.S. (2009) Biosensor based on enzyme coupled PVC reaction cell for electrochemical measurement of serum total cholesterol. *Sensors and Actuators B: Chemical*. 136 (1), pp. 235-241.

Ito, Y., Satoh, N., Ishii, T., Kumakura, J. and Hirano, T. (2014) Development of a homogeneous assay for measurement of high-density lipoprotein-subclass cholesterol. *Clinica Chimica Acta*. 427 (0), pp. 86-93.

Kane, J.P. (1983) Apoprotein B: structural and metabolic heterogeneity. *Annual Review of Physiology*. 45, pp. 637-650.

Kondo, A. (1999) Dynamic reaction in a homogeneous HDL-cholesterol assay visualized by electron microscopy. *Clinical Chemistry*. 45 (11), pp. 1974.

Kurosaki, Y. and Ogawa, Z. (2009) Reactivity of a detergent, polyoxyethylene derivative, with high density lipoprotein subclass. *Journal of Analytical Bio-Science*. 32, pp. 434-439.

Lee, E.J., Shin, S.D., Song, K.J., Kim, S.C., Cho, J.S., Lee, S.C., Park, J.O. and Cha, W.C. (2011) A point-of-care chemistry test for reduction of turnaround and clinical decision time. *American Journal of Emergency Medicine*. 29, pp. 489-495.

Lin, H., Gebhardt, M., Bian, S., Kwon, K.A., Shim, C., Chung, S. and Kim, D. (2007) Enhancing effect of surfactants on fexofenadine·HCl transport across the human nasal epithelial cell monolayer. *International Journal of Pharmaceutics*. 330 (1–2), pp. 23-31.

Matsui, H. and Ohta, M. (2008) *Method of Quantifying Cholesterol in High Density Lipoprotein and Reagent Compositions.* Denka Seiken Co., L. US 7348158 B2.

Miller, R., Fainerman, V.B., Makievski, A.V., Krägel, J., Grigoriev, D.O., Kazakov, V.N. and Sinyachenko, O.V. (2000) Dynamics of protein and mixed protein/surfactant adsorption layers at the water/fluid interface. *Advances in Colloid and Interface Science*. 86 (1–2), pp. 39-82.

Mitra, S. and Dungan, S.R. (2001) Cholesterol solubilisation in aqueous micellar solutions of quillaja saponin, bile salts, or nonionic surfactants. Journal of Agricultural and Food Chemistry. 49, pp. 384-394.

Murphy, L., Burrows, C., Bayly, S., Wilkinson, K. and Harris, J. (2009) *HDL Cholesterol Sensor using Selective Surfactant. US20090130696 A1*

Nakamura, M., Taniguchi, Y., Yamamoto, M. and Manabe, M. (2009) *Method of Pretreatment of Sample for Quantitating Cholesterol and Method for Quantitating Cholesterol in Specific Lipoproteins.* Daiichi Pure Chemicals Co., Ltd. 20090226945.

Nakanishi, K., Nakamura, M. and Hino, K. (1998) *Methods for Quantitating High-Density Lipoprotein Cholesterol* Sekisui Medical Co., L. EP2080810.

Okada, M., Matsui, H., Ito, Y. and Fujiwara, A. (2001) Direct measurement of HDL cholesterol:Method eliminating apolipoprotein E-rich particles. *Journal of Clinical Laboratory Analysis*. 15 (4), pp. 223-229.

Okada, M. (2001) Direct measurement of low-density-lipoprotein cholesterol is more effective than total cholesterol for the purpose of lipoprotein screening. *Preventive Medicine*. 32 (3), pp. 224-229.

Okada, M. (1998) Low-density lipoprotein cholesterol can be chemically measured: a new superior method. *The Journal of Laboratory and Clinical Medicine*. 132 (3), pp. 195-201.

Richmond, W. (1976) Use of Cholesterol Oxidase for Assay of Total and Free Cholesterol in Serum by Continuous Flow Analysis. *Clinical Chemistry*. 22 (10), pp. 1579-1588.

Richmond, W. (1973) Preparation and properties of a cholesterol oxidase from nocardia sp. and its application to the enzymatic assay of total cholesterol in serum. *Clinical Chemistry*. 19 (12), pp. 1350-1356.

Segrest, J.P., Jones, M.K., Loof, H.D., Brouillette, C.G. and Anantharamaiah, G.M. (1992) The amphipathic helix in exchangeable apolipoproteins: a review of secondary structure and function. *Journal of Lipid Research*. 33, pp. 141-166.

Segrest, J.P., Jones, M.K., Loof, H.D. and Dashti, N. (2001) Structure of apolipoprotein B-100 in low density lipoproteins. *Journal of Lipid Research*. 42, pp. 1346-1367.

Shih, W., Yang, M. and Lin, M.S. (2009) Development of disposable lipid biosensor for the determination of total cholesterol. *Biosensors and Bioelectronics*. 24 (6), pp. 1679-1684.

Tamura, H., Nishino, S., Yamaguchi, T. and Hino, K. (2009) *Test Piece for Measureing HDL- Cholesterol,* Daiichi Chemical Co., L. US7575884.

Tan, X., Li, M., Cai, P., Luo, L. and Zou, X. (2005) An amperometric cholesterol biosensor based on multiwalled carbon nanotubes and organically modified sol-gel/chitosan hybrid composite film. *Analytical Biochemistry*. 337 (1), pp. 111-120.

Tipton, K.F. (1992) *Principles of Enzyme Assay and Kinetic Studies*. Oxford, UK.

Tucker, I.G. and Florence, A.T. (1983) Interactions of ionic and non-ionic surfactants with plasma low density lipoprotein. *The Journal of Pharmacy and Pharmacology*. 35 (11), pp. 705-711.

Vidal, J.C., García, E. and Castillo, J.R. (1999) In situ preparation of a cholesterol biosensor: entrapment of cholesterol oxidase in an overoxidized polypyrrole film electrodeposited in a flow system: Determination of total cholesterol in serum. *Analytica Chimica Acta*. 385 (1–3), pp. 213-222.

Warnick, G.R., Nauck, M. and Rifai, N. (2001) Evolution of Methods for Measurement of HDL-Cholesterol: From Ultracentrifugation to Homogeneous Assays. *Clinical Chemistry*. 47 (9), pp. 1579-1596.

Yamamoto, S., Yamamoto, M. and Nakanishi, K. (2011) *Method of Measuring Lipid in Specific Lipoprotein.* Daiichi Chemical Co., L. US 2011/0091917.

Zoppi, F. and Fenili, F. (1976) Enzymatic determination of total serum cholesterol with the Vickers D-300 analyzer. *Clinical Chemistry*. 2 (5), pp. 690-691.

Chapter 4

Evaluation of the effect of HDL-C and TC assay reagents on the electrochemical measurement of H_2O_2 using modified silver electrodes

4.1 Introduction

The primary aim of this work was the development of an electrochemical biosensor for the selective measurement of cholesterols such as TC, HDL-C and, by calculation, non-HDL-C. This would be achieved by replacing the final enzymatic step of the spectrophotometric measurement of H_2O_2 in the presence of a colorimetric reagent with the amperometric detection of H_2O_2 generated in the final step of conversion of cholesterol to cholest-4-en-3-one which would take place at a printed, modified silver electrode which had been shown to have enhanced catalysis for the reduction of H_2O_2 (Gonzalez-Macia, Smyth and Killard, 2012, Gonzalez-Macia, 2011). Such an approach would reduce the complexity of the sensor system by reducing the number of enzymes involved from three to two, while also facilitating the use of a simple, rapid, low cost, disposable electrochemical sensing approach.

The general electrochemical behaviour these electrodes to reduce H_2O_2 was assessed, first in stirred batch mode with semi-infinite diffusion and convection, followed by their characterisation in a thin layer (encapsulated) cell suitable for low volume blood sampling. To use the modified electrodes for the detection of cholesterol, it was necessary to find out whether the electrochemical sensing principle was compatible with the cholesterol assays, e.g., impact of the assay reagents on the electrocatalysis. Since the electrocatalysis observed for modified electrodes in the presence of H_2O_2 is based on surfactant/salt solutions and due to the fact that assay reagent also contained a surfactant which is prepared in PBS, the evaluation of the effect of buffer and surfactant on modified electrodes were necessary before combining the cholesterol bioassay with a above mentioned electrochemical sensor for the measurement of H_2O_2. The assay reagents developed in Chapter 3 for HDL-C measurement were then evaluated for their effect on amperometric measurement of H_2O_2. The effects of buffer, surfactants, enzymes, HDL-C sample and delipidated serum on sensor behaviour were assessed and optimised, followed by evaluation of the effect of the final assay reagent formulation on the quantitative measurement of H_2O_2 in delipidated serum. The effect of Triton X-100 as a suitable surfactant for TC measurement on modified silver electrode was also evaluated.

4.2 Assessment of the electrochemical behaviour of the modified silver electrodes on the measurement of H_2O_2

Initial cyclic voltammetric and time-based amperometric experiments were carried out to compare the reduction of H_2O_2 at the silver paste electrodes before and after their modification with DBSA/KCl (Gonzalez-Macia, Smyth and Killard, 2011). Deposition of DBSA/KCl onto the silver electrodes was performed using inkjet printing in a manner similar to Gonzalez-Macia, Smith and killard (2012). A narrow potential window of -0.25 to 0 V (vs Ag/AgCl) for H_2O_2 measurement using modified electrodes suggested by Gonzalez-Macia *et al.* (2011) was used due to the oxidation of silver at more positive potentials and also oxygen interference at more negative potentials. The scan started and finished at -0.25 V vs. Ag/AgCl, with a scan rate of 100 mV/s (Fig. 4.1).

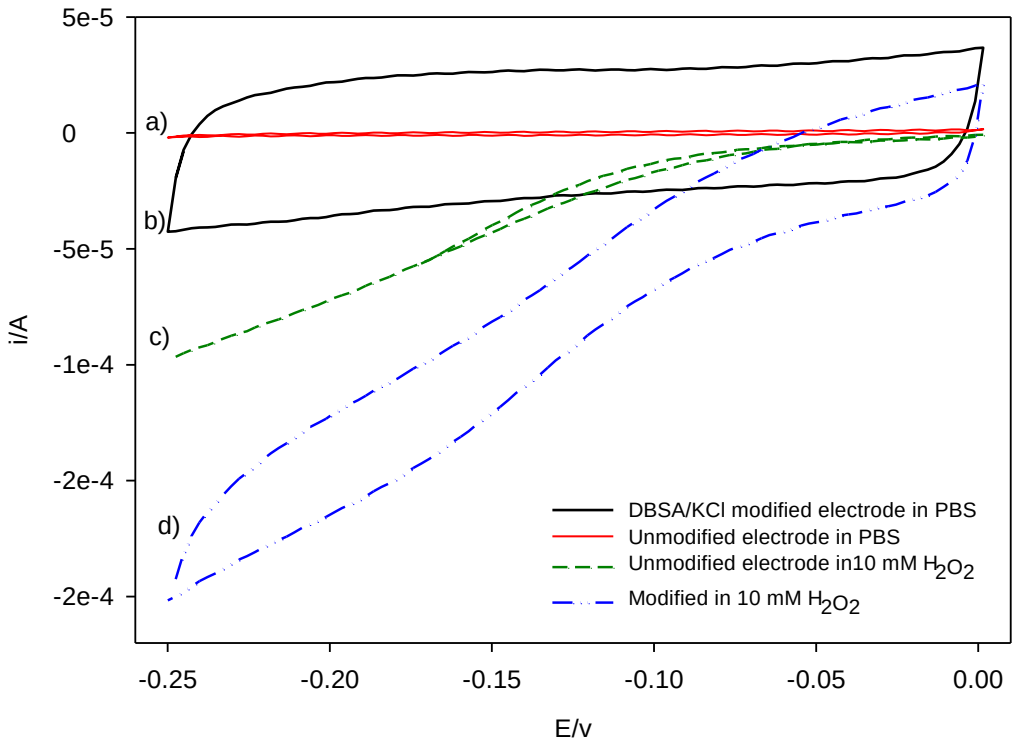

Fig. 4.1. Cyclic voltammograms of silver electrodes measured in PBS and 10 mM H_2O_2 (-0.25 to 0 to -0.25 V at 100 mV/s vs. Ag/AgCl): a) unmodified in PBS, b) modified in PBS, C) unmodified in 10 mM H_2O_2, d)modified in 10 mM H_2O_2.

It can be seen that the non-faradaic or charging currents for unmodified and modified electrodes at -0.1 V were 6.4×10^{-7} and 2.5×10^{-5} A, respectively. This was a confirmation of previous reports which showed a significant in 32-fold increase in double layer capacitance due to the deposition of the DBSA/KCl lyotropic layer on

the electrode surface (Gonzalez-Macia, Smyth and Killard, 2011; Gonzalez-Macia, Smyth and Killard, 2012). The cathodic current observed for the modified electrode at -0.1 V taken from cyclic voltammograms was approx. 5.2×10^{-5} A in the presence of 10 mM H_2O_2, after subtracting the background current in comparison with the unmodified electrode, which was 1.2×10^{-5} A. This represented a four-fold enhancement in catalysis, re-establishing the enhanced catalysis for H_2O_2 reduction due to the modification process seen in previous work (Gonzalez-Macia, Smyth and Killard, 2011).

Fig. 4.2 illustrates the amperometric responses for the modified and unmodified electrodes after addition of 1 mM injections of H_2O_2 (0 - 10 mM) in stirred batch solution at -0.1 V vs Ag/AgCl.

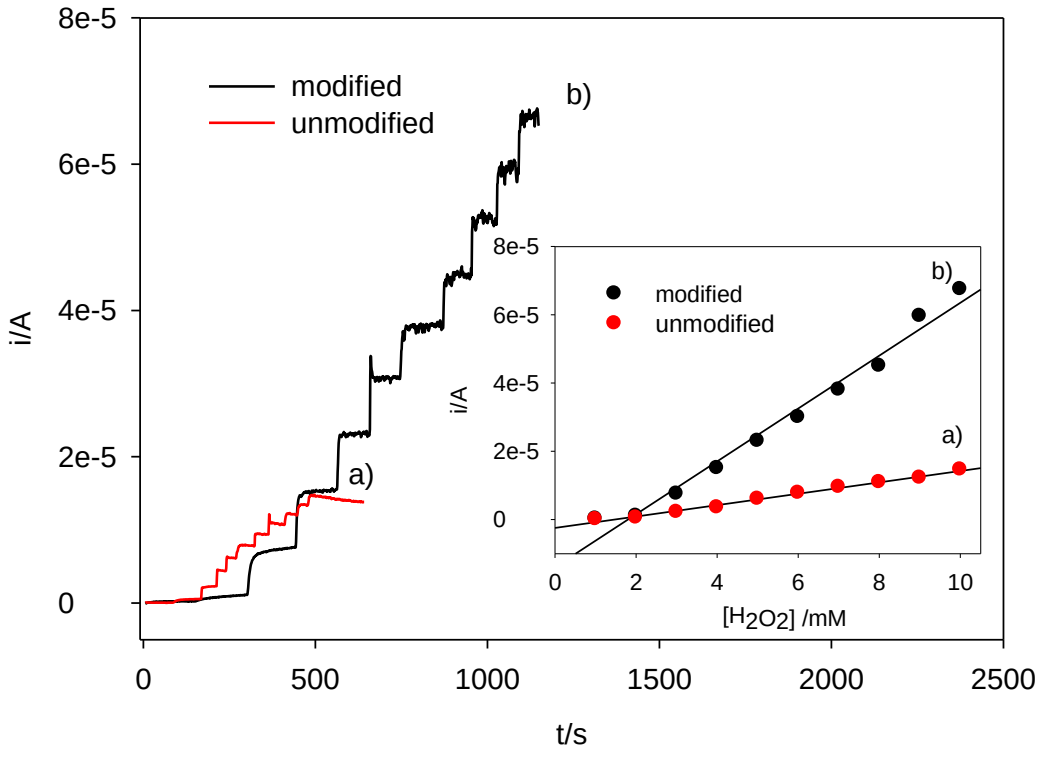

Fig. 4.2. Amperometric responses of silver paste electrodes at -0.1 V (vs Ag/AgCl) in 0 to 10 mM H_2O_2 in stirred batch solution: (a) unmodified and (b) modified, Inset: Plot of cathodic current vs the concentration of H_2O_2 at -0.1 V (vs Ag/AgCl) a) modified (slope=7.75×10^{-6}, r^2=0.98), b) unmodified (slope= 1.67×10^{-6}, r^2=0.99).

Although Fig. 4.1 showed that enhanced reduction began at -0.05 V and increased linearly up to the cathodic potential limit of -0.25 V, to avoid oxygen interference at more negative potentials, and based on previously reported work (Gonzalez-Macia, Smyth and Killard, 2012; Razmi and Habibi, 2009), -0.1 V was chosen for all the future amperometric studies, unless otherwise stated.

Amperometry also demonstrated an almost five-fold enhancement in electrocatalysis of H_2O_2 at the electrode following modification. The cathodic current observed for 10 mM H_2O_2 was 1.5×10^{-5} A and 7.3×10^{-5} A for unmodified and modified, respectively which represented a five-fold enhancement in catalysis. Cathodic current enhancement of modified silver electrodes was previously shown to be 80-fold higher which was significantly greater than that observed in this work. This might, in part, be due to the use of silver working electrodes with greater surface area of 12.6 mm^2 with a Ag/AgCl/NaCl (saturated) electrode and a platinum mesh electrode as reference and auxiliary electrodes (Gonzalez-Macia, Smyth and Killard, 2012). However, in the current work, silver paste electrodes with a surface area of 9 mm^2 were used as a working electrodes while screen printed Ag/AgCl and carbon overprinted on silver used as a reference and auxiliary, respectively. Some of this difference may also be due to the use of inkjet printing modification of the silver electrodes compared to those prepared using dip-coating which was reported previously. This was also shown by Gonzalez-Macia *et al.* (2012) that dip-coated modified silver electrodes gave cathodic currents two-fold greater than inkjet printed modified ones (Gonzalez-Macia, Smyth and Killard, 2012). Even the type of silver ink which was used for screen printing of silver electrodes can affect the catalytic activity of the electrodes towards H_2O_2 measurement. The silver paste ink consists of very fine silver particles dispersed in a thermoplastic resin as a binder which is suitable for screen printing (Electrodage®P410 data sheet). The nature of the binder was shown to have an impact on the catalysis and the formation of surfactant/salt lamellar structures (Gonzalez-Macia, Smyth and Killard, 2011) leading to different enhancements on catalytic activity of the modified electrodes depending on the type of the ink employed. Thus, these factors may have combined to reduce the catalysis from the previously determined 80-fold to the approximately five-fold demonstrated here. Since modified electrodes still showed improved amperometric responses to H_2O_2, the reproducibility of these electrodes was further assessed. Amperometric measurements of 1 to 10 mM H_2O_2 were performed in triplicate using stirred batch solution at -0.1 V (vs Ag/AgCl) leading to electrode responses with a slope of 1.87×10^{-6}, and linearity (r^2) of 0.99 and average RSD of 13.9%. As was mentioned earlier, the non-zero baseline current was approached using modified electrodes because of bachgrounf and non-Faradic currents produced after modification. Therefore, the cathodic current in this work decreases based on a diffusion layer dependent on time related to unstirred layer but did not approach zero due to the non-zero baseline current.

4.2.1 Evaluation of electrocatalysis in encapsulated electrodes

Current point of care diagnostic blood tests demand the use of small sample volumes to allow minimal discomfort with sampling typically performed by finger stick with measurement using capillary whole blood (Gubala *et al.,* 2012; Lee *et al.,* 2011), and this was an objective of the cholesterol biosensors being developed here, with a target of ≤10 µL of sample. Thus, electrodes were developed that were modified with a small volume sample chamber using a hydrophilic lid and spacer (25 µm), denoted 'encapsulated' electrodes. The final sample volume was found to be approx. 8 µL. However, the electrochemical behaviour of such a system differs significantly from the stirred batch system illustrated earlier, and so an understanding of the electrochemical behaviour of the encapsulated electrodes was required. For preliminary studies, sensor responses to H_2O_2 (0 to 3 mM) were investigated, as this was stoichiometrically equivalent to the amount of H_2O_2 that would be typically generated from samples containing HDL-C. However, later optimisation for measurement of TC would be in the range 0 to 7 mM. All measurements were subsequently performed at -0.1 V (vs Ag/AgCl).

As can be seen in Fig. 4.3, cathodic currents of 2.06×10^{-7}, 3.55×10^{-7}, 4.70×10^{-7}, 6.03×10^{-7} A at 420 s were observed in the presence of 0, 1, 2 and 3 mM H_2O_2, respectively, compared to currents obtained in the stirred batch system were 2.2×10^{-7}, 4.7×10^{-7}, 3.69×10^{-6}, and 7.9×10^{-6}A, illustrating an approximately ten-fold decrease in current due to the mass transport limitations introduced by the thin layer cell. This difference was more significant at higher concentrations of H_2O_2. The type of mass transport used in an electrochemical system has a direct effect on the current response.

The Faradic current is a direct measure of the rate of the electrochemical reaction taking place at the electrode. Further, the current itself is dependent upon the mass transport (the rate at which H_2O_2 gets from the bulk of solution to the electrode, and the rate at which electrons can transfer across the interface. Diffusion, migration and convection are the three basic mechanisms of mass transport. The total mass transport of H_2O_2, or *flux,* to an electrode is described for one dimension by the Nernst-Planck equation:

$$J_{(X,t)} = -[D(\partial C_{(X,t)}/\partial X)] - [(zF/RT)DC_{(X,t)}](\partial \emptyset_{(X,t)}/\partial X) + C_{(X,t)}v_{X(X,t)} \quad (4.1)$$

where J is the flux (mol cm^{-2} s^{-1}), D is the diffusion coefficient of the solution species (cm^2 s^{-1}), C is the concentration of H_2O_2 (mol/cm^3), $\partial C/\partial x$ expresses the concentration gradient and Φ is the electrostatic potential, and v_x is the hydrodynamic velocity. This equation shows that the flux of H_2O_2 towards the electrode surface is proportional to either of three slopes of concentration, electrostatic potential, or hydrodynamic velocity, all as a function of distance from the electrode surface.

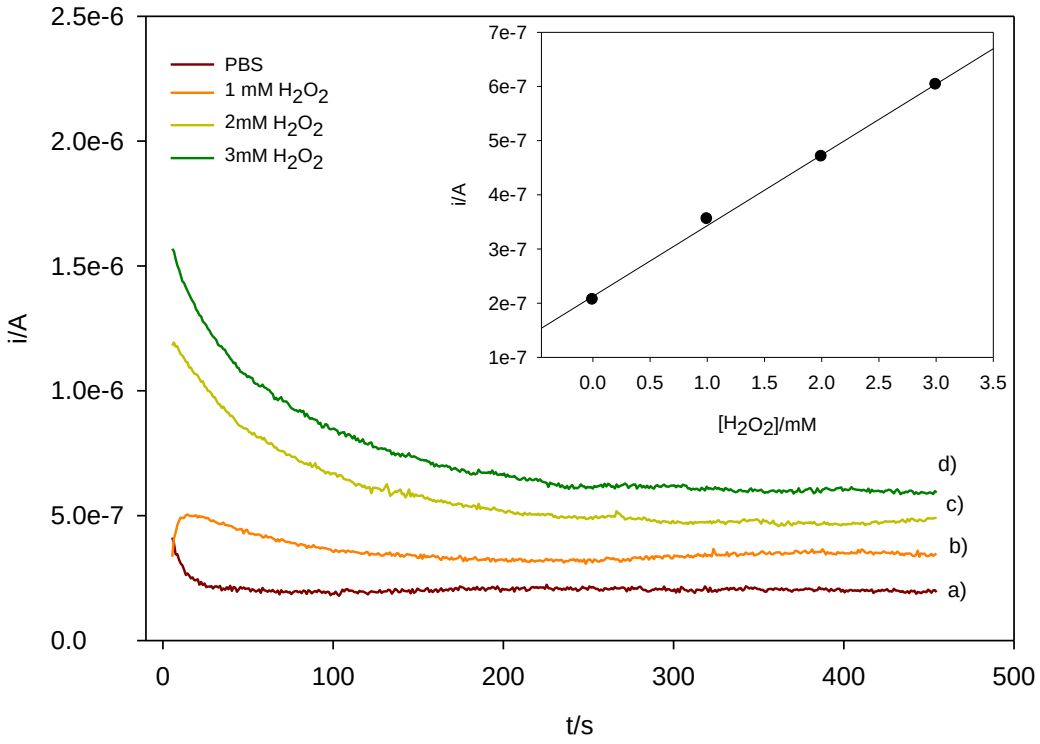

Fig. 4.3. Chronoamperometric responses of encapsulated modified Ag SPEs measured at -0.1V (vs Ag/AgCl) in a) 0.1 M PBS pH 6.8, b) 1 mM H_2O_2, c) 2 mM H_2O_2 and d) 3 mM H_2O_2; Inset: Plot of the current vs H_2O_2 concentration at t=300 s.

In stirred bulk solution, the flux of H_2O_2 towards the electrode surface is proportional to both diffusion and convection as a function of distance from the electrode surface. This means that convection will ensure the diffusion layer thickness is kept small and the bulk solution concentration will remain close to the initial value (Kissinger and Heineman, 1996). In other words, the bulk solution experiment is convection-diffusion controlled under semi-infinite diffusion conditions which allows setting up of a steady state condition provided the stirring rate is fast enough. Consequently the diffusion layer thickness is time independent, leading to a plateau in its current profile (Bott, 1996; Grieshaber, MacKenzie and Reimhul, 2008).

As was mentioned above, the stirred bulk solution experiment was performed under a semi-infinite diffusion condition which means that the diffusion layer thickness (d) was very small compared to the diffusion thickness of medium. In contrast, when the electrode is enclosed, the diffusion layer thickness is significant with respect to the thickness of the cell and the flux is related to the observed current at the working electrode by:

$$i_t = nFAD(\partial C_i/\partial X)|_{X=0} \quad (4.2)$$

where *A* is the electrode area (cm^2), *F* is 96,485, *n* is the number of electrons and other symbols as mentioned in Eq. 4.1. Considering that the system is finite, at longer time scales, the current response is dependent on the mass transport. Consequently, due to the longer time required for analyte to diffuse to the electrode surface to achieve a steady state, the current decreases with time. Therefore, in the encapsulated thin layer cell, the depth of the depletion zone becomes significant in comparison to the depth of the available cell thickness, whereas in bulk solution, this layer is essentially infinite. Therefore when the H_2O_2 concentration is reduced at the working electrode (in the encapsulated electrode), its bulk solution concentration will decrease significantly. This is dependent on the size of the working electrode compared to the volume of the solution and the rate of electrolysis.

In classical potential-step chronoamperometry, the signal decays to zero due to the total consumption of the reactant. Theoretically, the amperometric response would fall to 93% after $t = L^2/D$, where L is the diffusion layer thickness (25 µm) and D is the diffusion coefficient of H_2O_2. Based on a value of D of 1.71×10^{-5} cm^2 s^{-1}, this would occur after 365 ms. However, in this work, a pseudo steady-state response was evident after several hundred seconds. This is most likely due to a combination of barriers to diffusion slowing the process of complete reduction of the available H_2O_2 and the continued production of some H_2O_2 via the enzymatic catalysis of cholesterol.

To sum up, it was shown that modified silver electrodes were capable of measuring 0 to 10 mM H_2O_2 using stirred batch solution and 0 to 3 mM H_2O_2 using encapsulated electrodes. The next step was to assess if these electrodes could be used for the amperometric measurement of H_2O_2 produced from the enzymatic reaction of TC and HDLC.

4.3 Assessment of the impact of individual cholesterol assay reagents on the electrochemical behaviour of the modified electrode to H_2O_2 reduction

After assessing the suitability of the modified electrodes for the measurement of H_2O_2, the effect of the assay components used in the measurement of TC and HDL-C on the amperometric determination of H_2O_2 using the modified electrodes needed to be assessed. According to the spectrophotometric assay developed in section 3.2, for the final step of the assay, HRP, DMT and 4-AAP were used to measure the presence of H_2O_2. However, since the aim of this work was to measure H_2O_2 electrochemically, there was no need to evaluate the effect of these reagents on electrode behaviour. Consequently, the effect of assay surfactant, buffer, ChOx and ChEs were evaluated for their effect on modified silver electrode behaviour. Initial optimisation studies were performed in bulk stirred batch solution for reasons of experimental practicality and convenience and that, when optimised, were transferred to the encapsulated electrode configuration.

4.3.1 Effect of surfactant on amperometric measurement of H_2O_2 using modified electrodes

Firstly, the effect of Emulgen B-66 - as the surfactant used for the selective determination of HDL-C - on electrode performance was assessed. Fig. 4.4 shows the effect of a 1% (w/v) Emulgen B-66 on the electrocatalytic response of the modified electrode.

As can be seen, enhanced catalytic activity was observed in the presence of Emulgen B-66, particularly at lower concentrations of H_2O_2. A response of 8.1×10^{-7} A in the presence of 1 mM H_2O_2 was observed with a modified electrode without Emulgen B-66, whereas the current obtained by the same electrode in 1% (w/v) Emulgen B-66 reached approx. 3.4×10^{-6} A, demonstrating a four-fold increase in catalysis in the presence of Emulgen B-66. While this enhanced catalytic activity might assist in achieving a better detection limit of the sensors, such an enhancement would have to be controlled and reproducible, but should be considered as a potential enhancement of the performance of the device. It is, as yet, unclear why Emulgen B-66 appears to enhance the performance of the modified electrode, although similar effects have been observed with this electrode modification in the presence of a range of surfactants (Gonzalez-Macia, Smyth and

Killard, 2011). However, Emulgen B-66 is a polyoxyethylene (PEO) derivative compound and was reported that PEO and DBSA interact strongly with each other (Mohammad and Zarshad, 2009). In the presence of a polymer such as PEO, a decrease in critical micellar concentration (CMC) of DBSA is observed. Therefore, in the presence of the polymer, formation of micelles at lower concentration is facilitated. Formation of micelles and their interaction with the silver screen printed electrodes has been reported as the basis of the enhanced electrocatalysis of H_2O_2 (Gonzalez-Macia, Smyth and Killard, 2011; Gonzalez-Macia, Smyth and Killard, 2012).

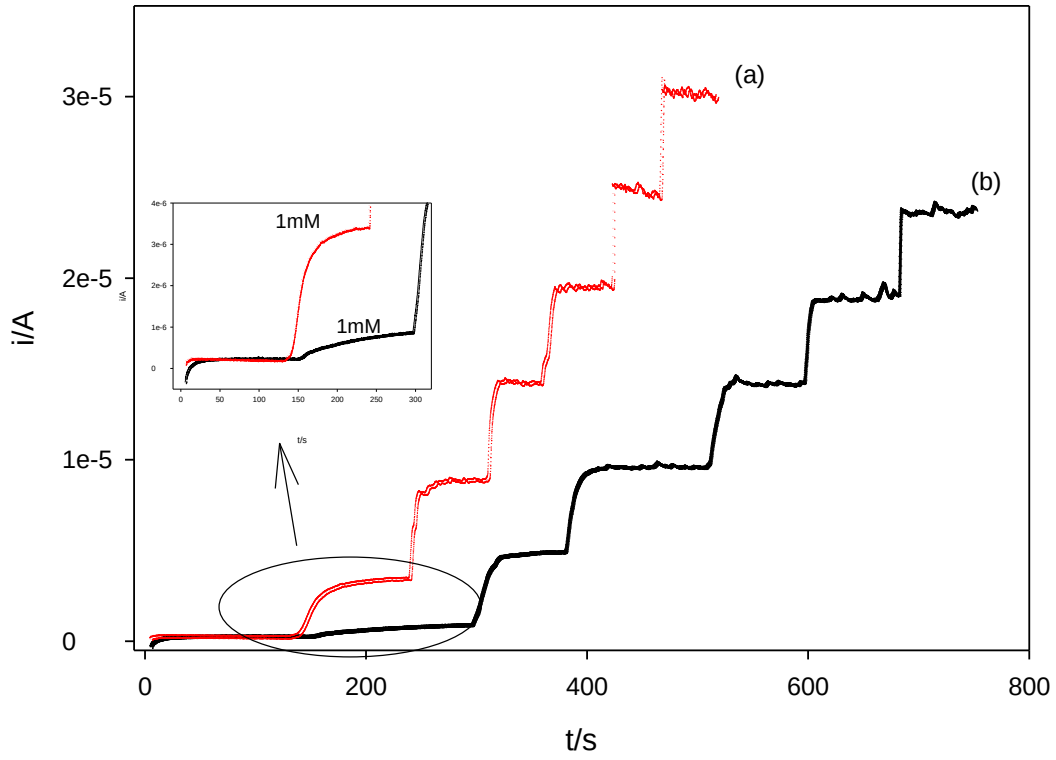

Fig. 4.4. Effect of Emulgen B-66 on the response of the modified electrode to the reduction of H_2O_2 (-0.1 V vs. Ag/AgCl). a) 1% (w/v) Emulgen B-66 in 0.1 M PBS pH 6.8; b) 0.1 M PBS pH 6.8. Inset: Detail of biosensor responses between 0 and 300 s.

4.3.2 Effect of buffer in amperometric measurement of H_2O_2 using modified electrodes

While the choice of buffer used in bioassay procedures can be a critical feature of their performance, the reagents employed in the spectrophotometric assay have been shown not to be particularly affected by the type of buffer used (Nakamura *et al.,* 2009). However, the choice of buffer may still have a significant impact on the

electrocatalysis of the modified electrodes. Gonzalez-Macia *et al*. (2011) demonstrated that the catalytic mechanism of H_2O_2 reduction at the modified electrodes was decreased in the presence of H^+, which is also in accordance with other models of H_2O_2 electroreduction (Brandt, 1983). However, since modified electrodes showed an acceptable response using a buffer with pH of 6.8 and blood pH is regulated within the range of 7.35 to 7.45, (Waugh and Grant, 2007), a buffer solution with physiological pH was deemed to be appropriate for any future sensor development. Based on this, several commonly used buffers operating at physiological pH were assessed for their effect on the behaviour of the sensor. These were Tris-HCl (0.1 M, pH 7.0), MOPS (50 mM, pH 7.3) and PBS (0.1 M pH 6.8); the latter which had been used for previous work. Tris-HCl has been used previously for the amperometric measurement of HDL-C (Kinoshita *et al.,* 1998) and Good's buffer - which is similar to MOPS - was also reported to be suitable for use in the spectrophotometric measurement of HDL-C (Nakamura *et al.,* 2009; Yamamoto, Yamamoto and Nakanishi, 2011).

A solution of 1% (w/v) Emulgen B-66 was prepared using these buffers and the amperometric responses of the modified electrodes were assessed in H_2O_2. Amperometric measurement of 0 to 6 mM H_2O_2 in MOPS and Tris-HCl are shown in Fig. 4.5A and B, respectively. In both experiments, a measurement of the electrode response to H_2O_2 in PBS was performed before and after measurement in either MOPS or Tris-HCl. Almost no cathodic response was observed in MOPS buffer (Fig. 4.5A).

This may be due to the fact that MOPS is an amphipathic molecule. MOPS also has a very similar structure to DBSA, having an aliphatic chain and a sulphonate group. This may result in interference with the surface modification of the DBSA/KCl lyotropic layer, while not contributing to the formation of such a layer and elimination of the enhanced electrocatalysis, The electrode showed a slight decrease in sensitivity after exposure to MOPS. This might be due to the transient disruption of K^+ and Cl^- structural organisation with the DBSA (and now Emulgen B-66) which is also an important part of the catalysis and which is restored upon removal of MOPS.

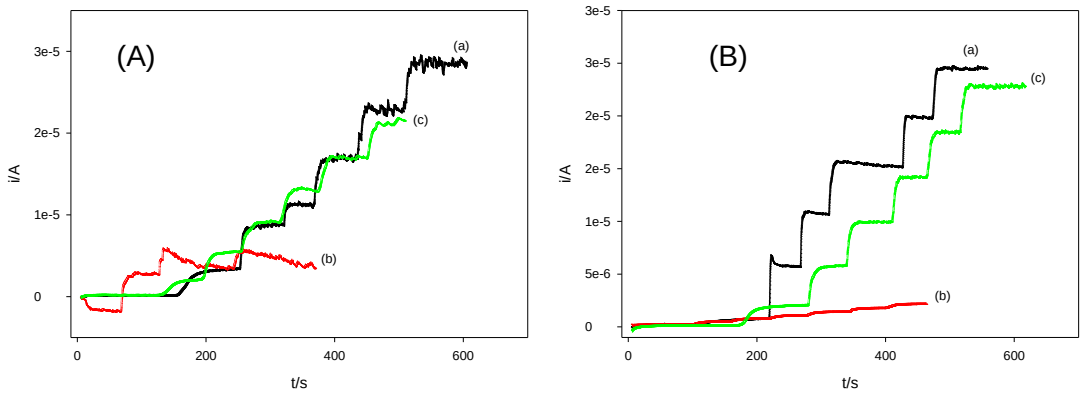

Fig. 4.5. Amperometric response of the modified electrodes to H_2O_2 (0-6 mM) in 1% (w/v) Emulgen B-66 prepared in (A) 50 mM MOPS pH 7.3 and (B) 0.1 M Tris-HCl pH 7.0. (a) Response in 0.1 M PBS pH 6.8 before testing in MOPS/Tris; (b) responses in MOPS/Tris; (c) Response in 0.1 M PBS after testing in MOPS/Tris.

Fig. 4.5B shows that, in the presence of Tris-HCl, the catalytic activity of the electrodes was also significantly reduced but was also restored after its replacement with PBS. This might be due to incompatibility of silver containing electrodes in the presence of Tris buffer (Ryan, 1969). Tris buffer solutions, when used with certain types of electrodes in particular Ag/AgCl reference electrodes, have been shown to cause undesirable side reactions. In addition, neutralising Tris buffer using HCl may affect the ionic strength of the solution, causing a negative effect on catalytic activity of the electrodes towards H_2O_2. A similar mechanism to that discussed for MOPS should also not be ruled out.

To sum up, while it has been reported that there is no limitation on the type of buffer used in cholesterol assays, the importance of evaluation of the type of buffer used in amperometric measurement was highlighted. Based on these findings, 0.1 M PBS pH 6.8 was used as the buffer for all further amperometric studies.

Having selected the appropriate buffer for further studies and evaluated the behaviour of the electrode to Emulgen B-66, the response of the modified electrode to H_2O_2 in 1% (w/v) Emulgen B-66 in 0.1 M PBS was evaluated over a wider range of H_2O_2 concentrations in two ranges from 0.05 to 0.55 mM and 1 to 6 mM using chronoamperometry at -0.1 V vs. Ag/AgCl. Fig. 4.6 shows the resulting amperograms from 5 to 125 s. Here, most of the early charging current and initial faradaic current had decayed away and after 20-40 s, the electrodes had reached a quasi-steady state from which current measurements at fixed time points could be made. The currents observed for 0, 0.05, 0.1 and 0.15 mM H_2O_2 at 120 s were

2.35×10^{-7}, 2.35×10^{-7}, 2.37×10^{-7} and 3.0×10^{-7} A, respectively. Currents rose above baseline at 0.1 mM concentration and showed good linearity up to 6 mM (slope=5.11×10^{-6}, r^2 =0.99).

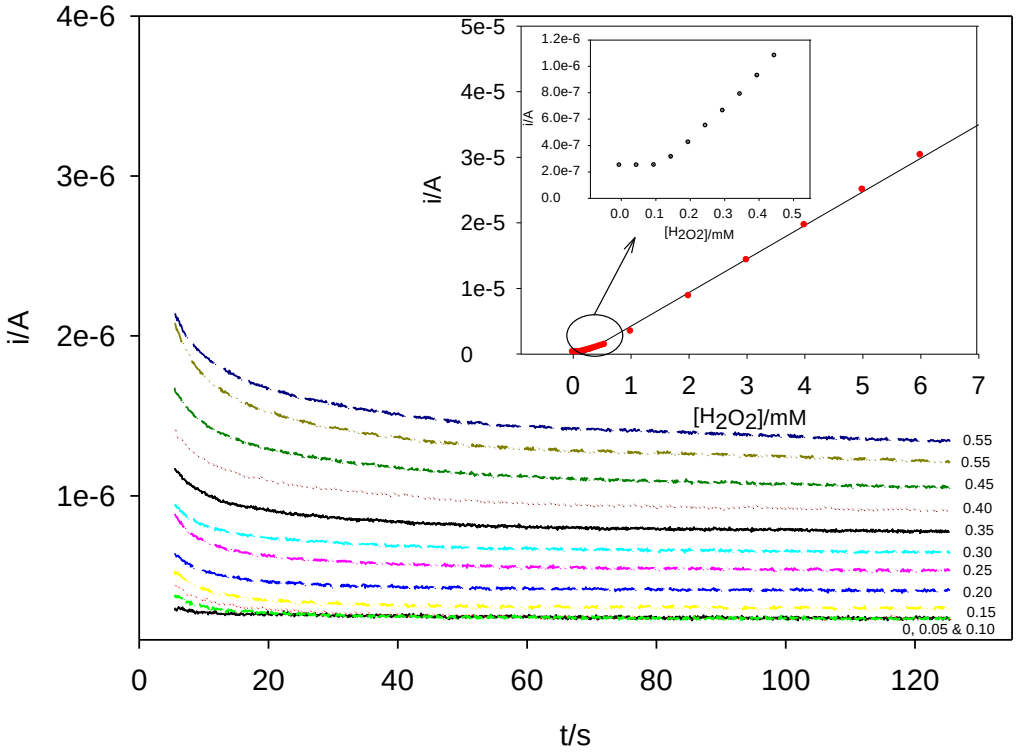

Fig. 4.6. Chronoamperometric measurement of H_2O_2 (0-0.55 mM) at modified electrodes in 0.1 M PBS pH 6.8, 1% (w/v) Emulgen B-66 at -0.1 V vs. Ag/AgCl. Inset: Current response at 120 s.

Based on this, the limit of detection was taken as 0.15 mM which is three times the background current. These preliminary amperometric experiments demonstrated that Emulgen B-66 prepared in 0.1 M PBS was suitable for the amperometric measurement of H_2O_2. H_2O_2 could be measured from 0.1 to 6 mM, with a lower detection limit of 0.15 mM. Since the minimum clinically relevant concentration of HDL-C is 1 mM and the stoichiometry of cholesterol and H_2O_2 is 1:1 for a complete reaction, this would suggest that, under these conditions, the electrode would be suitable for the measurement of HDL-C. In addition, due to the borderline high level of 5.2-6.2 mM for TC measurement, modified silver electrodes would also be capable of measuring TC as well as HDL-C. Having determined the effect of buffer and Emulgen B-66, the next step was to evaluate the effect of all other assay components through the amperometric measurement of H_2O_2 using the modified silver electrodes as the assay platform.

4.3.3 Effect of enzymes and HDL-C samples on H_2O_2 reduction

To evaluate the electrochemical response of the electrode towards H_2O_2 in the presence of enzymes and HDL-C isolated from serum, time-based amperometric measurement of H_2O_2 was performed in the presence of ChEs, ChOx and HDL-C and using 1% (w/v) Emulgen prepared in 0.1 M PBS pH 6.8. The effect of the enzymes (ChEs and ChOx) and the HDL-C sample on the sensor response to H_2O_2 was also investigated using amperometry in stirred batch solution. Fig. 4.7 shows the response of the modified sensor to H_2O_2 in the presence of 1% (w/v) Emulgen B-66 in PBS before, during and following exposure of the sensor to HDL-C, ChEs and ChOx. The response of the sensor was similar before (a) and after (b) the combined exposure of the electrode to these species. However, the response was reduced significantly in the presence of HDL-C (d) and ChOx (e) alone.

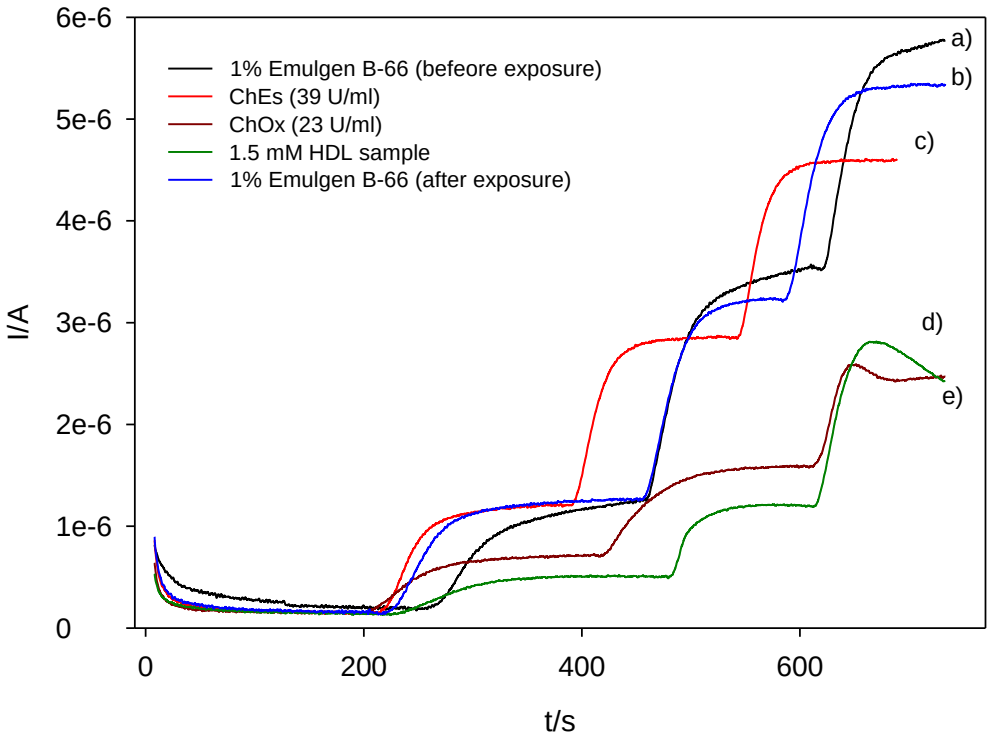

Fig. 4.7. Amperometric response of sensors to H_2O_2 (0.5 to 1.5 mM) in stirred solution containing 1% (w/v) Emulgen B-66: a) before exposure and; b) after exposure to a solution of ChEs (39 U/mL), ChOx (23 U/mL) and serum containing 1.5 mM HDL-C; c) in the presence of ChEs (39 U/mL); d) in the presence of serum containing 1.5 mM HDL-C; e) in the presence of ChOx (23 U/mL).

The presence of HDL or ChOx appeared to significantly disrupt sensor behaviour, possibly by disrupting the functioning of the lyotropic layer on the electrode surface.

In the case of HDL, it has already been clearly demonstrated that it interacts selectively with Emulgen B-66 and may disrupt the lyotropic layers formed at the electrode surface. In the case of ChOx, it has also been found to be capable of disrupting phospholipid membranes via the "active site lid" mechanism (Ghoshroy, Zhu and Sampson, 1997). Phospholipid membranes are also formed from the organisation of amphiphilic molecules and are analogous to the lyotropic phases formed by the interaction of Emulgen B-66 and electrolyte at the electrode surface. Disruption may also relate to the highly hydrophobic nature of the ChOx active site and the presence of additional hydrophobic domains on its surface (Vrielink, Lloyd and Blow, 1991). The sensor response was not significantly affected by the presence of ChEs. These results also demonstrated the reversible nature of the effects of both lipoprotein and ChOx on the electrocatalytic response of the sensor, further suggesting that the formed lyotropic phase is disrupted, but not eliminated.

In conclusion, while some reagents such as Emulgen B-66 increased H_2O_2 reduction, others such as ChOx and the HDL-C sample reduced this effect. Such influences must thus be considered when employing these assay components in the measurement of H_2O_2 following the release and enzymatic conversion of cholesterol from lipoproteins.

Preliminary studies on the effect of Emulgen B-66 on amperometric measurement of H_2O_2 using modified electrodes (see section 4.3.1) were performed at a concentration of 1% (w/v). However, later optimisation led to the selection of 6% (w/v) as the most suitable concentration which yielded maximum differentiation between HDL-C and LDL-C measurement. Therefore, the next step was to evaluate the effect of 6% (w/v) Emulgen B-66 in 0.1 M PBS pH 6.8 on the electrochemical measurement of H_2O_2 using the modified electrodes. Therefore, amperometric measurement of 0 to 3 mM H_2O_2 using unmodified and modified silver electrodes was this time performed in 6% (w/v) Emulgen/PBS solution. Significantly enhanced reduction currents (17-fold) were observed for unmodified electrodes in the presence of 6% (w/v) Emulgen B-66 compared to those in PBS (Fig. 4.8).

In the absence of surfactant, unmodified electrodes had a response of 1.33×10^{-7} A (curve a) while in the presence 6% (w/v) Emulgen B-66, there was a response of 2.2×10^{-6} A, showing an almost 17-fold enhancement in catalysis. A current response of 5.96×10^{-6} A was obtained for 3 mM H_2O_2 using DBSA/KCl modified

electrode in the presence of 6% (w/v) Emulgen B-66 (curve d), which was a 1.6-fold current increase over the one in PBS which was 3.6×10^{-6} A (curve c). This was almost the same as that observed for 3 mM H_2O_2 previously in the presence of 1% Emulgen B-66 which was 1.66-fold (section 4.3.1). In addition, by employing a new set of silver screen printed electrodes prepared with a new batch of silver ink, the catalytic enhancement was 27-fold greater for DBSA/KCl modified electrodes in PBS compared to the five-fold enhancement observed previously in section 4.2. This enhanced response might further assist in achieving a lower limit of detection of H_2O_2 with the sensor.

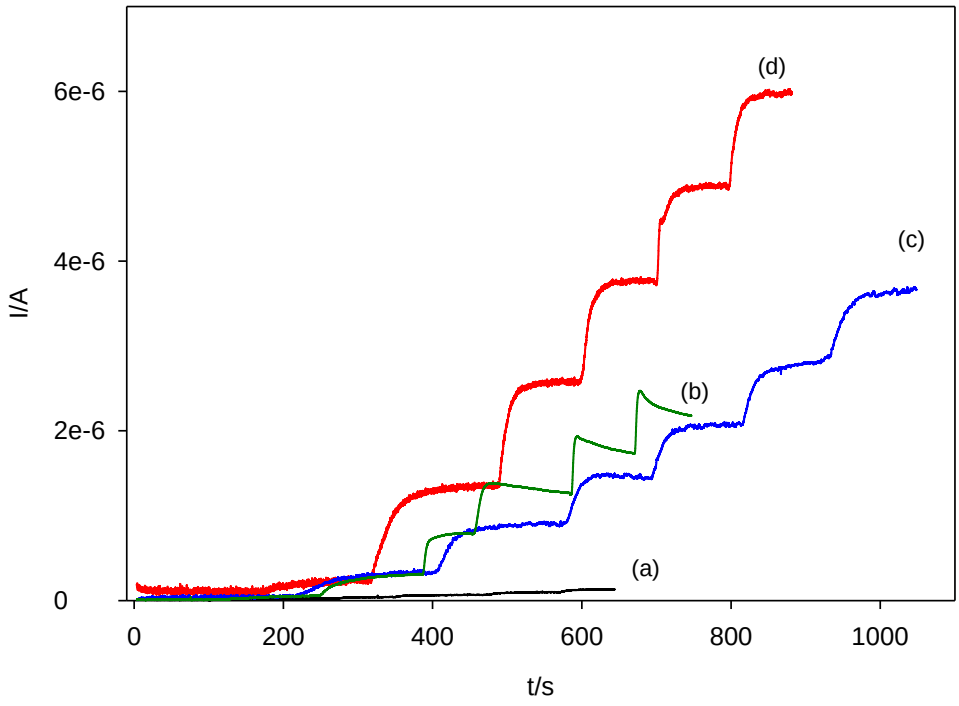

Fig. 4.8. Amperometric measurement of 0.5 to 3 mM H_2O_2 at -0.1 V vs. Ag/AgCl in 5 ml stirred batch solution using: a) unmodified silver electrode in PBS; b) unmodified silver electrode in 6% (w/v) Emulgen B-66, c) modified electrode in PBS and d) modified electrode in 6% Emulgen B-66.

To sum up, Emulgen B-66 is a nonionic surfactant which was prepared in an electrolyte solution of 0.1 M PBS pH 6.8. It is believed that an equivalent effect may be achieved by this combination as has previously been demonstrated with other surfactant/salt combinations. However, further enhancement appears to result from operation of the sensor in a solution of this surfactant and electrolyte, as opposed to the modification of the surface alone with DBSA and KCl. Further studies were performed on the suitability of using Emulgen B-66/PBS as an alternative modification solution which will be discussed in the following chapters.

4.3.4 Effect of delipidated serum in amperometric measurement of H_2O_2

In order to evaluate the effect of serum components on the amperometric measurement of H_2O_2 using encapsulated modified electrodes, the suitability of using delipidated serum was evaluated. The amperometric responses of 3 mM H_2O_2 prepared in PBS was compared to that observed for 3 mM H_2O_2 prepared in delipidated serum using encapsulated modified electrodes (Fig. 4.9 and Table 4.1).

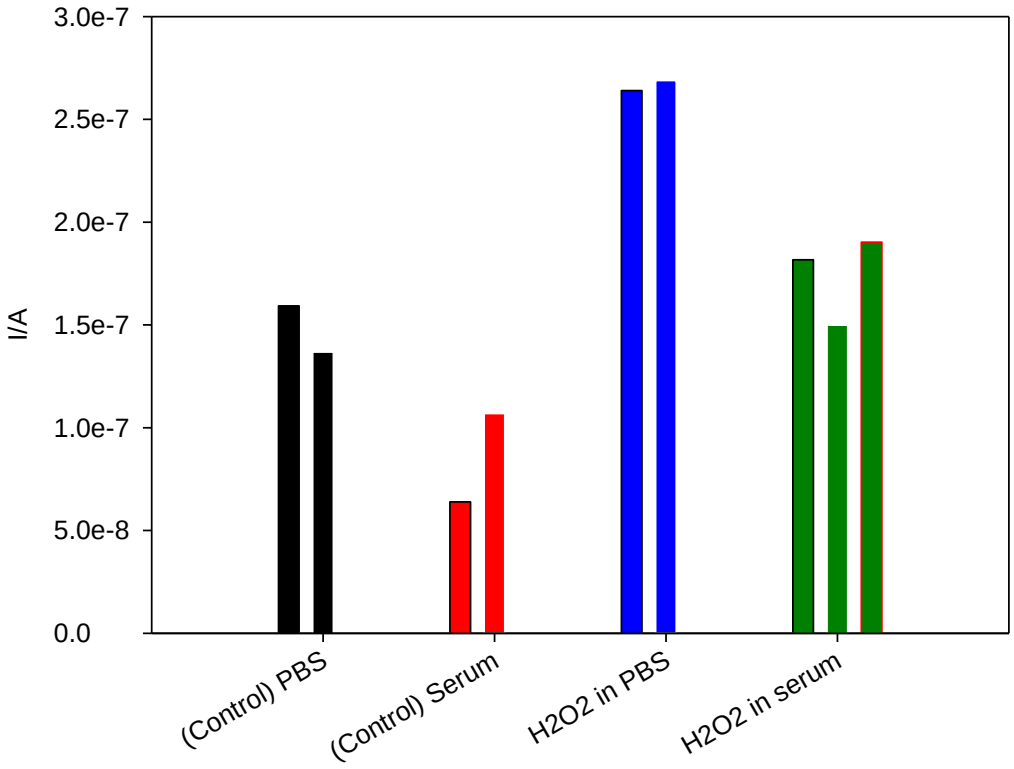

Fig. 4.9. Amperometric measurement of 3 mM H_2O_2 prepared in PBS compared to the one prepared in delipidated serum at 420 s.

As can be seen, in Fig. 4.9, although the cathodic current observed for the modified electrode in the presence of delipidated serum decreased in comparison with the one in PBS, the signal to background current (S/B) just showed a slight increase in the presence of delipidated serum showing that there would be no loss in surface modification in the presence of delipidated serum. This might be due to the slight change in ionic strength of sample once prepared in delipidated serum compared to that in PBS (Cham and Knowles, 1976; Murphy, Allen and Hill, 2010). Since no loss in sensitivity of modified electrode was observed in the presence of delipidated serum, it was accepted that delipidated serum would be a suitable sample matrix for further assay development.

Table 4.1. Signal to background (S/B) response of modified electrode to 3 mM H_2O_2 prepared in PBS and delipidated serum.

Substrate	I/A			Average S/B
	1st repeat	2nd repeat	3rd repeat	
Control (in serum)	6.39×10^{-8}	1.07×10^{-7}	-	-
Control (in PBS)	1.59×10^{-7}	1.55×10^{-7}	-	-
3 mM H_2O_2 in serum	1.82×10^{-7}	1.50×10^{-7}	1.90×10^{-7}	2
3 mM H_2O_2 in PBS	2.64×10^{-7}	2.69×10^{-7}	-	1.7

4.3.5 Effect of final assay reagent formulation on the quantitative measurement of H_2O_2 in delipidated serum

Based on the facts that some assay components enhance the electrocatalysis of the modified electrode and some diminish, which were found using stirred batch experiments, the response of the encapsulated biosensor system to 8 µL of H_2O_2 prepared in delipidated serum in the presence of all the assay components was assessed in the 0 to 10 mM range (Fig. 4.10).

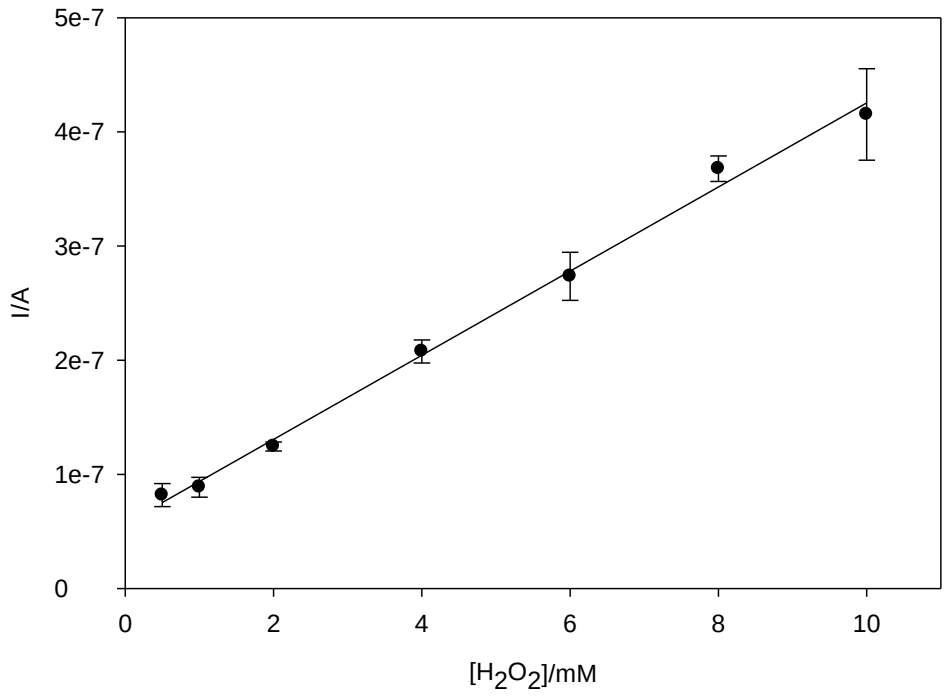

Fig. 4.10. Chronoamperometric response of modified electrodes to 8 µL of H_2O_2 (-0.1 V vs Ag/AgCl at 420 s) in the presence of the final assay reagent formulation and delipdated serum at 420 s, (slope=3.7×10^{-8} A/mM, r^2=0.99, RSD=7.2%).

The resulting responses took into account all processes which either enhanced the electrocatalysis such as the presence of Emulgen B-66, or which interfered with it,

such as HDL-C and ChOx. Chronoamperometric measurements were again performed at -0.1 V (vs Ag/AgCl) and the current responses recorded at 420 s. The sensor had excellent linearity from 0 to 10 mM H_2O_2 (slope=3.7×10^{-8}, r^2=0.99, RSD=7.2%), which would make it suitable to be used in direct determination of HDL-C in serum initially and then progressively for TC measurement in serum, based on an end point enzyme assay protocol such as the one developed in Chapter 3.

To employ this assay for the measurement of TC, the next step was to evaluate the effect of Triton X-100 as a non-specific surfactant for the measurement of TC compared to Emulgen B-66 in amperometric measurement of H_2O_2 using modified silver electrodes.

4.3.6 Effect of Triton X-100 on modified silver electrode behaviour

After assessing the effect of HDL-C specific surfactant on electrode behaviour, the effect of 0.5% (v/v) Triton X-100 as a non-specific surfactant for the measurement of TC was assessed. Therefore the response of the encapsulated sensor to 8 µL of 4 mM H_2O_2 prepared in delipidated serum in the presence of 0.5% (v/v) Triton X-100 and 6% (w/v) Emulgen B-66 was evaluated (Fig. 4.11).

Fig. 4.11 shows the results of chronoamperometric measurements performed at -0.1 V (vs Ag/AgCl) with current responses at 420 s. The same background current of 2.3×10^{-8} A was observed in the presence of both surfactants. The current responses in Triton X-100 were 1.13×10^{-7} and 1.07×10^{-7} A, compared to those in Emulgen B-66 which were 1.00×10^{-7} and 1.10×10^{-7} A, showing almost no difference in their effect on electrode behaviour which would make it suitable for specificity study which the results in different surfactants required to be assessed and compared with each other. Triton X-100 has also been reported to enhance silver screen printed electrodes response in the presence of KCl (Gonzalez-Macia, Smyth and Killard, 2011). Thus, 0.5% (v/v) in 0.1 M PBS may be suitable for the development of a TC biosensor which will be discussed in the following chapter.

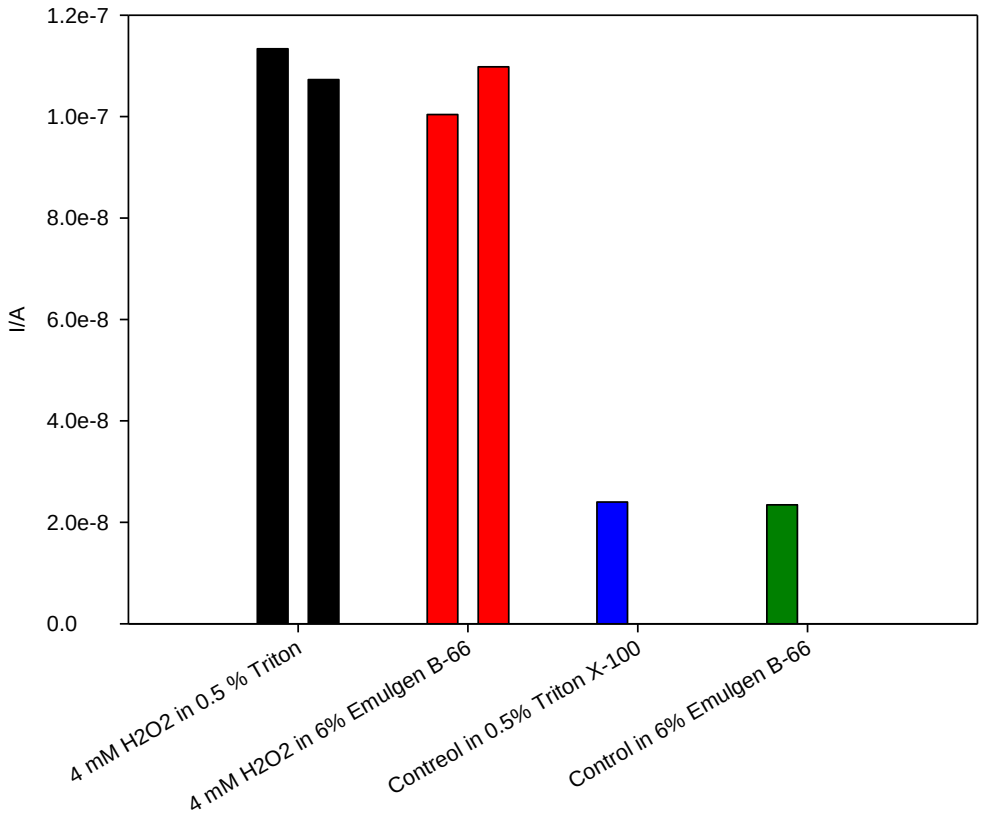

Fig. 4.11. Chronoamperometric response to 8 µL of 4 mM H_2O_2 (-0.1 V vs Ag/AgCl) in the presence of 0.5% (v/v) Triton X-100 and 6% (w/v) Emulgen B-66 at 420 s. Delipidated serum was used for controls.

4.4 Conclusions

DBSA/KCl-modified silver electrodes were evaluated for their ability to measure H_2O_2 in both stirred batch mode and using encapsulated electrodes using a non-enzymatic electrochemical determination. Direct measurement of H_2O_2 at the relatively moderate potential of -0.1 V (vs Ag/AgCl) and in absence of enzyme has the unique advantages of not requiring the complex optimisation and implementation of a biological component, as well as providing the possibility of designing a simpler miniaturised device which can broaden its application.

The effects of assay reagents such as buffer and surfactant on the electrode behaviour were assessed amperometrically in the presence of H_2O_2 solutions. Of three buffers evaluated, the electrodes showed a linear responses in 0.1M PBS (pH=6.8) with enhanced catalytic activity toward H_2O_2 in the presence of Emulgen B-66.

The effect of separate and combined cholesterol assay components on the DBSA/KCl modified silver electrodes was also assessed. Although HDL-C sample, ChOx and delipidated serum showed a negative effect on electrode response, reagents such as Emulgen B-66 and Triton X-100 had a positive effect on electrocatalysis. Using Emulgen B-66 - which would be suitable for the selective measurement of HDL-C - the sensor showed excellent linearity from 0 to 10 mM H_2O_2 with slope=3.7×10^{-8}, r^2=0.99, RSD=7.2%, which would make it suitable to be used for the direct determination of HDL-C in serum, based on an end point enzyme assay protocol such as that developed in Chapter 3. Finally, Triton X-100 had a comparable effect on the amperometric measurement of H_2O_2 to Emulgen B-66 which indicated that it would be suitable to form the basis of the amperometric measurement of TC.

References

Bott, A.W. (1996) Mass Transport. *Current Separations*. 14 (3/4), pp. 104-109.

Brandt, E.S. (1983) Electrochemistry of oxygen and hydrogen peroxide at clean and at halide-covered polycrystalline silver. *Journal of Electroanalytical Chemistry*. 150, pp. 97-109.

Cham, B.E. and Knowles, B.R. (1976) A solvent system for delipidation of plasma or serum without protein precipitation. *Journal of Lipid Research*. 17 (2), pp. 176-181.

Ghoshroy, K.B., Zhu, W. and Sampson, N.S. (1997) Investigation of membrane disruption in the reaction catalysed by cholesterol oxidase. *Biochem.* 36, pp. 6133-6140.

Gonzalez-Macia, L., Smyth, M.R. and Killard, A.J. (2011) Enhanced electrochemical reduction of hydrogen peroxide at metallic electrodes modified with surfactant and salt. *Electrochimica Acta*. 58, pp. 562-570.

Gonzalez-Macia, L., Smyth, M.R. and Killard, A.J. (2012) A Printed Electrocatalyst for Hydrogen Peroxide Reduction. *Electroanalysis*. 24 (3), pp. 609-614.

Grieshaber, D., MacKenzie, R. and Reimhul, E. (2008) Electrochemical Biosensors - Sensor Principles and Architectures. *Sensors*. 8, pp. 1400-1458.

Gubala, V., Harris, L.F., Ricco, A.J. and Tan MX, W., D.E. (2012) Point of care diagnostics: status and future. *Analytical chemistry.* 84, pp. 487-515.

Kinoshita, H., Torimura, M., Kano, K. and Ikeda, T. (1998) Amperometric determination of high-density lipoprotein cholesterol using polyethylene glycol-modified enzymes and a peroxidase-entrapped electrode. *Annals of Clinical Biochemistry*. 35, pp. 739-744.

Kissinger, P.T. and Heineman, W.R. (1996) *Laboratory Techniques in Electroanalytical Chemistry*. 2nd ed. Marcel Dekker, Inc.

Lee, E.J., Shin, S.D., Song, K.J., Kim, S.C., Cho, J.S., Lee, S.C., Park, J.O. and Cha, W.C. (2011) A point-of-care chemistry test for reduction of turnaround and clinical decision time. *American Journal of Emergency Medicine*. 29, pp. 489-495.

Mohammad, S.K. and Zarshad, A. (2009) Micellization of Dodecyl benzenesulfonic acid and its interaction with poly(ethylene oxide) polymer. *J. Chem. Soc. Pak*. 31 (5), pp.712-717.

Murphy, L.J., Allen, H. and Hill, O. (2010) *Reducing Signal Distortions.*Roche Diagnostics GmbH, EP 2171079 A1.

Nakamura, M., Taniguchi, Y., Yamamoto, M. and Manabe, M. (2009) *Method of Pretreatment of Sample for Quantitating Cholesterol and Method for Quantitating Cholesterol in Specific Lipoproteins.*Daiichi Pure Chemicals Co., Ltd. 20090226945.

Razmi, H. and Habibi, E. (2009) Nanomolar detection of hydrogen peroxide at a new polynuclear cluster of tin pentacyanonitrosylferrate nanoparticle-modified carbon ceramic electrode. *Analytical Biochemistry*. 392 (2), pp. 126-132.

Ryan, M.F. (1969) Unreliable results. *Science*. 165, pp. 851.

Vrielink, A., Lloyd, L.F. and Blow, D.M. (1991) Crystal structure of cholesterol oxidase from Brevibacterium sterolicum refined at 1.8 A°resolution. *Journal of Molecular Biology.* 219, pp. 533-554.

Waugh, A. and Grant, A. (2007) *Anatomy and Physiology in Health and Illness.* Churchill Livingstone Elsevier.

Yamamoto, S., Yamamoto, M. and Nakanishi, K. (2011) *Method of Measuring Lipid in Specific Lipoprotein.*Daiichi Chemical Co., L. US 2011/0091917.

Zhang, X., Ju, H. and Wang, J. (2008) *Electrochemical Sensors, Biosensors and their Biomedical Applications*. 1st ed. USA: Elsevier.

Chapter 5

Development of electrochemical biosensors for HDL-C and TC using external assay reagents

5.1 Introduction

In the previous chapter, the application of optimised assay reagents for the colorimetric measurement of HDL-C and TC was investigated. The next step was to transfer this assay protocol to the measurement of HDL-C and TC using an electrochemical sensor. In Chapter 4, a preliminary assessment was performed to investigate the effect of assay reagents on the electrochemical measurement of H_2O_2 using DBSA/KCl modified silver electrodes. Some interference by ChOx and serum components was observed on the amperometric determination of H_2O_2. However, the modified electrodes were found to be suitable for the quantitative measurement of 0 to 10 mM H_2O_2. DBSA/KCl modified silver electrodes had previously been used for amperometric determination of the H_2O_2 released from the enzymatic reaction of glucose in the presence of glucose oxidase and O_2 (Gonzalez-Macia, Smyth and Killard, 2012a). However, it had also been observed that Emulgen B-66, which was used for the selective determination of HDL-C, could also bring about enhanced catalysis in a manner similar to DBSA/KCl, which raised the prospect of replacing the DBSA/KCl modification entirely.

In this chapter, the optimised assay reagents established in Chapter 4 were used for the measurement of firstly H_2O_2, followed by cholesteryl acetate and finally HDL-C in PBS, followed by the measurement of H_2O_2, HDL-C and TC in delipidated serum. The linearity, sensitivity, and reproducibility of the sensor for determination of the released H_2O_2 were evaluated. Following this, the possibility of applying Emulgen B-66/PBS and Triton X-100/PBS were assessed as alternative electrode modifiers to DBSA/KCl for HDL-C and TC measurement, respectively and their suitability for the measurement of HDL-C and TC in delipidated serum was evaluated. Amperometric measurement of HDL-C and TC in clinically derived serum samples was also investigated.

5.2 Development of the HDL-C biosensor

Since DBSA/KCl modified electrodes showed an acceptable response to the amperometric measurement of 0 to 10 mM H_2O_2 in the presence of assay reagents (Section 4.3.5), in this section, these electrodes were first used to develop a HDL-C biosensor. Then, based on the fact that both un-modified silver electrodes and DBSA/KCl modified electrodes showed an enhanced catalytic activity towards H_2O_2 in the presence of 6% (w/v) Emulgen B-66 (Sections 4.3.1 and 4.3.4), the

possibility of employing Emulgen B-66/PBS as a modification solution to develop a HDL-C biosensor was evaluated.

5.2.1 Development of the HDL-C biosensor using DBSA/KCl modified electrodes

In order to develop the HDL-C biosensor, the assay reagents discussed in Chapter 4 were used first to measure 0 to 6 mM H_2O_2, followed by 0 to 6 mM cholesteryl acetate and finally 0 to 6 mM HDL-C, all prepared in PBS. As was shown in Chapter 3, final concentrations of 23 U/mL ChOx and 39 U/mL ChEs were found to be suitable for the spectrophotometric measurement of 0 to 4 mM cholesteryl acetate with acceptable linearity up to 4 mM. While the concentration of 6% (w/v) Emulgen B-66 was eventually found to be most optimal for measuring HDL-C over LDL-C in later studies, a concentration of 1% (w/v) was used for preliminary investigations here. Encapsulated DBSA/KCl modified electrodes were used for amperometric measurement, unless otherwise stated. Fig. 5.1 shows the amperograms from 0 to 420 s for 0 to 6 mM H_2O_2 in the presence of enzymes and surfactant using encapsulated electrodes.

The amperometric responses of the biosensor to H_2O_2 and cholesteryl acetate at 420 s are compared in Fig. 5.2. Both plots show good linearity of r^2=0.97 up to 6 mM, with cholesteryl acetate showing a slightly lower slope compared to H_2O_2. This might be due the fact that for cholesteryl acetate measurement, the H_2O_2 is released after two enzymatic reaction steps leading to the delayed release of H_2O_2 during the assay period.

HDL-C was also measured in the presence and the absence of assay reagents. Amperometric measurements in the absence of enzymes were used as controls. As can be seen in Fig. 5.3, the electrode responses were linear across the measured range showing a slope of 2.78×10^{-8} A/mM which was approximately 2-fold and 1.5-fold less than those observed for H_2O_2 and cholesteryl acetate measurements, respectively. This was also expected as the use of HDL-C as a substrate adds an additional kinetic step to the assay. Once again, despite the negative effect of the HDL-C sample on the electrode response which was discussed in Chapter 4, the method showed good linearity across the measured range. The specificity of the method for measuring HDL-C over LDL-C in PBS was then assessed.

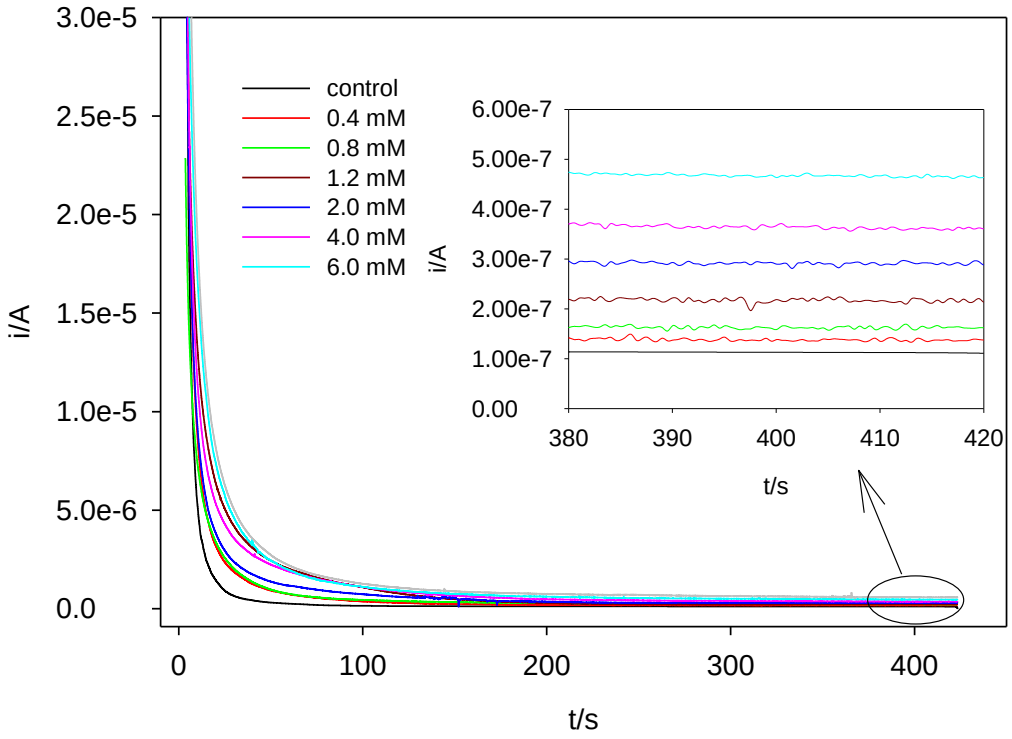

Fig. 5.1. Chronoamperometric measurement of H_2O_2 (0-6 mM) using encapsulated electrodes in the presence of HDL-C assay reagent at -0.1 V (vs Ag/AgCl).

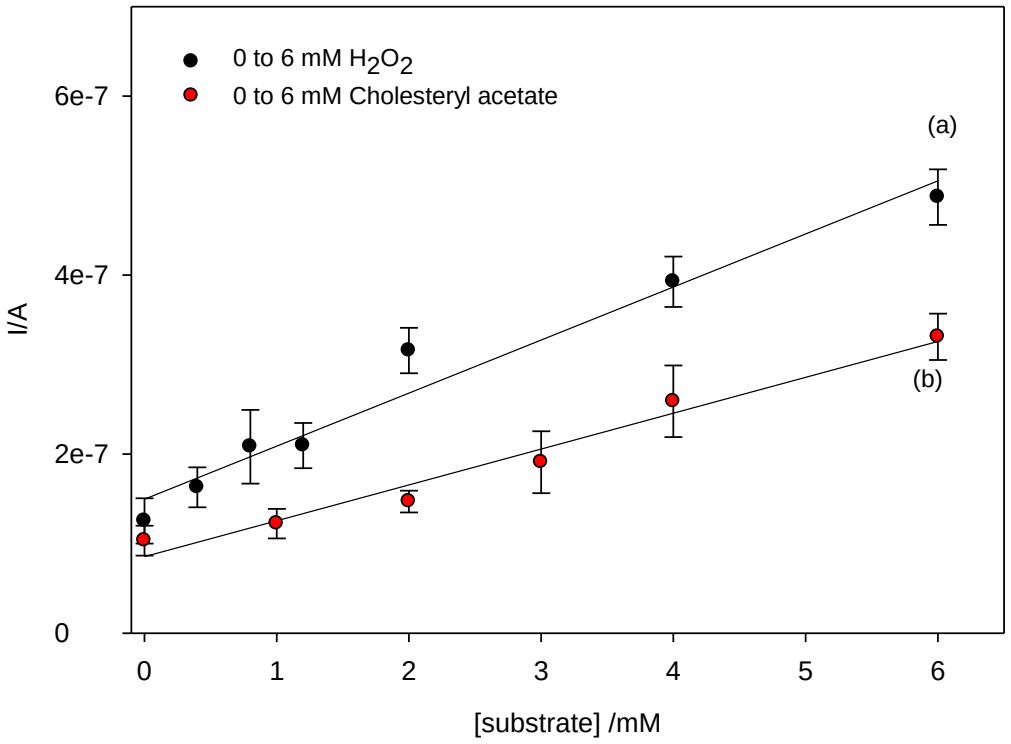

Fig. 5.2. Chronoamperometric response to 8 µL of (a) 0 to 6 mM H_2O_2 and (b) 0 to 6 mM cholesteryl acetate (-0.1 V vs Ag/AgCl at 420 s) in the presence of HDL-C assay reagents at 420 s, (slope=5.93×10^{-8} A/mM, r^2=0.97 for H_2O_2 and slope=4.01×10^{-8} A/mM, r^2=0.97 for cholesteryl acetate).

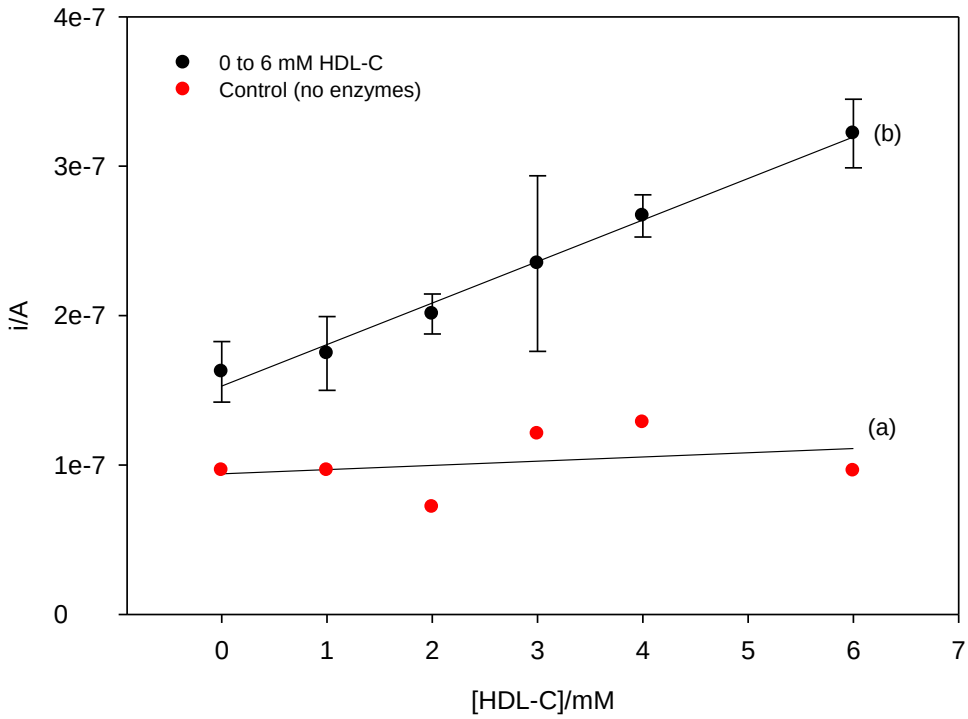

Fig. 5.3. Biosensor response to 0-6 mM HDL-C in PBS at -0.1 V (vs Ag/AgCl). a) 0 mM in the presence of ChEs and ChOx and 1 to 6 mM in the absence of enzymes; b) in the presence of 1% (w/v) Emulgen B-66, 39 U/mL ChEs and 23 U/mL ChOx; (slope=2.78×10^{-8} A/mM, r^2=0.99).

5.2.1.1 Measurement of the selectivity of the biosensor for measurement of HDL-C over LDL-C

It was shown previously in Chapter 3 that Emulgen B-66 was capable of bringing about the selective measurement HDL-C over LDL-C, while use of Triton X-100 allowed the measurement of TC. To assess whether this remained the case with amperometric measurement, HDL-C (1 mM) in the presence or absence of LDL-C (1 mM) was used to measure the HDL-C and TC content of the samples in the presence of HDL-C selective and non-selective surfactants. 1% (w/v) Emulgen B-66 and 0.5% (v/v) Triton X-100 were used for HDL-C and TC measurements, respectively (Fig. 5.4 and Fig. 5.5). Almost the same cathodic current was observed in the presence of Emulgen B-66 and Triton X-100 for the amperometric measurement of 1 mM HDL-C (1.7×10^{-7} A and 1.8×10^{-7}A, respectively), while a mixture of 1 mM HDL-C and 1 mM LDL-C showed an approx. 1.1-fold greater cathodic current in the presence of Emulgen B-66 for its HDL-C measurement (1.9×10^{-7} A), which might be due to the presence of some FC present in the LDL-C sample. However, the current observed for the mixture of HDL-C and LDL-C in the

presence of Triton X-100 (2.45×10^{-7} A) was significantly higher compared to that observed for HDL-C measurement, demonstrating that Emulgen B-66 was selectively measuring HDL-C over LDL-C in the biosensor.

The selectivity according to Murphy *et al.,* (2009) can be calculated as that the ratio of the G_{LDL} in the presence of specific surfactant vs of the G_{LDL} in its absence which should be less than 1 and more preferably around 0.3 where G is the gradient of the measured response which is the current measured verus the known concentrations of the sample. Therefore, the selectivity according to this method after removing the background current observed in the presence of each surfactant (controls in Fig. 5.5), was calculated to be 0.44 which was within the acceptable limit reported by Murphy *et al.,* (2009).

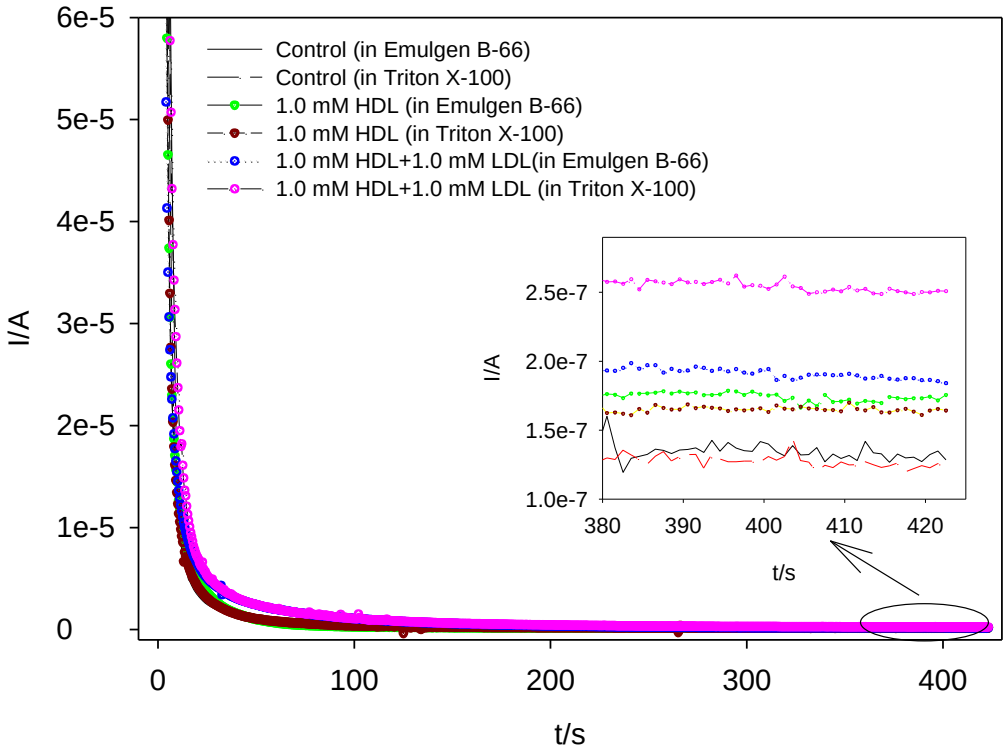

Fig. 5.4. Chronoamperometric measurement of 1 mM HDL-C in the presence and absence of 1 mM LDL-C at -0.1 V (vs Ag/AgCl), Emulgen B-66 and Triton X-100 were used for HDL-C and TC measurements, respectively. Inset: Detail of biosensor responses between 380 and 420 s.

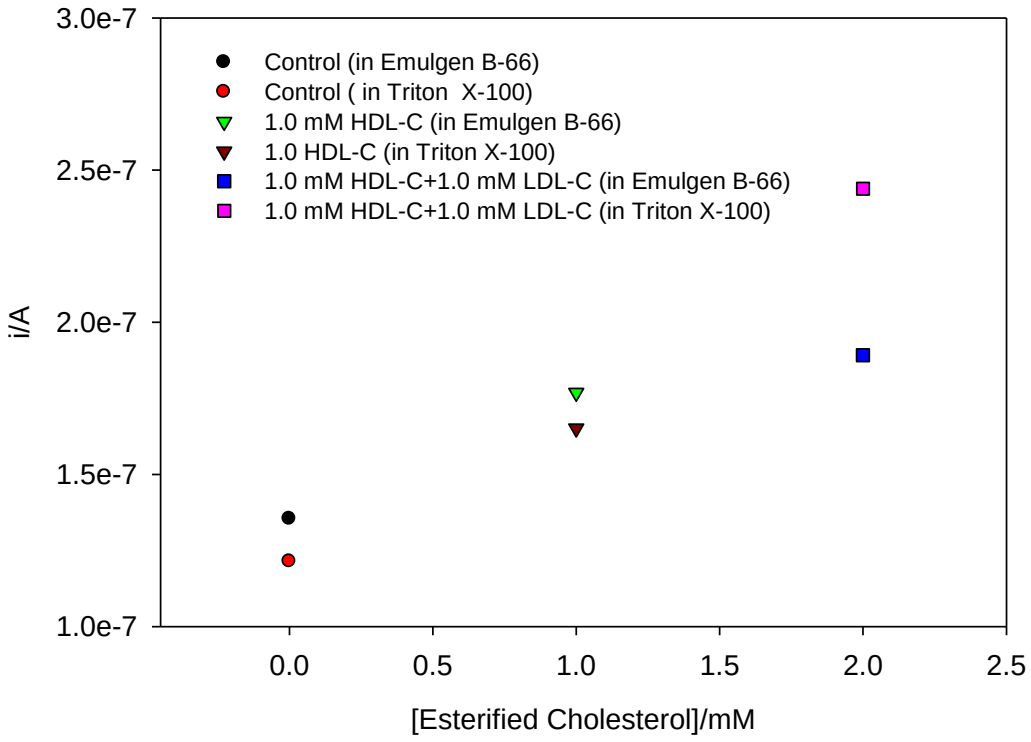

Fig. 5.5. Chronoamperometric response at 420 s (-0.1 V (vs Ag/AgCl)) to 1 mM HDL-C in the presence and the absence of 1 mM LDL-C using selective and non-selective surfactants.

5.2.1.2 Effect of Emulgen B-66 concentration on the selectivity of the HDL-C biosensor

The effect of a number of surfactants and their concentration on the spectrophotometric measurement of HDL-C over LDL-C in PBS was assessed in Chapter 3 showing that the selectivity of HDL-C over LDL-C was improved by increasing the concentrations of Emulgen B-66 from 1 to 2.5 and 6% (Section 3.3.3). While 1% (w/v) Emulgen B-66 showed acceptable selectivity towards HDL-C in PBS during amperometric measurement (Section 5.1.2), in this section, the effect of higher concentrations of the surfactant was investigated, initially using DBSA/KCl modified electrodes for the measurement of 0 to 5 mM H_2O_2 in stirred batch solution and then for HDL-C and LDL-C measurement in delipidated serum using encapsulated biosensors. As can be seen in Fig. 5.6, increased concentrations of 2.5 and 6% (w/v) Emulgen B-66 had no significant effect on biosensor response to different concentrations of H_2O_2.

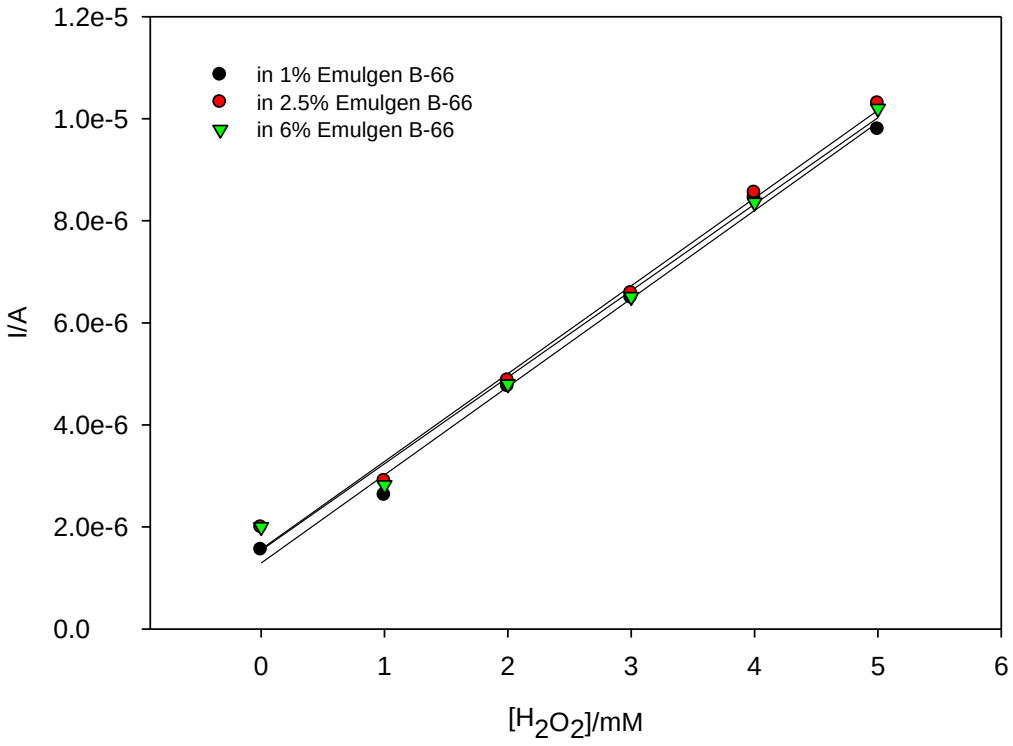

Fig. 5.6. Chronoamperometric measurement of H_2O_2 in 1, 2.5 and 6% (w/v) Emulgen B-66 (-0.1 V vs Ag/AgCl) in stirred batch solution.

Therefore, HDL-C biosensors containing 2.5 and 6% (w/v) Emulgen B-66 were further used to measure 2 and 4 mM LDL-C and 2 mM HDL-C to assess their selectivity towards HDL-C when measured amperometrically. Delipidated serum was used to prepare HDL-C and LDL-C solutions and was also used as a control (see Section 4.3.4). Fig. 5.7 and Table 5.6 illustrate that the background current did not change significantly for delipidated serum in the presence of different surfactant concentrations, showing that both Emulgen B-66 and Triton X-100 had similar effects on sensor response, which is likely to be due to the similar non-ionic properties of both detergents (see Section 4.5). HDL-C (2 mM) in the presence of both surfactants showed almost the same current responses, which again confirmed the result previously obtained from the spectrophotometric assay performed in PBS. LDL-C at 2 and 4 mM showed hardly any recovery in the presence of 6% (w/v) Emulgen B-66. In conclusion, 0.5% (v/v) Triton X-100 and 6% (w/v) Emulgen B-66 were found to be suitable for the amperometric measurement of HDL-C and TC prepared in delipidated serum.

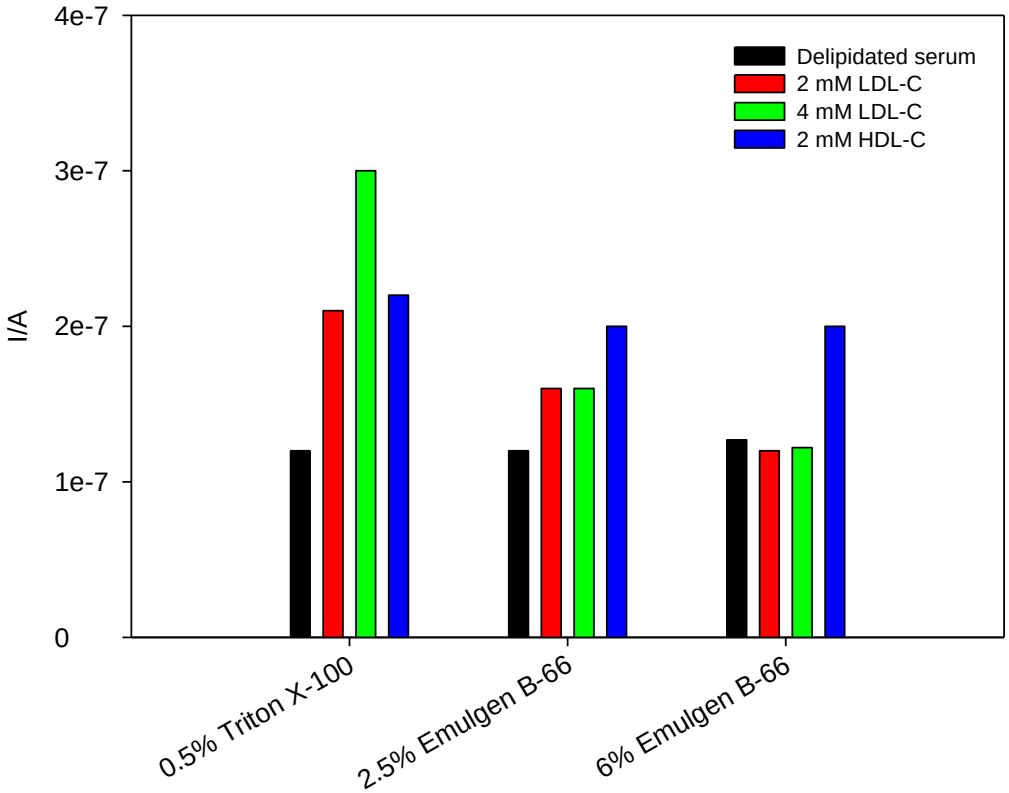

Fig. 5.7. Biosensor responses of 2 mM HDL-C, 2 and 4 mM LDL-C in the presence of HDL-C selective and non-selective surfactants at 420 s and after 180 s incubation time.

Table 5.1. Current response of the biosensor to 2 mM HDL-C and 2 and 4 mM LDL-C prepared in delipidated serum in the presence of HDL-C selective and non-selective surfactants at 420 s after 180 s incubation.

Sample	I/A		
	2.5% (w/v) Emulgen B-66	6.0% (w/v) Emulgen B-66	0.5% (v/v) Triton X-100
Delipidated serum	1.368×10^{-7}	1.242×10^{-7}	1.249×10^{-7}
2 mM HDL-C	2.044×10^{-7}	1.95×10^{-7}	2.18×10^{-7}
2 mM LDL-C	1.603×10^{-7}	1.176×10^{-7}	2.148×10^{-7}
4 mM LDL-C	1.647×10^{-7}	1.221×10^{-7}	2.607×10^{-7}

To sum up, the DBSA/KCl modified silver electrodes were found to be suitable for the amperometric measurement of 0 to 6 mM HDL-C in PBS, and they were also found to be capable of measuring HDL-C over LDL-C amperometrically in PBS and delipidated serum with optimised selectivity in the presence of 6% (w/v) Emulgen B-66. As was discussed in Chapter 4, delipidated serum best represents the sample matrix for further assay development, having as it does a normal

composition of serum components except lipids. Therefore amperometric measurement of HDL-C in the 0 to 4 mM range was next assessed, this time using delipidated serum with 6% (w/v) Emulgen B-66.

5.2.1.3 Amperometric measurement of HDL-C in delipidated serum

The biosensor was first applied to the measurement of HDL-C in PBS and linearity and reproducibility studies were performed (Section 5.2.1). However, since the final aim of the project is to measure HDL-C in real serum samples, in this section the performance of the biosensor to the amperometric measurement of HDL-C prepared in delipidated serum using 6% (w/v) Emulgen B-66 was evaluated (Fig. 5.8, Fig. 5.9 and Table 5.2).

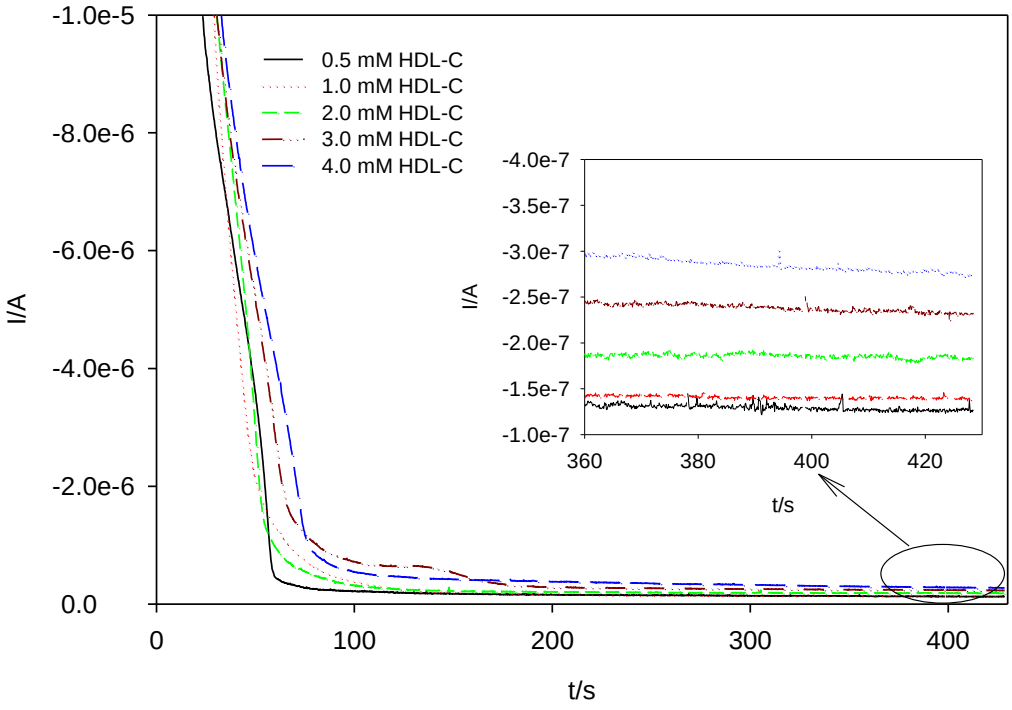

Fig. 5.8. Chronoamperometric response of encapsulated biosensors to 0 to 4 mM HDL-C in delipidated serum at -0.1V (vs Ag/AgCl). Inset: Detail of biosensors response between 360 and 420 s.

The amperometric response was taken at 420 s, this time following 180 s incubation time with an additional 60 s which was taken prior to amperometric measurement to equilibrate the three screen printed electrode system (240 s total reaction time) (Grennan, Killard and Smyth, 2001).

Fig. 5.9 shows the unsubstracted chronoamperometric responses of the biosensor across the HDL-C measurement range. Controls (no enzyme and 0 mM HDL-C) show consistent background currents of <1 x 10^{-7} A, which is an acceptable value

of electrochemical background, most likely due to a combination of non-faradaic processes, most notably from the double layer charging effects of the DBSA/KCl surface modification. The cathodic current observed at 420 s was also found to be proportional to the HDL-C concentration and the encapsulated modified electrodes had a linear response of 4.5×10^{-8} A/mM (r^2 = 0.998, n=3) between 0.5 and 4 mM HDL-C with an average RSD of 7.5 % (Table 5.2). A comparison of these results with those performed in PBS (Section 5.2.1) illustrated a 1.6-fold increase in slope in the presence of delipidated serum compared to PBS (slope=2.78×10^{-8} A/mM) while the linearity did not change. Better reproducibility was observed after introducing the 60 s equilibration period before the start of amperometric measurement (RSD of 11.8% in PBS and 7.5% in delipidated serum). The current observed for 4 mM HDL-C in PBS was 2.67×10^{-7} A which was very similar to that observed in delipidated serum (2.76×10^{-7} A). However, the S/B observed for 4 mM HDL-C in PBS was calculated to be 2.0 while this was 2.9 in delipidated serum, showing an improvement in S/B current observed in the presence of delipidated serum, leading to a potentially better detection limit of the biosensor. This effect was also observed previously in Chapter 4 for the amperometric measurement of 3 mM H_2O_2 in serum (Section 4.3.4). However, it can not be discounted that the improvement in slope might also be due to variations in the batches of screen printed electrodes used throughout the research which used different batches of silver ink (Gonzalez-Macia, Smyth and Killard, 2011).

Table 5.2. Analytical data of amperometric measurement of 0 to 4 mM HDL-C in delipidated serum at 420 s after 240 s incubation using encapsulated biosensors.

HDL-C (mM)	Average current (A)	S/B	SD	RSD (%)
0	9.67×10^{-8}	-	8.89×10^{-9}	9.1
0.5	1.13×10^{-7}	1.17	1.32×10^{-8}	11.6
1	1.41×10^{-7}	1.46	9.77×10^{-9}	6.9
2	1.79×10^{-7}	1.85	1.11×10^{-8}	6.2
3	2.16×10^{-7}	2.23	2.08×10^{-8}	9.6
4	2.76×10^{-7}	2.85	4.99×10^{-9}	1.8
Average RSD (%)				7.5

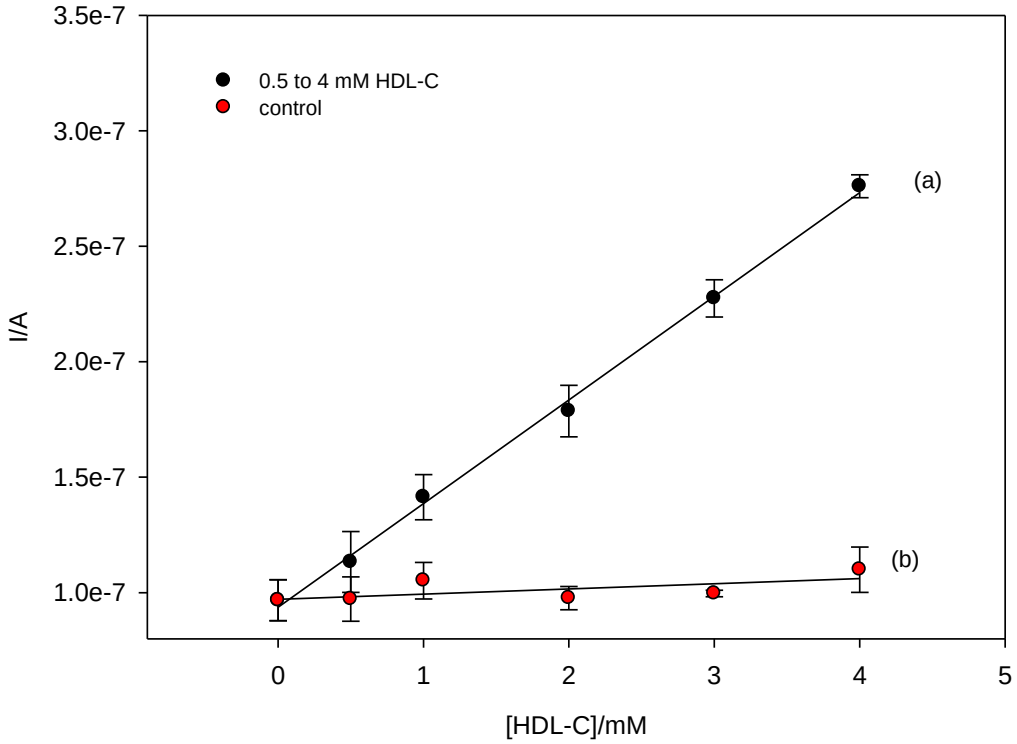

Fig. 5.9. Biosensor response to HDL-C in delipidated serum at -0.1 V (vs Ag/AgCl); a) Biosensor containing 6% (v/v) Emulgen B-66, 39 U/mL ChEs and 23 U/mL ChOx; (slope=4.49×10^{-8}, r^2=0.998, n=3) (240 s incubation time, due to 1 min for equilibrium); b) Biosensor controls in the absence of ChEs and ChOx (0.5 to 4 mM HDL-C) and in the presence of all assay components at 0 mM (delipidated serum).

In conclusion, the biosensor was capable of measuring HDL-C in delipidated serum with acceptable linearity, with reproducibility nearly always below 10% RSD. However, it was necessary to again establish that the biosensor was capable of selective measurement of HDL-C in the presence of LDL-C in a serum matrix. In the next section, amperometric measurement of HDL-C (up to 3 mM) prepared in delipidated serum containing 4 mM LDL-C was evaluated. 4 mM LDL-C was chosen as it is clinically a high borderline limit for LDL-C measurement.

5.2.1.4 Selective amperometric measurement of HDL-C in mixtures of HDL-C and LDL-C in delipidated serum

Selectivity of HDL-C over LDL-C in PBS was previously investigated in Section 5.2.1.1 using 1% (w/v) Emulgen B-66. However 6% (w/v) Emulgen B-66 was shown to have better selectivity towards HDL-C over LDL-C in delipidated serum (5.2.1.2) and also demonstrated good sensitivity and linearity in measuring 0 to 4 mM HDL-C in delipidated serum (5.2.1.3). In this section, the amperometric measurement of HDL-C (0 to 3 mM) was investigated in delipidated serum

containing 4 mM LDL-C with a 240 s incubation time using the encapsulated biosensors.

Fig. 5.10 illustrates the amperometric measurement of delipidated serum containing 4 mM LDL-C in the presence of Emulgen B-66 and Triton x-100. The presence of FC in the LDL-C serum sample was also assessed by omitting ChEs and surfactant. As can be seen, a comparable response was observed for FC and HDL-C measured in 4 mM LDL-C, confirming that the slight increase in current observed in the presence of Emulgen B-66 might be due to FC present in the LDL-C. The different concentrations of HDL-C did not show any change in measured FC, suggesting that there was minimal FC present in the HDL-C material used (Fig. 5.11). The presence of a small quantity of FC has been reported by Nakamura *et al.,* (2009) to be a source of uncertainty in HDL-C measurement using spectrophotometric methods (Nakamura *et al.,* 2009). The response of the biosensor at 420 s to concentrations of HDL-C is shown in Fig. 5.12, showing a slope of 4×10^{-8} A/mM (r^2=0.93). A slight decrease in sensitivity and linearity was observed in the presence of LDL-C compared to HDL-C alone (slope=4.50×10^{-8} A/mM, r^2=0.998) (5.2.1.3).

As shown in Table 5.3, some increase in the RSD (approx. 5%) was observed compared to samples prepared without LDL-C (Table 5.2) which might be due to some variability in sensor batches, as well as a slight negative effect on the sensor due to the presence of 4 mM LDL-C and finally a slight change in sample matrix which might be due to the presence of some insoluble LDL-C aggregates which may effect the way the produced H_2O_2 reaches the electrode surface (Gonzalez-Macia, Smyth and Killard, 2011; Helm, Jalukse and Leito, 2010; Jeroschewski, Steuckart and Kulhl, 1996; Kondo, 1999; Segrest *et al.,* 2001). Kondo *et al.,* (1999) used electron microscopy technique to investigate the reaction in Daiichi HDL-C homogeneous assay kit containing polyanion-polymer/detergent showing that almost every lipoprotein aggregates in the presence of polyanion-polymer while only HDL-C aggregates was capable of being resolved and undergone the enzymatic reaction (Kondo, 1999).

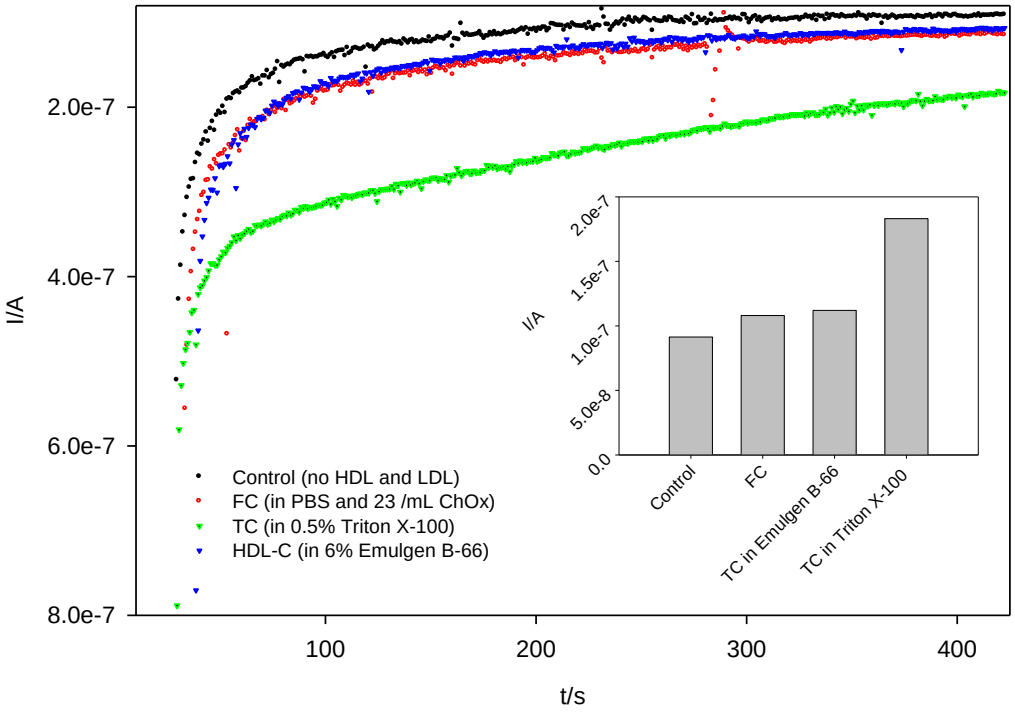

Fig. 5.10. Chronoamperometric measurement of TC, HDL-C and FC present in delipidated serum containing 4 mM LDL-C using the developed biosensor. Inset: Biosensor response at 420 s in delipidated serum was used as control.

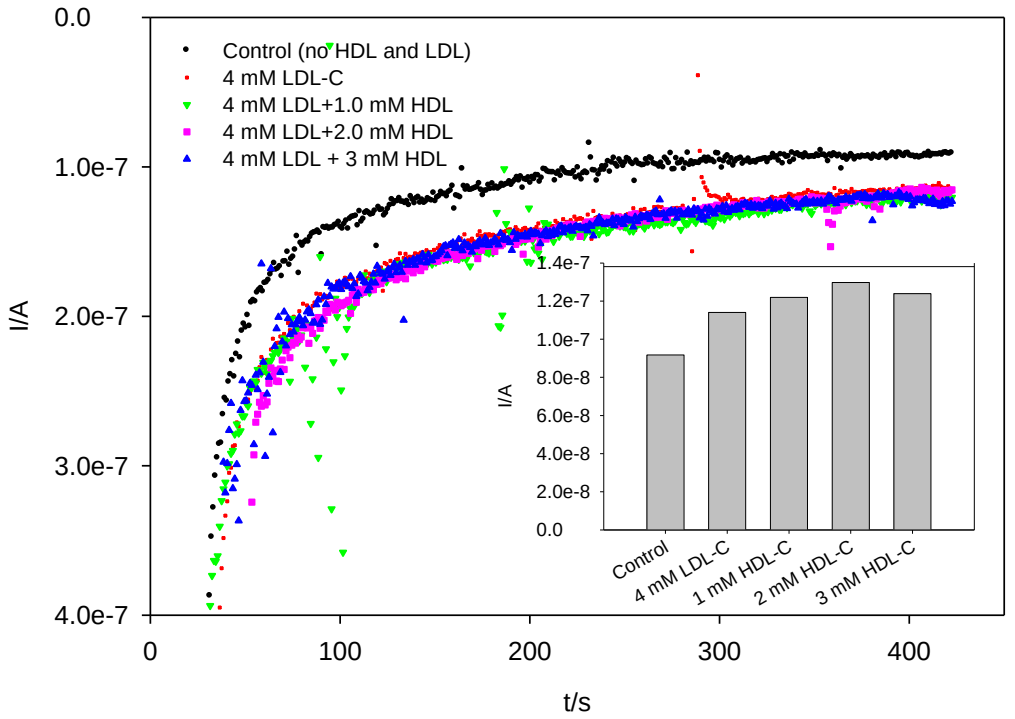

Fig. 5.11. Chronoamperometric measurement of FC present in delipidated serum containing 0 to 3 mM HDL-C and 4 mM LDL-C. Inset: Biosensor response at 420 s in delipidated serum was used as control.

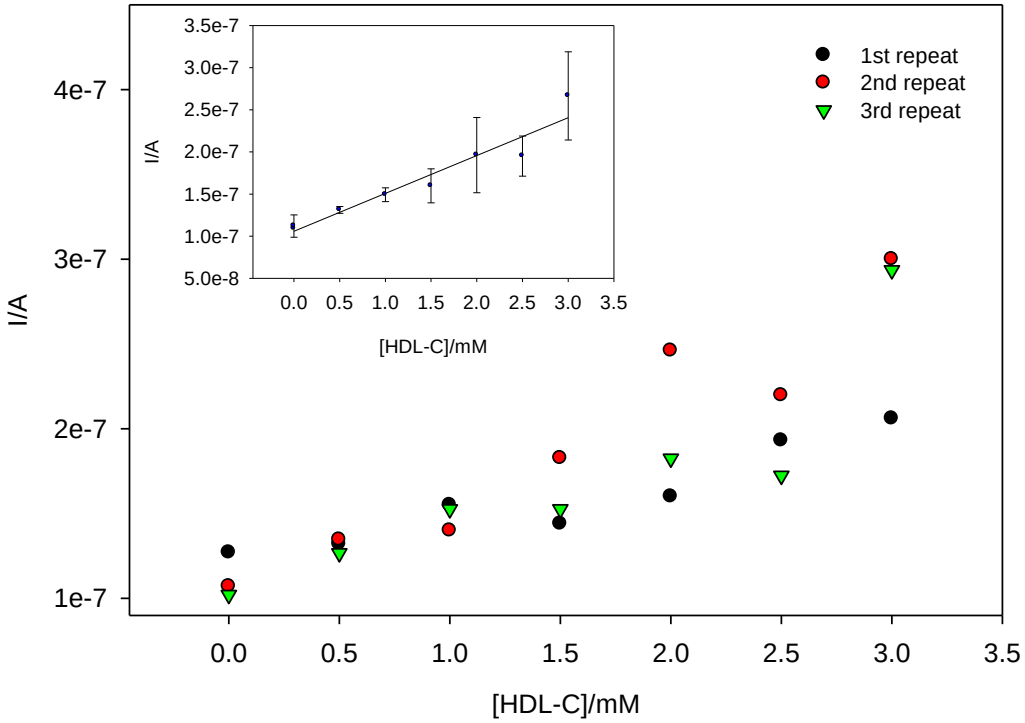

Fig. 5.12. HDL-C biosensor response to delipidated serum containing 0 to 3 mM HDL-C and 4 mM LDL-C. Inset: calibration curve (slope: 4×10^{-8} A/mM, r^2=0.93, n=3).

Table 5.3. Amperometric responses of the HDL-C biosensor to delipidated serum containing 0 to 3 mM HDL-C and 4 mM LDL-C at 420 s.

HDL-C (mM)	I/A (average current at 420 s)	SD	RSD (%)
0	1.12×10^{-7}	1.32×10^{-8}	11.8
0.5	1.31×10^{-7}	4.04×10^{-9}	3.1
1	1.49×10^{-7}	8.11×10^{-9}	5.4
1.5	1.60×10^{-7}	2.03×10^{-8}	12.7
2	1.96×10^{-7}	4.46×10^{-8}	22.7
2.5	1.95×10^{-7}	2.37×10^{-8}	12.2
3	2.66×10^{-7}	5.25×10^{-8}	19.7
Average RSD			12.5

To sum up, the biosensor based on the DBSA/KCl modified silver electrode was successfully used to measure HDL-C in delipidated serum while in the presence of LDL-C. However, it was also observed that employing the surfactant Emulgen B-66 in PBS resulted in a similar catalytic enhancement effect to the DBSA/KCl modification. It was hypothesised that the DBSA/KCl modification step could be removed and replaced entirely by Emulgen B-66. The next step, therefore, was to evaluate the effect of 6% (w/v) Emulgen B-66/PBS as an alternative electrode modifier to DBSA/KCl. Therefore in following section, this combination of

surfactant/salt was used to assess its effect on the modification of the silver screen printed electrodes and their sensitivity towards H_2O_2.

5.2.2 Development of the HDL-C biosensor using Emulgen B-66/PBS modified electrodes

As was mentioned by Gonzalez *et al.,* (2011), a combination of surfactant/salt was found to enhance the catalytic activity of silver electrode towards H_2O_2. KCl in combination with DBSA was an example showing that the presence of salt was important factor to enhance catalytic activity of silver electrodes towards H_2O_2 (Gonzalez-Macia, Smyth and Killard, 2011; Gonzalez-Macia, Smyth and Killard, 2012b). However, the enhancement was also demonstrated for a range of surfactant/salt combinations. This effect was also observed using 6% (w/v) Emulgen B-66 in combination with 0.1M PBS on silver paste electrodes in stirred batch solution, as shown in Fig. 5.13.

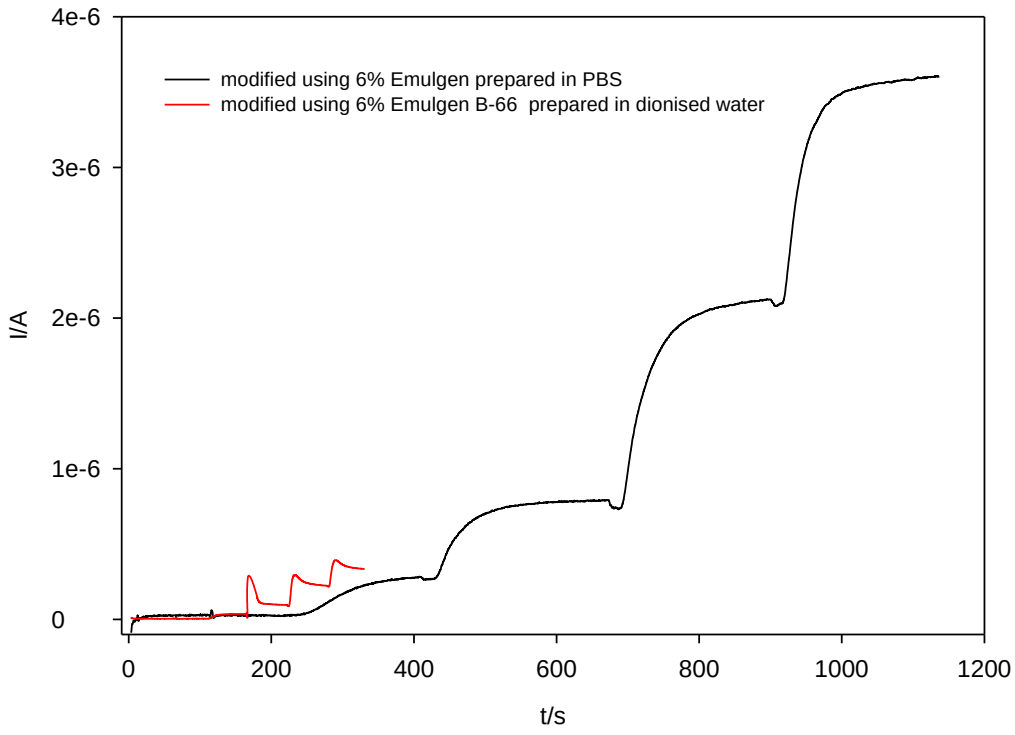

Fig. 5.13. Amperometric measurement of 0 to 3 mM H_2O_2 using modification solution containing: a) 6% (w/v) Emulgen B-66 prepared in deionised water and b) 6 % (w/v) Emulgen B-66 prepared using 0.1 M PBS.

The dependency of the effect on the presence of supporting electrolyte is clearly demonstrated here and further validates the nature of the effects previously observed which required the formation of electrolyte stabilised surfactant structures such as lamellar phases which modify the electrode surface.

The suitability of Emulgen B-66/PBS as a catalytic modifier was further investigated using different deposition techniques such as dip-coating, drop coating and finally inkjet-printing, the latter of which was proven to be a reproducible, quick and simple technique for patterning and depositing defined volumes of the modification solution. To begin with, a three electrode system containing screen printed silver working electrode was dipped into 6% (w/v) Emulgen B-66/PBS solution for 0, 5, 60 and 180 min and then studied using cyclic voltammetry. As is shown in Fig. 5.14, by increasing the exposure time of the silver electrodes to the modification solution, the non-Faradic or charging current increased, showing that surface modification was occurring and was increasing in a time-dependent manner (Gonzalez-Macia, Smyth and Killard, 2011).

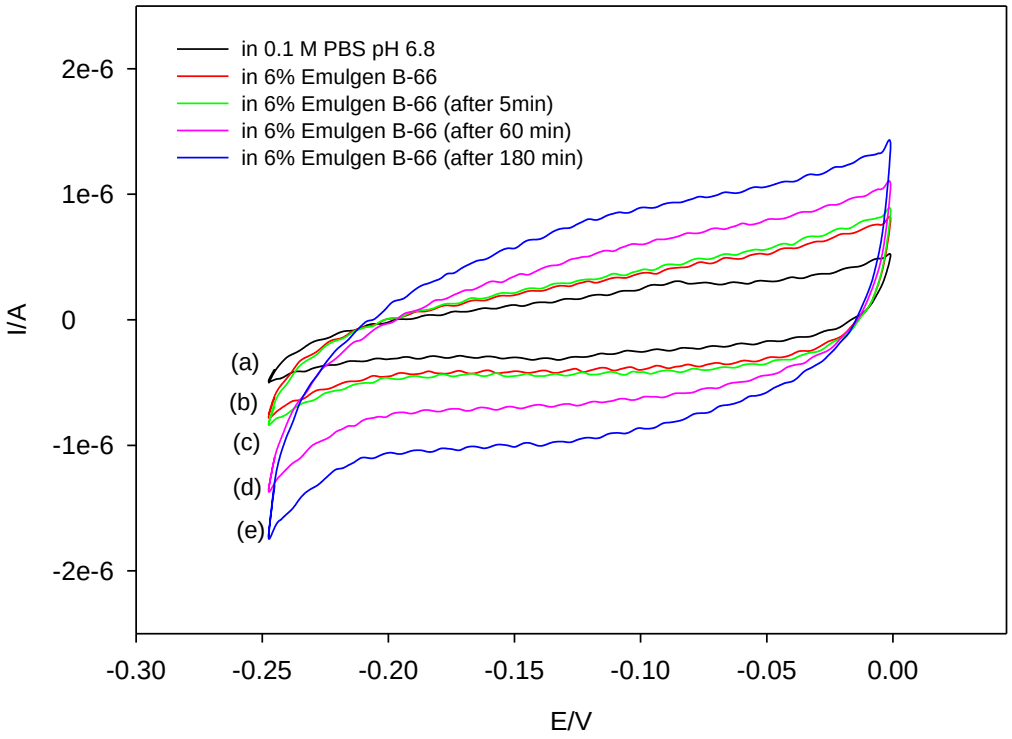

Fig. 5.14. Cyclic voltamograms of a) unmodified silver electrodes measured in PBS following exposure to 6% (w/v) Emulgen B-66/PBS for b) 0 min, c) 5 min, d) 60 and e) 180 min.

Following this, 3 µL of 6% (w/v) Emulgen B-66/PBS was drop-coated onto silver screen printed electrodes and allowed to incubate for 180 min. Drop coating using a defined volume of the modification solution was performed to see whether it was possible to move towards an inkjet-printing process, as had been demonstrated for DBSA/KCl previously (Gonzalez-Macia, Smyth and Killard, 2012b). Electrodes modified by inkjet printing were then prepared by printing five layers of 6% (w/v) Emulgen B-66/PBS at a nozzle voltage of 26 V using a 16-nozzle head cartridge

(approx. 1.2 µL reagent deposition). The electrode was allowed to dry for 5 min and the excess modification solution was washed using deionised water before cyclic voltammetric experiment was performed (Fig. 5.15).

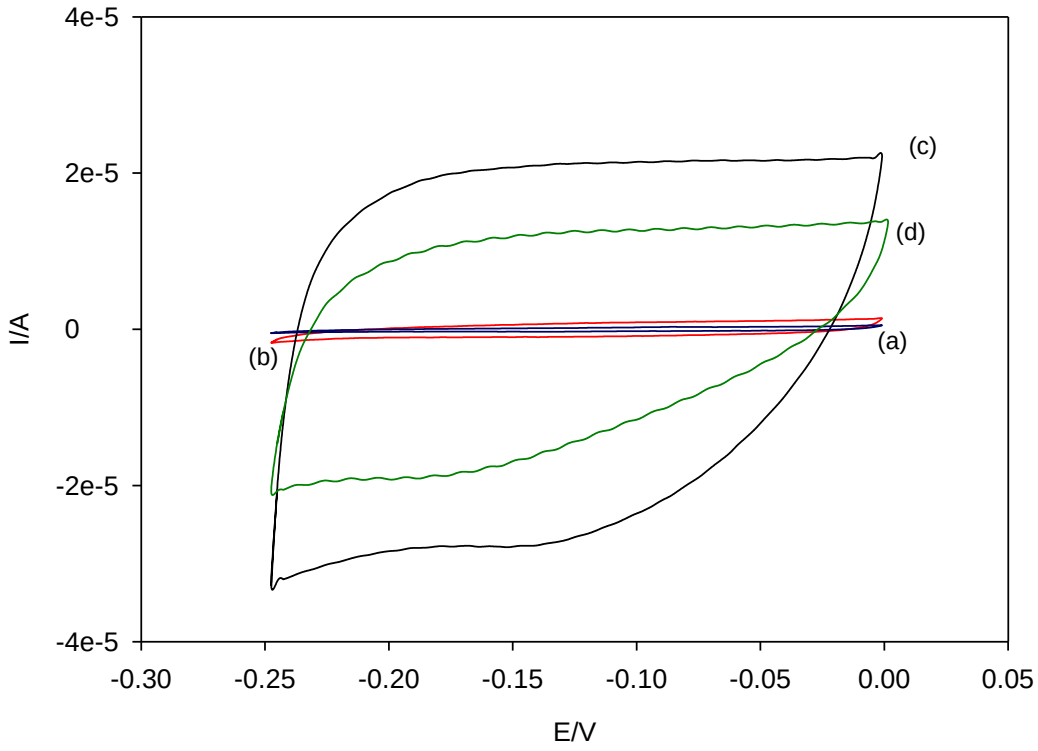

Fig. 5.15. Cyclic voltamograms of unmodified silver electrode measured in 6% (w/v) Emulgen B-66 using: a) un-modified electrode in PBS, b) electrodes dipped in 6% (w/v) Emulgen B-66/PBS for 180 min, c) drop coated electrode using 3 µL of 6% (w/v) Emulgen B-66/PBS, d) inkjet-printed electrode using 5 layers 6% Emulgen B-66 (1.2 µL)

The non-Faradic current observed at -0.1 V (vs Ag/AgCl) after 180 min modification time, was 2.37×10^{-5} A for drop-coated modified silver electrode and 8.62×10^{-7} A for electrode being dipped in 6% (w/v) Emulgen B-66/PBS while 2.54×10^{-7} A was observed for an unmodified silver electrode in PBS. This was up to 900 and 300 fold greater non-faradaic current for drop-coated compared to unmodified and dip-coated modified electrodes, respectively showing again a significant modification of the surface with a capacitive double layer formed by Emulgen B-66 and PBS. The non-Faradic current observed for inkjet-printed modified electrodes was 1.16×10^{-5}A which was two-fold lower than that for the drop-coating technique, which might be due to the smaller volume of reagent used. The fact that modified electrodes prepared using the drop coating and inkjet printing techniques showed massive increases in their non-Faradic current and therefore on surface modification compared to those dipped into modification solution for 180 min, without the

requirement for a subsequent drying step, illustrates that the micellisation due to surfactant/salt aggregation which occurs at greater surfactant concentrations after deposition on the surface after inkjet-pinting and drop-coating may lead to better modification in comparison with dip-coating which requires diffusion of surfactant molecules from the bulk to the surface and then adsorption which depends on the surfactant concentrations (Eastoe, 2010).

Amperometric measurement of H_2O_2 using un-modified electrodes and those modified using the inkjet-printing technique were then performed in both PBS and a solution of 6% (w/v) Emulgen B-66/PBS in stirred batch solution. The response of the inkjet-print modified electrodes showed the greatest sensitivity (2.56×10^{-6} A/mM) and linearity (r^2=0.998), in a solution of 6% (w/v) Emulgen B-66/PBS while this was 9.51×10^{-8} A/mM with r^2 of 0.924 for un-modified electrode in PBS (Fig. 5.16). The current observed using unmodified electrodes in PBS was 22-fold lower than unmodified electrodes in 6% (w/v) Emulgen B-66/PBS which shows better catalysis in the presence of Emulgen B-66/PBS solution. However, this was still 8-fold greater than the current observed for 3 mM H_2O_2 using unmodified electrodes in PBS. However, the current observed for 3 mM H_2O_2 in 6% (w/v) Emulgen B-66 using modified electrodes were only 1.3-fold greater than that using unmodified electrodes. This demonstrated that 6% (w/v) Emulgen B-66/PBS could be employed as a modification of silver electrode for amperometric measurement of H_2O_2 in an assay solution which also contained 6% (w/v) Emulgen B-66/PBS. Such an approach would be optimal, firstly for an optimised biosensor based on externally applied assay reagents, as well as for a biosensor in which the assay reagents have been fully integrated through printing (Chapter 6).

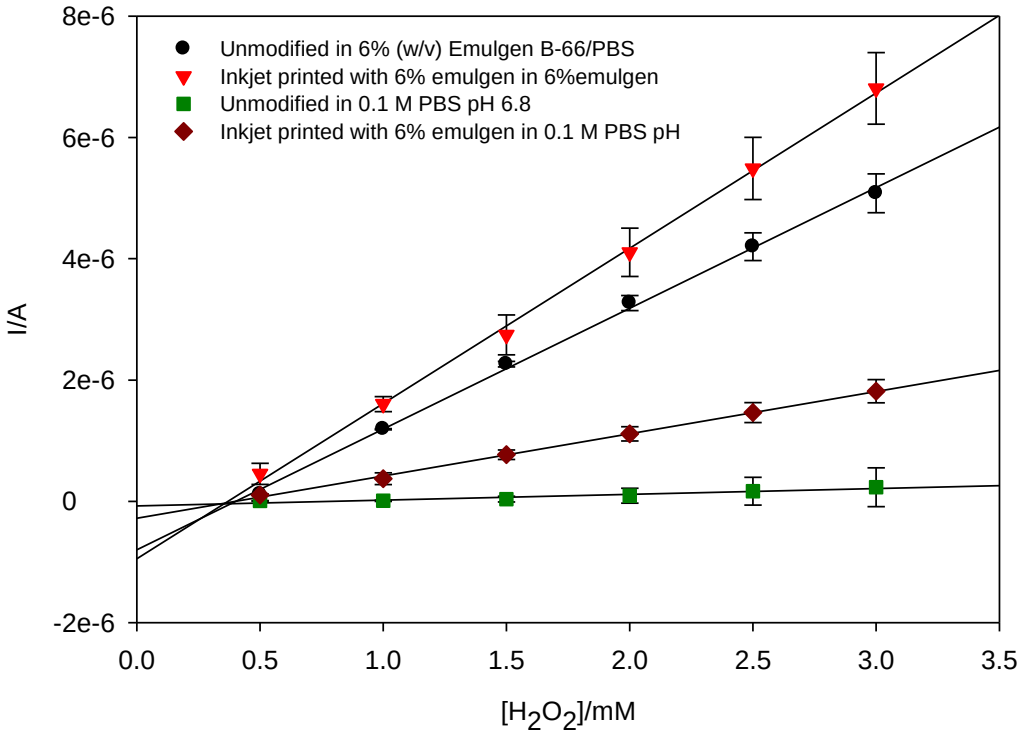

Fig. 5.16. Amperometric response of un-modified electrodes and those modified using inkjet-printed of 6% Emulgen B-66/PBS to 0 to 3 mM H_2O_2 in both PBS and 6% Emulgen B-66/PBS (stirred batch experiment).

After demonstrating the possibility of employing of 6% (w/v) Emulgen B-66 for the modification of the silver electrodes, the next step was to optimise the volume of modification solution deposited using inkjet-printing. Inkjet printing is capable of rapid and reproducible deposition of very small volumes of ink and is suitable for mass production of modified electrodes at low cost. This is dependent on the physical properties of the ink. Ejection volume and nozzle voltage are also important factors for the optimisation of inkjet printing deposition (Hutchings and Martin, 2013; Magdassi, 2008; Tekin, Smith and Schubert, 2008). Since nozzle voltage did not show any significant affect on the formation of the catalytic surface (data not shown), and based on the density of ink solution, 26-27 V was found to be a suitable voltage for ejecting through a 16 nozzles printer cartridge. Considering the pattern of 3.2×3.2 mm with 20 µm spacing which provides 25,921 drops per layer, 0.24 µL of 6% (w/v) Emulgen B-66 was deposited on the silver electrode surface in each printed layer. By increasing the number of printed layers, the mass of deposited material will increase, while the concentration of solution does not change. Therefore, 5, 10 and 15 layers of 6% (w/v) Emulgen B-66/0.1 M PBS were printed on the surface of the silver electrodes and allowed to be dried for 5 min after printing. The excess modification solution was washed using deionised water

and then cyclic voltammetry and amperometric studies were performed using these electrodes in H_2O_2 solutions (0 to 3 mM). As can be seen in Fig. 5.17 A, the non-Faradic current at -0.1 (vs Ag/AgCl) for 0, 5 and 10 prints was 4.35×10^{-7} to 1.2×10^{-5} and 2.12×10^{-5} A, respectively, confirming that surface modification had occurred. By further increasing the print layers to 15, the shape of the voltammogram changed and the charging current of 9.23×10^{-6} was observed which was approx. 1.3 and 2.2 times less than those observed for 5 and 10 layers of prints. The current observed for 3 mM H_2O_2 at -0.1 V (vs Ag/AgCl) (Fig. 5.17B), using 15 layers inkjet-printed modified electrodes was also slightly lower than that observed for 10 layers.

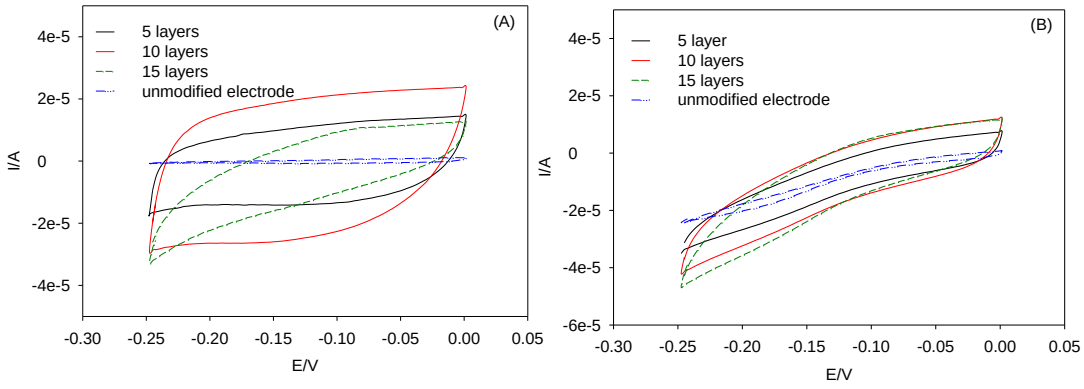

Fig. 5.17. Cyclic voltammogram of 0, 5, 10 and 15 layers inkjet-printed 6% (w/v) Emulgen B-66 modified silver electrodes in A) 6% (w/v) Emulgen B-66/PBS and B) 3 mM H_2O_2 prepared in 6% (w/v) Emulgen B-66/PBS solutions.

A possible explanation for this behaviour might be due to the volume of modification solution required to be adsorbed to the silver electrode surface and the importance of the concentration of deposited modification solution on the electrode surface. The volume of deposited liquid on the electrode surface was 1.2, 2.4 and 3.6 µL for 5, 10 and 15 layers, respectively. Since the same drying time of 5 min was used after printing of all different numbers of print layers before washing the excess modification solution, the extra modification solution in 15 layers prints may interfere with micellisation and aggregation of modification solution on the electrode surface in a similar way observed in electrode prepared by dipping into the modification solution (Eastoe, 2010). Killard and coworkers (2012b) also reported the similar effect at higher volume of modification solution.

The effect of the number of print layers on the amperometric response to H_2O_2 prepared in 6% (w/v) Emulgen B-66 is shown in Fig. 5.18 and detailed analytical data are illustrated in Table 5.4.

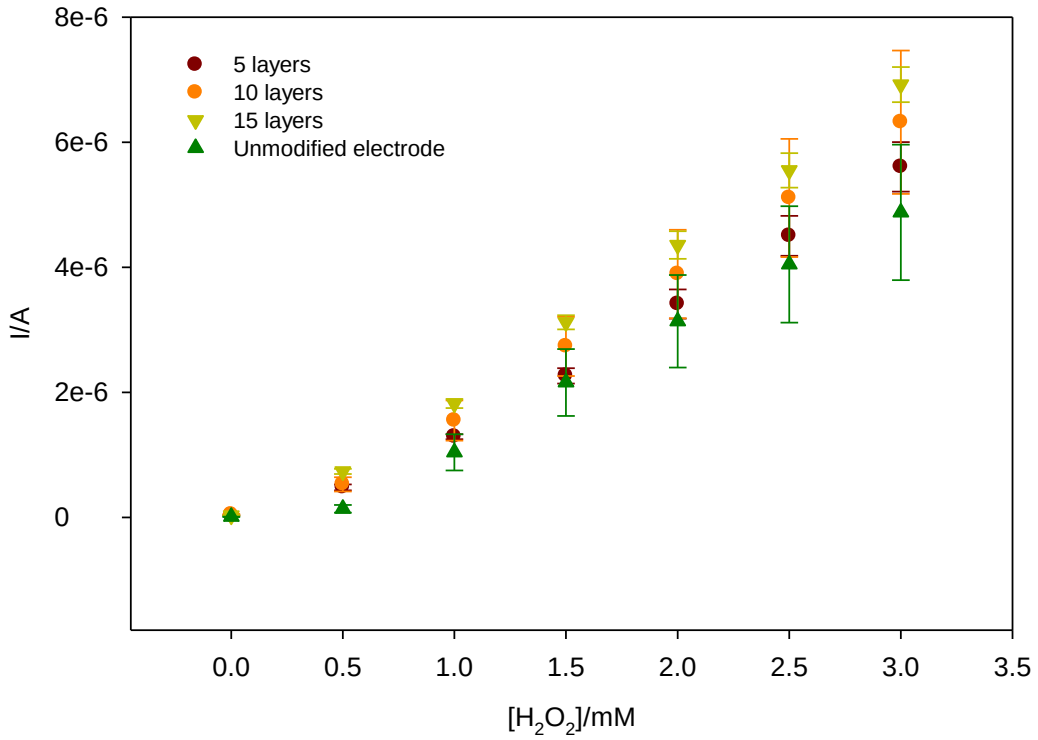

Fig. 5.18. Amperometric measurement of 0 to 3 mM H_2O_2 in 6% (w/v) Emulgen B-66 using 0, 5, 10 and 15 layers inkjet-printed 6% (w/v) Emulgen B-66/PBS modified electrodes (n=3) (stirred batch experiment).

As can be seen, by increasing the number of prints from 5 to 10 and 15, sensitivity increased slightly from 2.08×10^{-6} to 2.33×10^{-6} and 2.48×10^{-6}, while linearity remained almost the same (r^2=0.99). Random standard error was significantly improved after modification, with good reproducibility in 5 and 15 print layers. The error increased from 8% at 5 layers to 19.6% at 10 layers which might be due some variability in one of the silver electrodes out of the three used for this experiment. Since 15 layers did not show any significant improvement on silver electrode catalytic activity and based on the fact that 5 layers produced good linearity and reproducibility as well as presenting good catalytic activity, 5 prints were used for any future experiments. 5 print layers were also reported previously to provide an optimum modification solution for catalytic activity of silver surface (Gonzalez-Macia, Smyth and Killard, 2012b). The fact that unmodified electrode in 6% (w/v) Emulgen B-66/0.1M PBS demonstrate slightly lower sensitivity than modified electrodes may be a good case for development of integrated biosensor which will be discussed in Chapter 6.

Table 5.4. Detailed analytical data of amperometric measurement of H_2O_2 (0 to 3 mM) using 0, 5, 10 and 15 layers Emulgen B-66 modified electrodes (N=3).

H_2O_2 (mM)	5 layers (I/A)	SD	RSD (%)	10 layers (I/A)	SD	RSD (%)	15 layers (I/A)	SD	RSD (%)	unmodified electrode (I/A)	SD	RSD (%)
0	3.28×10^{-8}	5.52×10^{-9}	16.8	4.75×10^{-8}	1.08×10^{-8}	22.8	3.16×10^{-8}	6.39×10^{-9}	20.2	1.13×10^{-8}	3.29×10^{-9}	29.2
0.5	4.82×10^{-7}	4.78×10^{-8}	9.9	5.31×10^{-7}	1.15×10^{-7}	21.6	7.37×10^{-7}	4.07×10^{-8}	5.5	1.39×10^{-7}	6.04×10^{-8}	43.4
1	1.29×10^{-6}	3.98×10^{-8}	3.1	1.55×10^{-6}	3.21×10^{-7}	20.7	1.82×10^{-6}	7.27×10^{-8}	4	1.04×10^{-6}	2.90×10^{-7}	27.8
1.5	2.26×10^{-6}	1.23×10^{-7}	5.4	2.74×10^{-6}	4.74×10^{-7}	17.3	3.12×10^{-6}	1.13×10^{-7}	3.6	2.16×10^{-6}	5.35×10^{-7}	24.8
2	3.41×10^{-6}	2.34×10^{-7}	6.9	3.89×10^{-6}	7.07×10^{-7}	18.2	4.36×10^{-6}	2.21×10^{-7}	5.1	3.14×10^{-6}	7.39×10^{-7}	23.6
2.5	4.50×10^{-6}	3.19×10^{-7}	7.1	5.11×10^{-6}	9.45×10^{-7}	18.5	5.55×10^{-6}	2.75×10^{-7}	5	4.05×10^{-6}	9.31×10^{-7}	23
3	5.61×10^{-6}	3.95×10^{-7}	7	6.32×10^{-6}	1.15×10^{-6}	18.1	6.92×10^{-6}	2.81×10^{-7}	4.1	4.88×10^{-6}	1.08×10^{-6}	22.2
Average RSD			8.0	Average RSD		19.6	Average RSD		6.8	Average RSD		27.7

A final comparison study was performed between amperometric measurements of H_2O_2 in stirred batch mode using DBSA/KCl modified silver electrodes and those modified with 6% (w/v) Emulgen B-66/PBS which is shown in Fig. 5.19. Signal to background data for each concentration is detailed in Table 5.5.

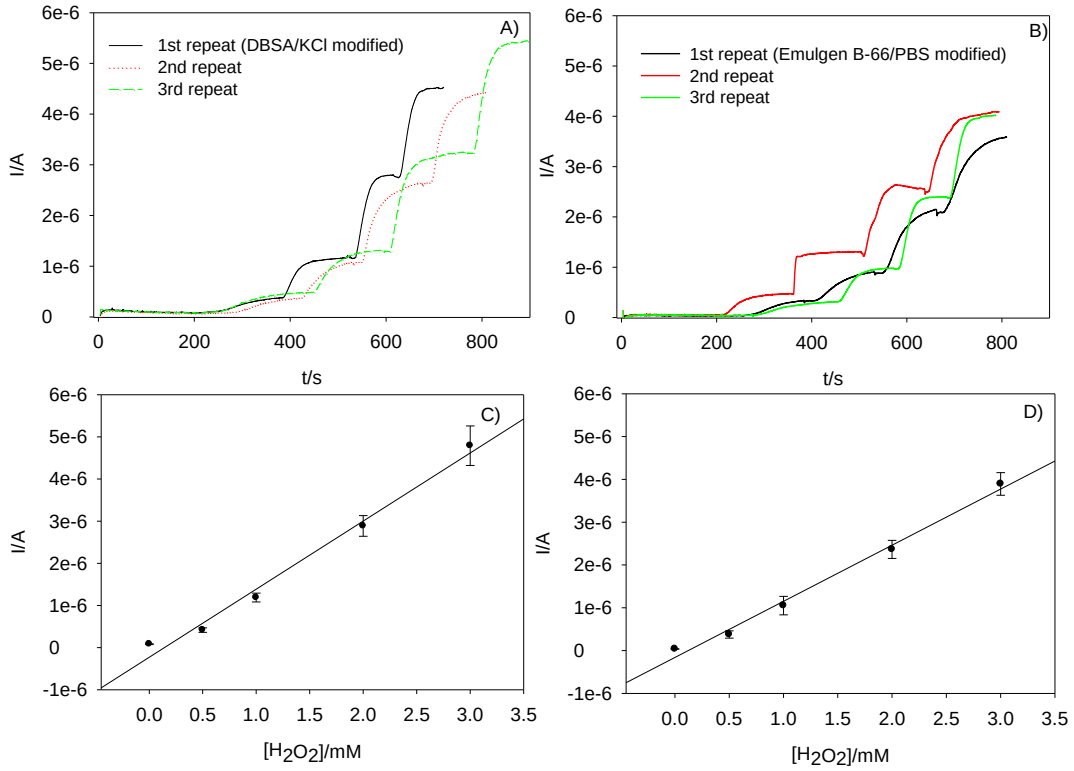

Fig. 5.19. Comparison of electrode response to H_2O_2 with modification using DBSA/KCl or Emulgen-B66/PBS: A) DBSA/KCl modified electrodes and B) Emulgen B-66/PBS modified electrodes; Amperometric response to H_2O_2 using: C) DBSA/KCl modified electrodes (Slope=1.61×10^{-6}A/mM; r^2=0.988) and D) Emulgen B-66/PBS modified electrodes (slope=1.31×10^{-6}A/mM; r^2=0.991). -0.1 V vs. Ag/AgCl.

Table 5.5. Signal to background (S/B) data of amperometric measurement of 0 to 3 mM H_2O_2 using DBSA/KCl modified electrodes and those modified using 6% Emulgen B-66/PBS both in 6% (w/v) Emulgen B-66/PBS.

H_2O_2 (mM)	DBSA/KCl modified electrode in 6% Emulgen B-66		6% Emulgen B-66/PBS modified electrode in 6% Emulgen B-66	
	I/A (Average)	S/B	I/A (Average)	S/B
0	8.15×10^{-8}	-	3.71×10^{-8}	-
0.5	4.15×10^{-7}	5.1	3.75×10^{-7}	10.1
1	1.19×10^{-6}	14.6	1.05×10^{-6}	28.3
2	2.89×10^{-6}	35.4	2.36×10^{-6}	63.6
3	4.79×10^{-6}	58.8	3.89×10^{-6}	104.8

Although no significant change was observed in the amperometric response of the modified electrodes to H_2O_2 as was shown in Fig. 5.19 using either of the modification methods, the signal to background responses observed for 6% (w/v) Emulgen B-66/PBS were almost two-fold higher compared to those modified with DBSA/KCl (Table 5.5). This might be due to the fact that most of the early charging current and initial faradaic current had decayed away for Emulgen B-66/PBS modified electrodes within 10 s and the electrodes had reached a quasi-steady state from which current measurements at fixed time points could be made while for DBSA/KCl modified electrodes this was around 150 s showing higher background current (Fig. 5.20). Therefore, better signal to background response as well as better sensitivity at lower concentration (0.5 mM) which is important in HDL-C measurement was obtained for Emulgen B-66/PBS modified electrodes which made them a suitable alternative for amperometric measurement of HDL-C. In addition, the ability to employ Emulgen B-66/PBS as a modification solution instead of DBSA/KCl may reduce the number of preparation step in the development of a biosensor with integrated reagents, which will be discussed in next chapter.

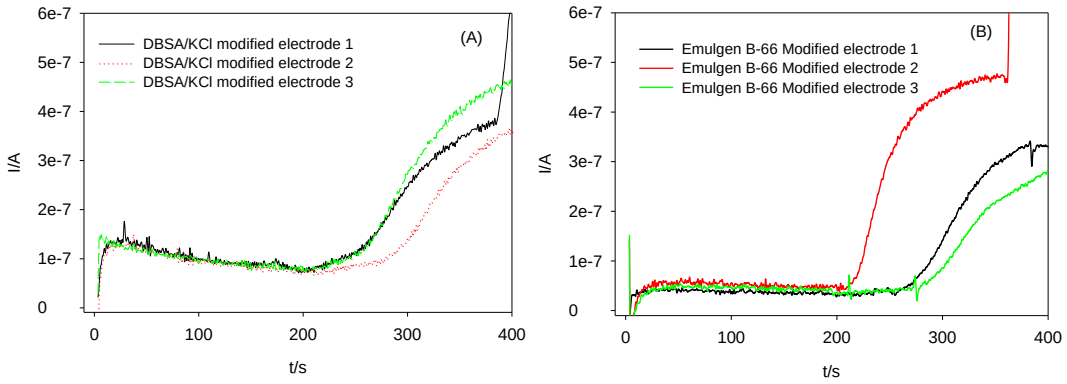

Fig. 5.20. Background current and the current observed for 0.5 mM H_2O_2 in 6% (w/v) EmUlgen B-66/PBS solution using: A) DBSA/KCl modified electrodes and B) Emulgen B-66/PBS modified electrodes (Stirred batch experiment).

To summarise, five layers of inkjet-printed 6% (w/v) Emulgen B-66/PBS showed an acceptable analytical performance for the measurement of H_2O_2 in stirred batch experiments in the presence of 6% (w/v) Emulgen B-66/PBS, making it to be suitable as an alternative modification method to be used for the amperometric measurement of HDL-C. The next step was then to see if sensors prepared in this way would still be capable of measuring H_2O_2 in the presence of all other assay reagents.

5.2.2.1 Amperometric measurement of H_2O_2 in the presence of assay reagents using encapsulated electrodes

After assessing the amperometric response of modified electrodes in stirred batch solution, the amperometric response of encapsulated electrodes modified using Emulgen-B66/PBS to 0 to 10 mM H_2O_2 in the presence of assay reagents was assessed and compared to those performed using DBSA/KCl modified electrodes (Fig. 5.21, Table 5.6).

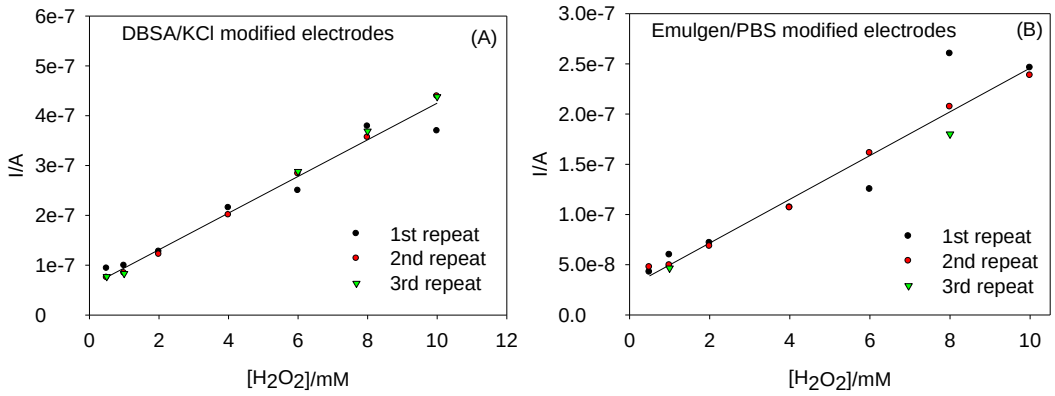

Fig. 5.21. Amperometric measurement of H_2O_2 at 420 s in the presence of assay reagents using A) DBSA/KCl modified electrodes and B) Emulgen B-66/PBS modified electrodes (240 s incubation time).

Table 5.6. Signal to background (S/B) data of amperometric measurement of 0 to 10 mM H_2O_2 at 420 s using encapsulated DBSA/KCl and 6% (w/v) Emulgen B-66/PBS modified electrodes (240 s incubation time).

H_2O_2/ mM	DBSA/KCl modified electrodes (I/A)	S/B	Emulgen B-66/PBS modified electrodes (I/A)	S/B
0	7.76×10^{-8}	-	4.09×10^{-8}	-
0.5	8.18×10^{-8}	1.05	4.50×10^{-8}	1.10
1	8.87×10^{-8}	1.14	5.18×10^{-8}	1.27
2	1.24×10^{-7}	1.60	6.98×10^{-8}	1.70
4	2.08×10^{-7}	2.68	1.07×10^{-7}	2.62
6	2.74×10^{-7}	3.53	1.43×10^{-7}	3.50
8	3.68×10^{-7}	4.74	1.94×10^{-7}	4.74
10	4.15×10^{-7}	5.35	2.42×10^{-7}	5.92

The slope of 2.1×10^{-8} with linearity (r^2) of 0.994 was observed for encapsulated Emulgen B-66/PBS modified electrodes which was 1.76-fold lower compared to the one observed for encapsulated DBSA/KCl modified electrodes (3.7×10^{-8}) with

almost the same linearity for amperometric measurement of 0.5 to 10 mM H_2O_2. However, a slightly better S/B was observed for Emulgen B-66/PBS modified electrodes compared to those modified with DBSA/KCl (Table 5.6). To sum up, the encapsulated Emulgen B-66/PBS modified electrodes were capable of measuring H_2O_2 in the presence of assay reagents with acceptable linearity and sensitivity. Therefore, the next step was to evaluate the amperometric response of these electrodes to the amperometric measurement of HDL-C in delipidated serum.

5.2.2.2 Measurement of HDL-C using a biosensor based on Emulgen B-66/PBS modified electrodes

The response of Emulgen B-66/PBS modified electrodes to 8 µL of 0 to 4 mM HDL-C in the encapsulated electrodes is shown in Fig. 5.22 and Table 5.7.

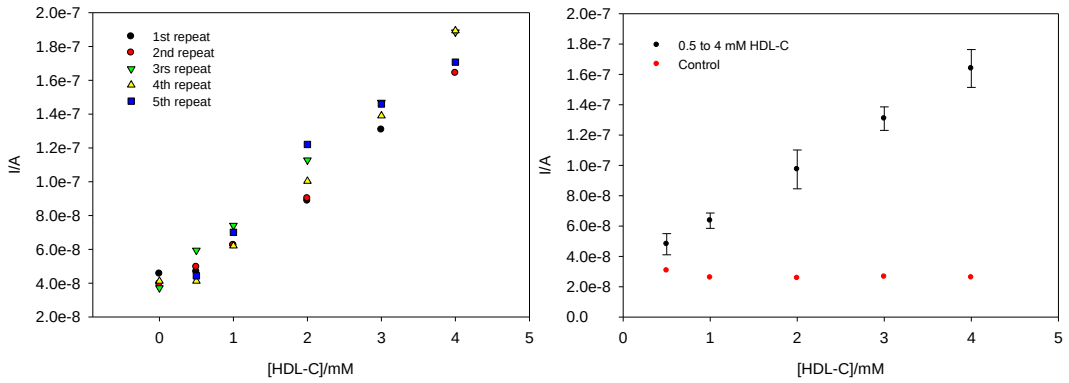

Fig. 5.22. Biosensor response to HDL-C in delipidated serum at -0.1 V (vs Ag/AgCl). a) Biosensor controls in the absence of ChEs and ChOx; b) Biosensor containing 6% (w/v) Emulgen B-66, 39 U/mL ChEs and 23 U/mL ChOx; (slope=3.32×10^{-8}, r^2=0.999, n=5).

The amperometric response taken at 420 s and after 240 s incubation time was found to be proportional to the HDL-C concentration. The encapsulated biosensor showed a slope of 3.32×10^{-8} A/mM with linearity (r^2) of 0.9999 for five repeats between 0.5 to 4 mM HDL-C prepared in delipidated serum while DBSA/KCl modified electrodes had a slope of 4.5×10^{-8} A/mM with linearity (r^2) of 0.998 for three repeats. Once again, slight decrease (1.36-fold) in slope was observed using Emulgen B-66/PBS modified electrodes compared to modification using DBSA/KCl. However, better signal to background data and consequently better sensitivity was observed for amperometric measurement using Emulgen B-66/PBS modified electrodes compared to that observed for DBSA/KCl. This might be due to the smaller and more stable background currents observed for Emulgen B-66 modified electrodes, which was discussed previously in Section 5.2.2. Average RSD

observed for five repeat measurements using Emulgen B-66/PBS modified electrodes was 9.5% while this was 7.5% for three repeats using DBSA/KCl modified electrodes.

Table 5.7. Detailed analytical data of amperometric measurement of 0 to 4 mM HDL-C using encapsulated Emulgen B-66/PBS modified electrodes.

HDL-C (Mm)	Average current (A)	S/B	SD	RSD (%)
0	4.07×10^{-8}	-	3.09×10^{-9}	7.6
0.5	4.80×10^{-8}	1.18	6.96×10^{-9}	14.5
1	6.35×10^{-8}	1.56	5.05×10^{-9}	7.9
2	9.74×10^{-8}	2.39	1.28×10^{-8}	13.2
3	1.31×10^{-7}	3.22	7.77×10^{-9}	5.9
4	1.64×10^{-7}	4.02	1.25×10^{-8}	7.6
Average RSD				**9.5**

Kinoshita *et al.,* (1998) developed one of the first amperometric HDL-C sensors based on a homogeneous assay in which a peroxidase-entrapped and ferrocene-embeded carbon paste electrode was used to measure the H_2O_2 produced after the enzymatic reaction of HDL-C. PEG-modified enzymes in the presence of α-cyclodextrin sulphate and $MgCl_2$ were employed to impart selectivity to the measurement of HDL-C. This method was performed at 37°C and was only linear up to 0.04 mM. However, since it is important to measure HDL-C directly up to at least 2 mM preferentially at room temperature, their method would be unsuitable for the development of a point of care device.

Foster, Cassidy and O'Donoghue, (2000) developed an electrochemical device for HDL-C based on a precipitation methodology in which phosphotungstic acid (PTA) and $MgCl_2$ were employed as the precipitation reagents. However, incorporation of this method on a disposable platform has also proved challenging. Murphy *et al.,* (2009) in their patent also reported the use of a surfactant selected from sucrose esters and maltoside to selectively measure HDL-C. This method is based on using coenzyme (NAD^+) and a redox agent capable of accepting electrons from the coenzyme and transfers it to the electrode. They have also employed delipidated serum for control measurement and the current response at 154 s was used to produce a calibration curve. Using a mediatorless sensor as a platform such as in the sensor developed in this research may lead to simpler device compared to those in the presence of redox mediator.

To sum up, by changing the modification method from DBSA/KCl to Emulgen B-66/PBS, an improvement on the signal to background of the biosensor was observed with no other significant changes on HDL-C biosensor parameters. Since use of Emulgen B-66/PBS modified electrode may remove a preparation step for an integrated HDL-C biosensor, this modification solution was used for all future experiments for the analysis of clinical samples.

5.2.3 Clinical HDL-C sample measurement

The application of the HDL-C biosensor was evaluated in serum samples. Two sets of samples were used. One set was referred to as 'calibrators' and were spiked human serum samples. The other set was composed of donor samples which were un-manipulated 'off the clot' serum samples from a donor population in San Diego, USA (Table 5.8).

Table 5.8. Detailed analytical results of serum samples as per certificate of analysis and those measured using Abcam assay kit. Calibrators highlighted in grey.

Samples ID	TG (mg/dL)	TC (mM)	FC (mM) colorimetric value	GLU (mg/dL)	HDL-C(mM)	
					Certified value	Colorimetric value
1	39.5	1.47	0.18	26	0.3	0.31
2	138	3.57	0.45	48	0.72	0.53
3	163.5	4.23	0.63	70	0.95	0.88
4	183.5	4.93	0.68	92	1.2	1.12
5	127.5	4.89	0.52	113	1.39	0.98
6	127	4.17	0.42	136	1.7	1.2
7	126.5	3.63	0.41	183	2.175	1.21
8	160.5	5.56	0.63	231	0.699	0.58
9	211	6.34	0.63	267	1.24	0.87
10	174	7.5	0.7	316	0.44	0.66
11	205	8.96	0.72	360	0.62	0.61
1085S	104.1	5.5	0.7	73	2.33	1.14
1087S	94.1	4.11	0.57	128.8	1.2	0.63
1106s	289.9	3.7	0.64	120.7	0.74	0.29

The HDL-C concentration in all the samples provided was measured using a spectrophotometric precipitation assay methodology (Abcam, UK) at the same time as the developed biosensor as HDL-C levels have been shown to decline in samples during storage and deviated from their certificated values (Ignatius *et al.,* 2009; Valentine Charlton-Menys, 2007). According to Table 5.8, samples 1, 2, 5, 7

and 10 with HDL-C concentrations of 0.31, 0.53, 0.98, 1.21 and 0.66 were used to prepare a calibration curve and the rest were used for HDL-C sample measurements (Fig. 5.23).

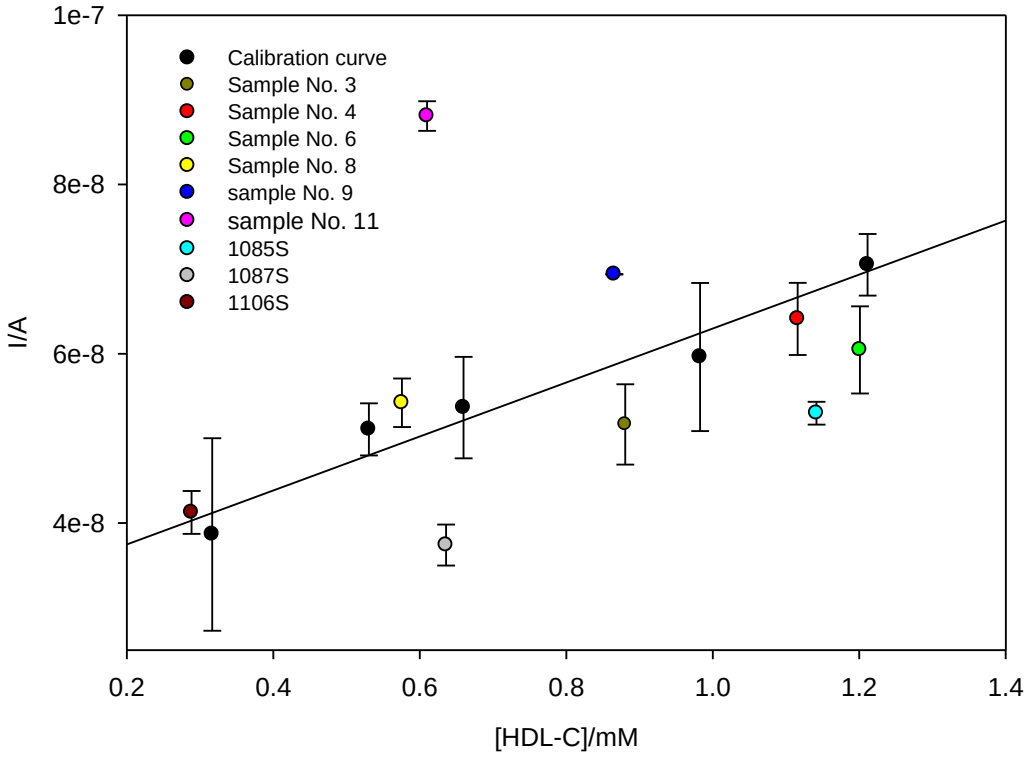

Fig. 5.23. Amperometric responses of the HDL-C biosensor to clinical serum samples.

The calibration curve showed a sensitivity of 3.18×10^{-8} A/mM with r^2 of 0.95. The linearity was decreased compared to that prepared using HDL-C in delipidated serum which might be due to some variability in serum matrix as a result of presenting different concentrations of triglyceride, glucose and other serum constituents in each sample (Helm, Jalukse and Leito, 2010; Zhang, Ju and Wang, 2008). The correlation between the HDL-C biosensor and Abcam assay kit had a slope of 0.87 and a Pearson correlation coefficient of 0.76 (n=13) (Fig. 5.24 and Table 5.9).

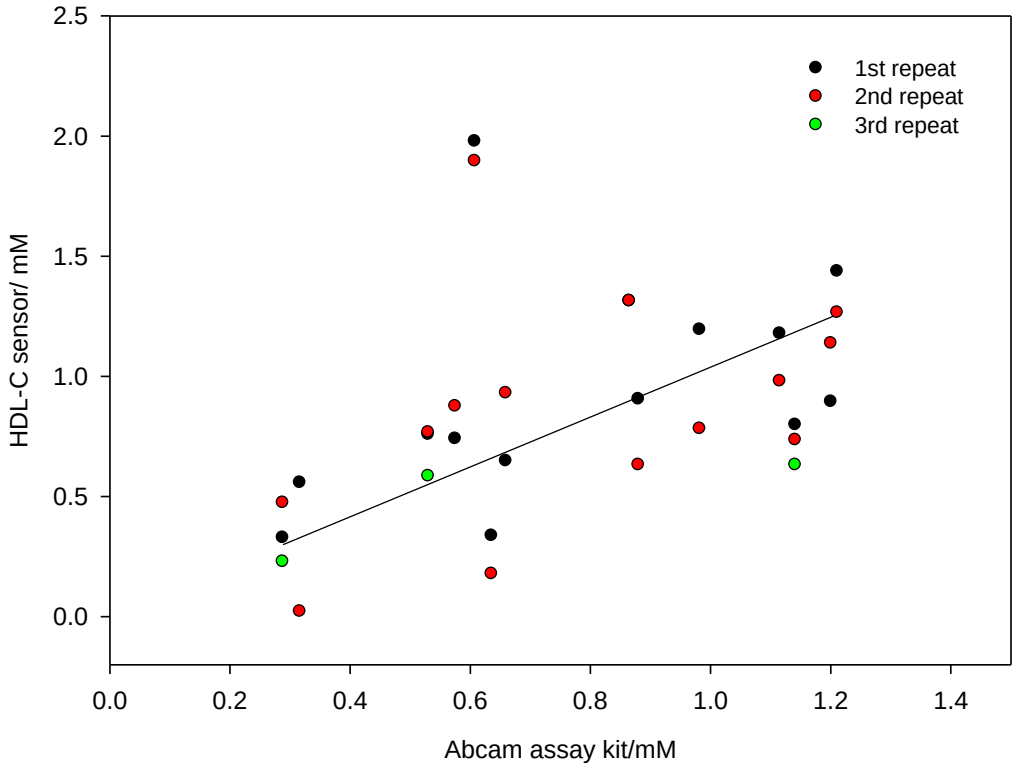

Fig. 5.24. Correlation of HDL-C in serum measured by the biosensor and the Abcam assay kit (n=13) (slope= 0.87, r=0.76).

Table 5.9. HDL-C concentrations measured using HDL-C sensor in comparison with the result measured using Abcam assay kit.

sample ID	Assay kit/ mM	HDL-C sensor/ mM		
		1st repeat	2nd repeat	3rd repeat
1	0.32	0.56	0.02	-
2	0.53	0.76	0.77	0.58
5	0.98	1.19	0.78	-
7	1.21	1.44	1.26	-
10	0.66	0.65	0.93	-
3	0.88	0.90	0.63	0.33
4	1.12	1.18	0.98	-
6	1.20	0.89	1.14	-
8	0.58	0.74	0.87	-
9	0.87	1.31	1.31	-
11	0.61	1.98	1.90	-
1085S	1.14	0.80	0.73	0.63
1087S	0.64	0.34	0.18	1.26
1106S	0.29	0.33	0.47	0.23

Sample 11 showed very high HDL-C in its both measurements. Although the reason for that is unknown, this might be due to the presence of high levels of FC.

Falsely elevated HDL-C in patients with type III hyperlipoproteinaemia was reported for the Roche assay in serum when it was compared with phosphotungstate magnesium precipitation which was due to the VLDL sub-fraction (Lackner and Schmitz, 1998). Since there was no more information about the samples other than those detailed in Table 5.9, by discarding the results for sample 11, the Bland–Altman plot which calculates the mean difference between the two methods of measurement is shown in Fig. 5.25. No significant bias was observed between the two methods across the measured range with most of the measurements within two standard deviations of the mean (Bland and Altman, 1999).

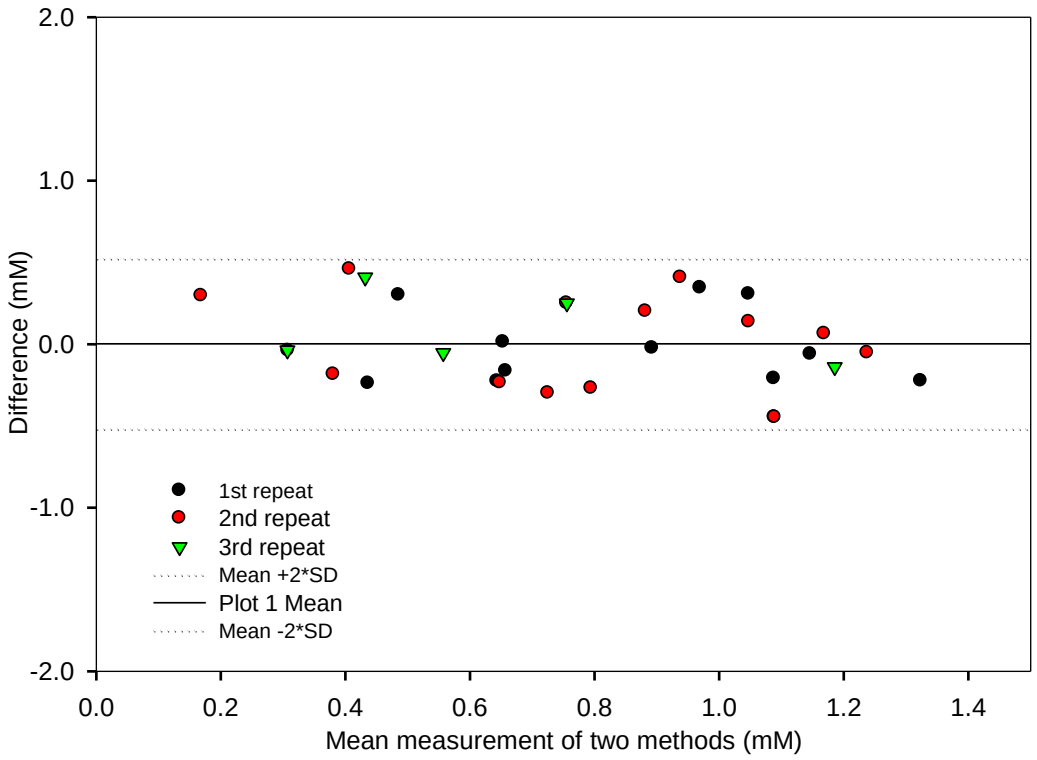

Fig. 5.25. Bland–Altman plot of the difference between sensor/abcam assay kit against the mean measurement of two methods in the 26 measurements

The mean of differences shown in the Bland-Altman plot is the estimated bias (difference between methods) which was 0.029 mM for HDL-C biosensor developed in this research and the standard deviation (SD) according to the Bland-Altman plot was approx. 0.26 mM which presents random fluctuations around its mean (Owiredu, Teye and Quaye, 2013). If the limits of agreement which is the mean difference ± 2SD between two methods are not clinically important, the two methods are comparable. However, according NCEP accuracy guideline, the mathematical mean of biases for HDL-C measurement should be less than 1 mg/dL (0.0259 mM) when compared with CDC method (Warnick, 1995). While the method

developed in this research showed agreement with the precipitation method used for comparison, its estimated bias (0.029) was 1.1-fold greater than the NCEP accepted higher bias level of 0.0259 mM.

Kinoshita *et al.,* (1998) reported the only previous example of an externally mixed amperometric HDL-C sensor which was used to measure HDL-C in 34 human serum samples showing an mean difference between methods of -0.05 mM and SD of approx. 0.3 mM for its Bland–Altman plot compared to the sensor developed in this research which had a mean difference of 0.029 mM and SD of approx. 0.26 mM.

By looking at two HDL-C point of care testing systems such as CardioCheck PA and Cholestech LDX with CRMLN certification, the correlation coefficient of 0.77 (n=101) and 0.95 (n=119) were reported against IL 600 and Kodak 700 analyser, respectively (Batki *et al.,* 1995; Batki *et al.,* 2005; Patel *et al.,* 2011). IL 600 and Kodak 700 were used as NECP certified methods. The CardioCheck PA and Cholestech LDX are the two smallest POCT HDL-C and total cholesterol measurement systems that support the NHS health check vascular risk assessment. Cardiocheck PA is based on a spectrophotometric method and the measurement of the light reflected off a test strip that has changed colour after applying blood. The analyzer converts this reading into an HDL result and displays it (CardioCheck PA user manual). The Cholestech LDX System combines the enzymatic methodology and solid-phase technology to measure TC, HDL-C, TGs, glucose, and others. The sample is applied to a Cholestech LDX cassette. The cassette is then placed into the Cholestech LDX analyzer that can measure the resultant colour by reflectance photometry (Cholestech LDX user manual). Both of the machines are used as a near patient POC device and require an expert for perfuming test (Batki *et al.,* 2005; Panz *et al.,* 2005).

In general, the biosensor indicated slightly higher HDL-C concentrations compared to the assay kit. Although the exact reason for that is not known, it may be due to varying levels of free cholesterol present in the samples, as this has been shown to affect the response of this type of assay (Yamamoto, Yamamoto and Nakanishi, 2011). Nakamura *et al.,* suggested a method employing a reaction accelerator such as Flufenamic acid in combination with ChOx as a sample pretreatment to remove free cholesterol before measuring cholesterol contained in specific lipoprotein (Nakamura *et al.,* 2009).

On the other hand, HDL-C measurement is very challenging due to very small differences in its concentrations which can lead an even small analytical error to misclassification. Even newer assay kit showed a poor agreement with designated comparison methods (DCM) (Contois, 2012). Miller *et al.,*(2010) also reported that out of eight homogeneous HDL-C assays, six of them failed to meet NCEP total error goals in the healthy control group, while all of them failed to meet goals for the donors with cardiovascular disease or lipoprotein disorders (Miller *et al.,* 2010). It has been also reported that most homogeneous assay methodologies give positive predictive values due to the presence of intermediate density lipoproteins (IDL) in the serum, or in the presence of high levels of Lp(a) (Nakamura *et al.,* 2006). While the biosensor was developed based on the homogeneous assay methodology, the Abcam assay kit is based on the precipitation principle (Arranz-Peña, 1998; Bairaktari *et al.,* 1999; Cobbaert *et al.,* 1998; Harris *et al.,* 1997). Therefore, discrepancies between the two methods are very likely to be due to the differences in the methods and how they process and respond to the complex mixtures of lipids and lipoproteins in the sample.

To sum up, an electrochemical biosensor was developed for the selective measurement of HDL-C. The sensor was able to achieve both selectivity and sensitivity enhancements using the surfactant Emulgen B-66. The presence of this surfactant was shown to selectively dissolve HDL over LDL. In addition, it also resulted in an enhanced electrocatalysis of H_2O_2 which is produced following the release of cholesterol ester and cholesterol from HDL and its catalysis by ChEs and ChOx, respectively. The biosensor was shown to have good linearity across the diagnostically relevant range. Despite some deviation which was observed in some of the samples, the biosensor was successfully applied to the measurement of HDL-C in real samples. Following development of the HDL-C biosensor by mixing assay reagents externally, the next step was to evaluate whether the same strategy could be used to develop TC biosensor by substituting Emulgen B-66 with Triton X-100 which will be discussed in the following section.

5.3 Development of a TC biosensor

The first step in the enzymatic reaction of TC in serum samples is through the disruption of all the lipoproteins carrying serum cholesterol using Triton X-100 which was shown to successfully disrupt lipoproteins and make their cholesterol ester available for enzymatic reaction with cholesterol esterase (Deacon and Dawson, 1979; Kinoshita *et al.,* 1998). Initial investigations were performed to find the optimal Triton X-100 concentration suitable for the enzymatic reaction of TC (Section 3.3) and its effect on the electrocatalysis of the DBSA/KCl modified electrodes was evaluated via amperometric measurement of H_2O_2 (Section 4.3.6). As was the case of the HDL-C biosensor, the DBSA/KCl modified silver electrode was used initially as a platform for the TC biosensor. However, the possibility of using an alternative modification solution of 0.5% (v/v) Triton X-100/PBS for development of the TC biosensor was evaluated and its application to the amperometric measurement of TC in serum samples was assessed.

5.3.1 Development of the TC biosensor using DBSA/KCl modified electrodes

Initially, the same concentrations of the enzymes applied for HDL-C measurement were used to measure TC. LDL-C stock solution with a TC concentration of 151.67 mM, FC concentration of 34.88 mM and HDL-C concentration of 5.26 mM was used to prepare 2 to 16 mM TC in delipidated serum. Encapsulated DBSA/KCl modified electrodes were used to measure TC in the presence of the same concentrations of enzymes as was mentioned previously in section 5.2 and 0.5% (v/v) Triton X-100. The amperometric responses of biosensors to each concentration were then measured at 420 s after 240 s incubation at room temperature and are shown in Fig. 5.26. As can be seen, a linear response was observed from 2 to 8 mM cholesterol, but which plateaued with a slight decrease at concentrations above 10 mM. Since the final aim of the project was to measure TC directly and without any dilution, ChEs and ChOx concentrations were re-optimised in order to improve linearity up to 10 mM TC (5 mM final solution concentration). Therefore, the effect of different enzymes concentration on the amperometric response of the biosensor to 8 µL of delipidated serum containing LDL-C (0 to 10 mM) was evaluated.

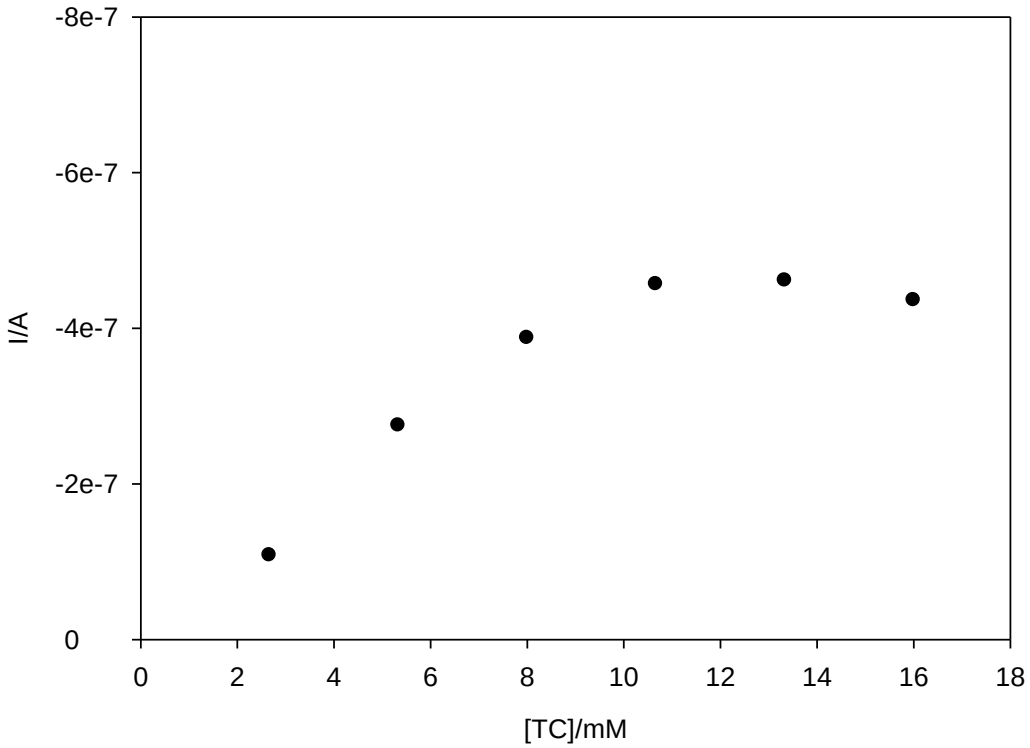

Fig. 5.26. Amperometric response of the TC biosensor at 420 s to 2 to 16 mM TC in the presence of 23 U/mL ChOx, 39 U/mL ChEs and 0.5% (v/v) Triton X-100 (240 s incubation time was applied)

As was mentioned in Chapter 3, enzymatic assays can be performed either when the reaction has gone to completion, or kinetically, by looking at the rates of reaction during the earlier phase of the assay (Copeland, 2000; Tipton, 1992). Therefore, for optimisation purposes, and based on the fact that this is a coupled enzyme reaction, different combinations of ChEs/ChOx as summarised in Table 5.10 were investigated and amperometric measurement was performed following 240 s incubation at room temperature and the current responses at 200 s and 420 s were assessed. The results for each ChEs/ChOx combination are shown in Table 5.10 and plotted as shown in Fig. 5.27.

As can be seen, measurement at 200 s did not show acceptable linearity or reproducibility, which might be due to some interference caused by non-Faradic current at the beginning of the measurement as well as incompletion of the reaction at higher TC concentrations, while the biosensor showed better linearity and reproducibility at 420 s resulting in it being used as a suitable measurement time in further experiments. As can be seen in Fig. 5.27, almost all the ChEs/ChOx combinations failed to give linearity greater than 0.96 for up to 11 mM TC measurements. Only the combination in Fig. 5.27 G (156 U/mL ChEs and 60 U/mL ChEs) showed the greatest slope of 2.86×10^{-8} A/mM with linearity of 0.978 (r^2) for

up to 11 mM TC concentration using the encapsulated DBSA/KCl modified electrodes. Further increase in ChOx concentrations in the presence of 156U/mL ChEs (Fig. 5.27 H) led to a significant decrease in both linearity and slope which might be due the coupled enzymes characteristic of the reaction. This was also reported by Noma and Nakayama (1976). As is summarised in Table 5.10, no significant improvement was observed by changing the concentrations of ChEs and ChOx. This might be due to the coupled enzyme properties of the reaction which, in the first step, cholesterol ester in the presence of ChEs produces non-esterified cholesterol. Then non-esterified cholesterol in the presence of ChOx produces H_2O_2. In this coupled assay, the concentration of non-esterified cholesterol depends on its rate of formation by ChEs and the rate of removal by ChOx. If the concentration of cholesterol ester is greater than its K_m for ChEs (3×10^{-5} M), the first reaction is zero order. Therefore the concentration of esterified cholesterol does not change significantly when non-esterified cholesterol is formed. The concentration of non-esterified cholesterol then increases to reach a steady-state concentration which is high enough that the rate of ChOx becomes almost equal to the rate of ChEs. Reaching of non-esterified cholesterol to its steady-state concentration very much depends on its Michaelis constant for ChOx and the concentration of ChOx presents. The time which non-esterified cholesterol is building up, results in a lag phase in enzymatic reaction.

Table 5.10. ChEs and ChOx concentrations applied for optimisation study in amperometric measurement of TC and biosensor analytical response at 200 and 420 s.

Sample ID	ChEs (U/mL)	ChOx (U/mL)	at 200 s		at 420 s	
			Slope	linearity (0-11 mM)	Slope	linearity (0-11 mM)
A	78	20	2.13×10^{-8}	0.948	2.62×10^{-8}	0.957
B	78	40	5.18×10^{-8}	0.923	1.90×10^{-8}	0.948
C	78	60	3.74×10^{-8}	0.482	2.50×10^{-8}	0.883
D	78	100	4.53×10^{-8}	0.885	2.48×10^{-8}	0.750
E	156	20	4.04×10^{-8}	0.603	2.75×10^{-8}	0.889
F	156	40	5.35×10^{-8}	0.873	2.43×10^{-8}	0.905
G	156	60	3.42×10^{-8}	0.509	2.82×10^{-8}	0.953
H	156	100	2.41×10^{-8}	0.362	2.21×10^{-8}	0.774

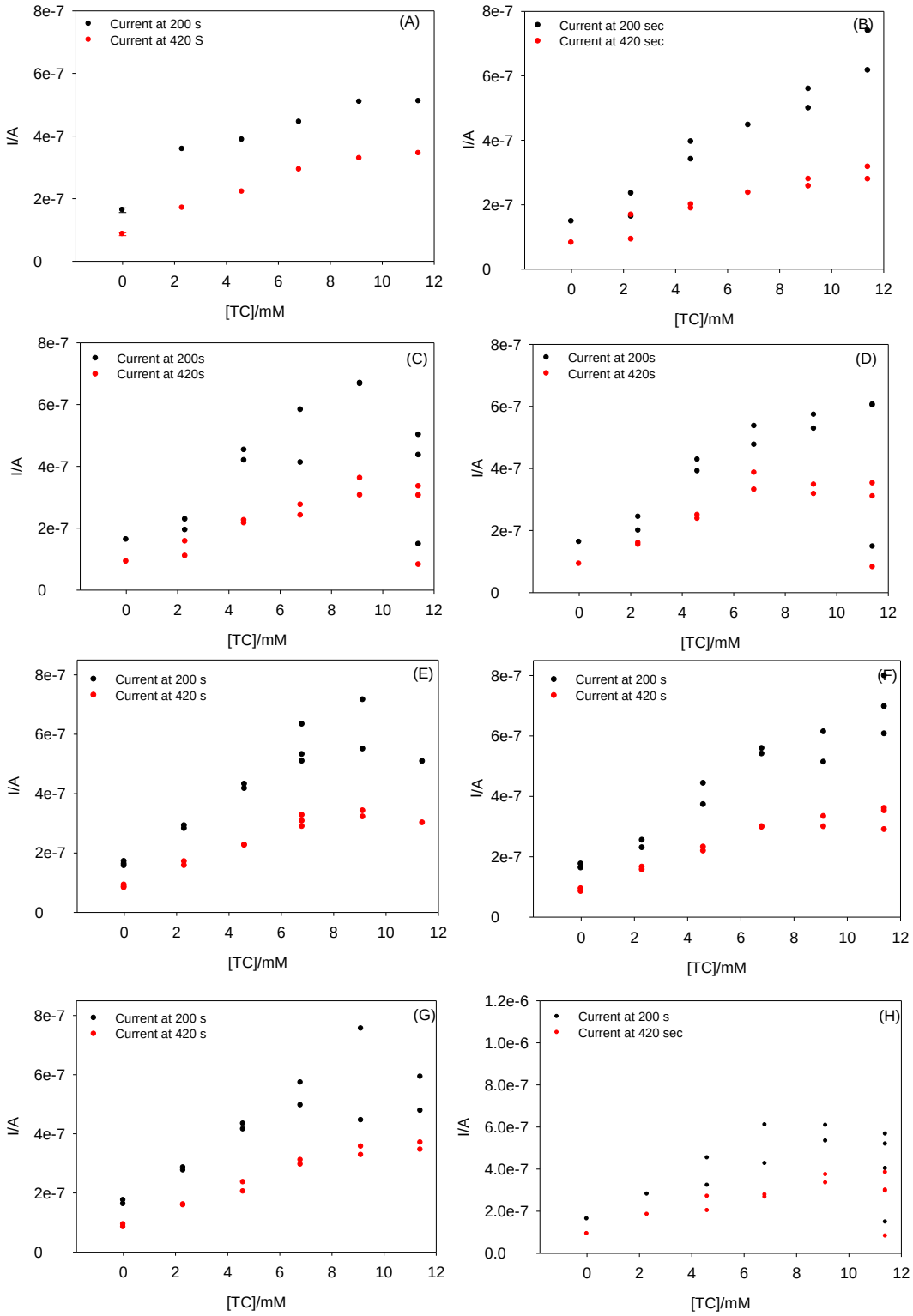

Fig. 5.27. Amperometric response of the TC biosensor at TC concentrations of 0 to 11 mM at t=200 s and 420 s in the presence of different ChEs/ChOx combinations: A) 78/20, B) 78/40, C) 78/60, D) 78/100, E)156/20, F) 156/40, G) 156/60 and H) 156/100 U/mL.

As can be seen this type of assay has unusual feature of having a long lag phase which the amount of second enzymes can influence the rate of the first enzyme as

well as kinetic characteristics of the reaction. So depends on the activity of the enzymes (ChEs and ChOx) and the lag phase of coupled enzymatic reaction, the amount of H_2O_2 produced may be vary (Copeland, 2000; Tipton, 1992). The optimum slope and linearity was thus observed in the presence of 156 U/mL ChEs and 60 U/mL ChOx up to 11 mM TC concentration as shown in Table 5.10.

To assess whether a shorter incubation time lead to better linearity on the DBSA/KCl modified electrode response, due to coupled characteristic of the reaction, an assay employing the optimum enzyme concentrations was used to measure TC (0 to 11 mM) in the presence of 0.5% (v/v) Triton X-100, this time following 180 s incubation (Fig. 5.28).

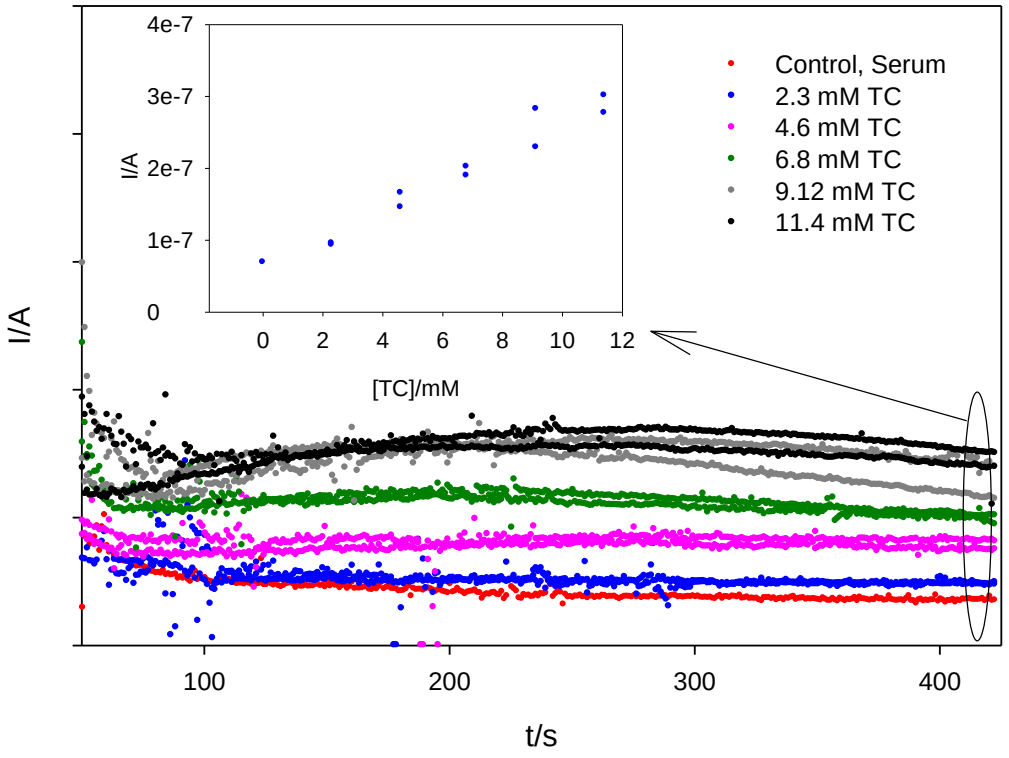

Fig. 5.28. Amperometric measurement of 0 to 11 mM TC in the presence of 156 U/mL ChEs and 60 U/mL ChOx and after 180 s incubation time. Inset: Biosensor response to 0 to 11 mM TC concentration at 420 s.

As can be seen the current response at 420 s had a sensitivity of 2.1×10^{-8} A/mM which was 1.4-fold less than that observed following 240 s incubation, while linearity was similar with r^2=0.960. Therefore, since 240 s provided better sensitivity compared to 180 s, while having comparable linearity this incubation time was employed for future TC measurements.

Noma and Nakayama (1976) reported the first polarographic method based on the consumption of oxygen produced after the enzymatic reaction of serum cholesterol using ChEs, ChOx and 0.1% (v/v) Triton X-100 in phosphate buffer pH 7.0. This method was unsuitable for the clinical laboratory as the measurement of oxygen consumption was not reliable. The most suitable enzymatic methods for TC measurement as described in Chapter 1 have been the spectrophotometric measurement of H_2O_2 using a HRP, 4 AAP and a Trinder's dye. The first enzymatic determination of TC in serum using cholesterol ester hydrolysis and ChOx was reported by Allain *et al.,* (1974) which used a 100-fold sample dilution and was linear up to 15 mM (Allain *et al.,* 1974). ChEs from *Pseudomonas* sp. appeared to be the preferred enzyme with significant improvement in specificity for the enzymatic reaction of TC (Rifai, Warnick and Dominiczak, 2001). While most methods employing reagent additions are based on spectrophotometric methods, almost all the recent reports for TC biosensors have been based on methods on which the enzymes are immobilised on the electrode surface (Arya, Datta and Malhotra, 2008). Since TC measurement is critical for calculation of non-HDL-C and LDL-C, development of a TC biosensor using the same platform as HDL-C was found to be very essential.

To sum up, enzyme concentrations of 156 U/mL ChEs and 60U/mL ChOx were found to be optimum for the enzymatic reaction of TC using DBSA/KCl modified electrodes in the presence of 0.5% (v/v) Triton X-100. However, in a manner analogous to that already established for the HDL-C assay, it was hypothesised that a TC biosensor could also be based on the silver paste electrodes modified with Triton X-100/PBS rather than DBSA/KCl, so removing an unnecessary preparation step in the assay and facilitating more effective integration of a fully printed biosensor as will be demonstrated in Chapter 6.

5.3.2 Development of the TC biosensor using Triton X-100/PBS modified electrodes

It was shown in the previous chapter (Section 4.3.6) that 0.5% (v/v) Triton X-100 had almost the same effect on the electrocatalysis of modified electrodes towards H_2O_2 prepared in delipidated serum as that observed in 6% (w/v) Emulgen B-66. Furthermore, Triton X-100 has been reported to enhance the response of silver screen printed electrodes in the presence of KCl (Gonzalez-Macia, Smyth and Killard, 2011). While Gonzalez-Macia *et al.,* (2011) reported the use of 33 mM

Triton X-100 (approx. 2% (w/v)) in the presence of a lower salt concentration (0.1 mM KCl) as achieving optimal electrocatalysis, a concentration of 0.5% (v/v) Triton X-100 was found to be optimal for measurement of TC. Thus, the effect of 0.5% (v/v) Triton X-100/PBS as a modification solution was compared to DBSA/KCl modified electrodes. Five layers of 0.5% (v/v) Triton X-100/PBS were inkjet-printed on silver paste electrode using a similar method employed for DBSA/KCl. Cyclic voltammetric experiments was performed using both modified electrodes in 0.5% (v/v) Triton X-100 in PBS followed by 3 mM H_2O_2 in the same solution (Fig. 5.29). Although Triton had a slightly different double layer charging, especially around -0.1 V, which might reduce the signal to background, DBSA/KCl showed marginally better electrocatalysis.

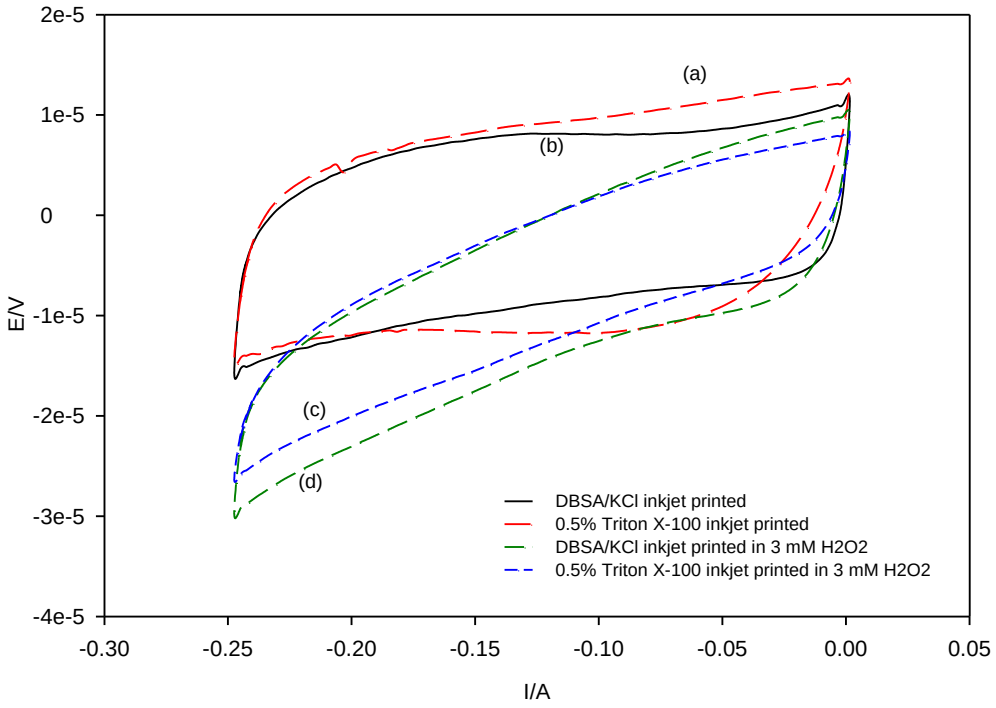

Fig. 5.29. Cyclic voltammograms of electrodes modified in Triton X-100/PBS solution (a and c) or DBSA/KCl (b and d), measured in Triton/PBS (a and b) or Triton/PBS and 3 mM H_2O_2 (c and d) (vs. Ag/AgCl).

0.5% (v/v) Triton X-100/PBS was then assessed as an electrode modifier and its effect on the cyclic voltammetric and amperometric measurement of H_2O_2 in stirred batch solution on silver paste electrodes was investigated In a similar way as was mentioned in Section 5.2.2. Five layers of 0.5% (v/v) Triton X-100/PBS solution were inkjet-printed onto silver paste electrodes and their electrocatalytic response

to H_2O_2 studied in stirred solution (Fig. 5.30). Detailed analytical data are shown in Table 5.11.

Table 5.11. Detailed analytical results of amperometric measurement of 0 to 5 mM H_2O_2 using unmodified and Triton X-100/PBS modified electrodes.

$[H_2O_2]$ /mM	unmodified in PBS	Unmodified in Triton X-100/PBS		Triton X-100/PBS modified in PBS	Triton X-100/PBS modified in Triton X-100	
1	5.42×10^{-8}	8.80×10^{-7}	1.06×10^{-6}	8.31×10^{-7}	1.76×10^{-6}	1.76×10^{-6}
2	1.83×10^{-7}	2.60×10^{-6}	3.11×10^{-6}	2.25×10^{-6}	4.54×10^{-6}	4.58×10^{-6}
3	3.22×10^{-7}	3.94×10^{-6}	5.21×10^{-6}	3.92×10^{-6}	7.29×10^{-6}	7.10×10^{-6}
4	4.65×10^{-7}	5.34×10^{-6}	7.17×10^{-6}	5.59×10^{-6}	1.01×10^{-5}	9.73(10-6
5	5.91×10^{-7}	6.55×10^{-7}	9.13×10^{-7}	7.30×10^{-7}	1.30×10^{-7}	1.24×10^{-5}

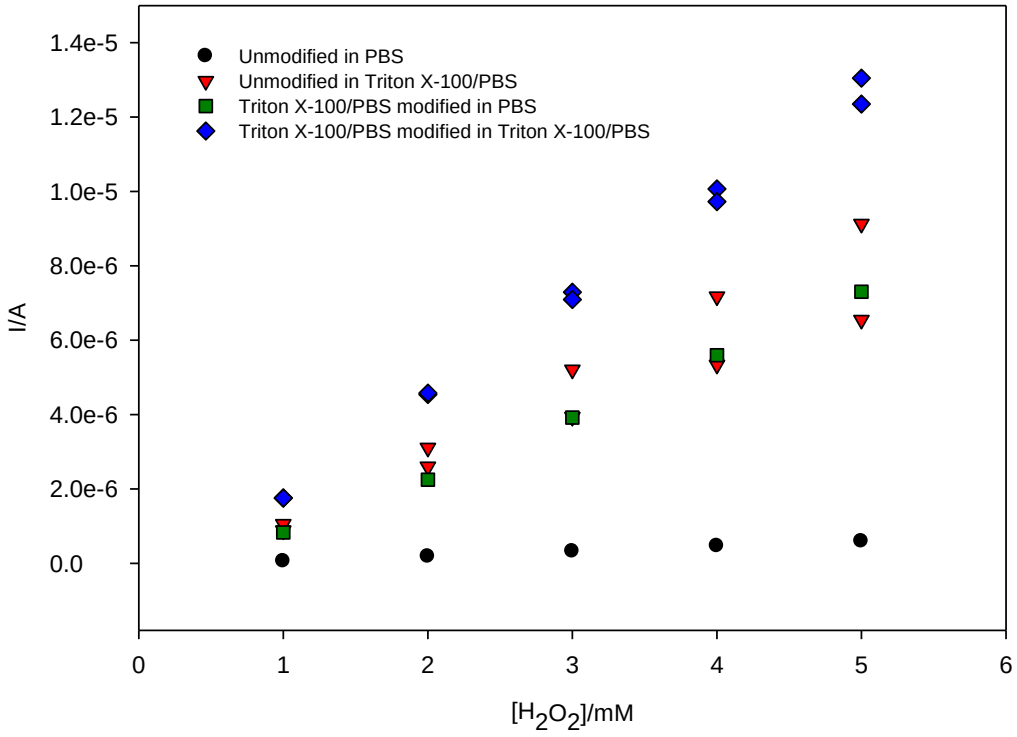

Fig. 5.30. Amperometric measurement H_2O_2 in stirred solution at 420 s using unmodified and Triton X-100/PBS modified electrodes in both PBS and 0.5% (v/v) Triton X-100/PBS solution.

The current observed using an electrode modified in Triton X-100/PBS and measured in Triton/PBS was 1.28×10^{-5} A for 5 mM H_2O_2 which was approx. 216-fold greater than that observed for unmodified electrode in PBS (5.91×10^{-7} A) illustrating a significant catalytic effect when the electrode was both modified with Triton/PBS using inkjet printing and when measurement was performed in the

presence of Triton/PBS. Unmodified electrodes which were used to measure H_2O_2 in Triton/PBS solution showed almost the same catalysis as that observed for modified electrodes in PBS showing that the presence of Triton X-100/PBS is essential for enhanced catalysis. Amperometric measurement of H_2O_2 in 0.5% (v/v) Triton X-100/PBS solution was also assessed using both electrodes modified with both DBSA/KCl and Triton/PBS in stirred batch solution (Fig. 5.31). Slightly better current responses were observed for H_2O_2 concentrations using the DBSA/KCl modified electrodes compared to 0.5% (v/v) Triton X-100/PBS which might be due to the use of Triton X-100 and PBS at concentrations optimised for TC measurement, rather than for electrocatalysis. However, only a slight reduction was observed due to this difference, demonstrating the suitability of using Triton/PBS as an alternative modification method to DBSA/KCl.

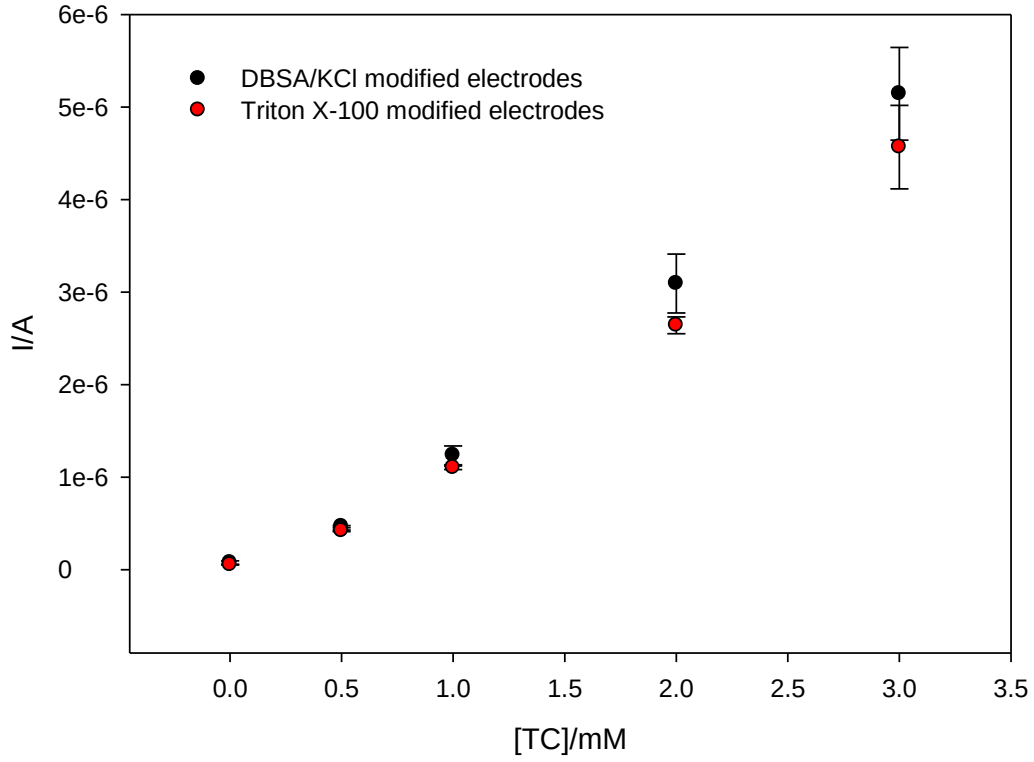

Fig. 5.31. Amperometric response of electrodes modified using DBSA/KCl or Triton/PBS to H_2O_2 in stirred batch solution at -0.1 V (vs. Ag/AgCl) (n=3).

A further experiment was performed to find the optimum volume of modification solution to achieve maximum electrocatalysis and electrode reproducibility. Electrodes were modified by inkjet-printing of 5, 10 or 15 layers of 0.5% (v/v) Triton X-100/PBS at a nozzle voltage of 26 V using a 16-nozzle head cartridge (approx. 1.2 µL reagent deposition). The electrodes were allowed to dry for 5 min and the excess modification solution was then washed using deionised water. A cyclic

voltammetric study was then performed. Fig. 5.32 A shows increases in charging current of 13-fold for 5 and 10 layers and 23-fold for 15 layers compared to un-modified electrodes. CVs in 5 mM H_2O_2 showed increased cathodic currents following modification (Fig. 5.32 B).

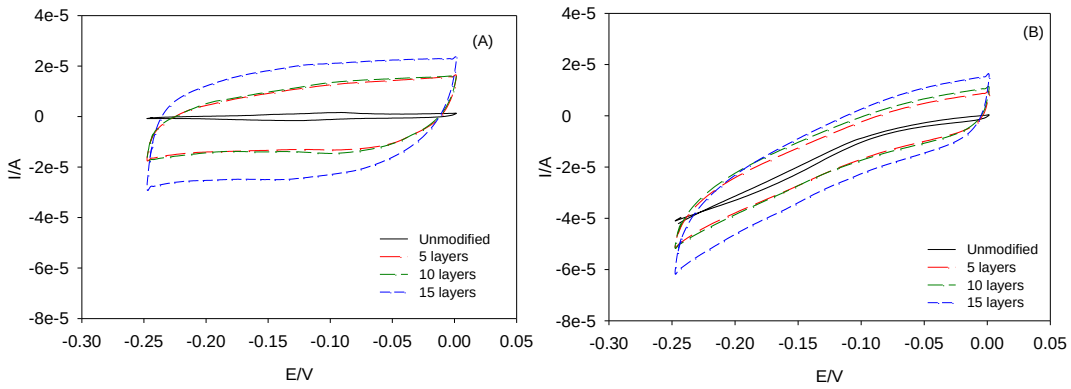

Fig. 5.32. Cyclic voltammograms of unmodified electrodes and those modified with 5, 10 and 15 inkjet-printed layers in: A) 0.5% (v/v) Triton X-100/PBS solution and B) 5 mM H_2O_2.

Table 5.12. Detailed analytical data of the amperometric measurement of H_2O_2 in stirred batch solution using different volumes of inkjet-printed modification solution.

$[H_2O_2]$/ mM	Unmodified	5 layers		10 layers		15 layers	
	I/A	I/A		I/A		I/A	
0	1.8×10^{-8}	4.9×10^{-8}	6.5×10^{-8}	4.7×10^{-8}	6.2×10^{-8}	6.1×10^{-8}	4.5×10^{-6}
1	7.5×10^{-7}	1.4×10^{-6}	1.6×10^{-6}	1.2×10^{-6}	1.6×10^{-6}	1.4×10^{-6}	1.7×10^{-6}
2	2.6×10^{-6}	3.5×10^{-6}	4.0×10^{-6}	2.9×10^{-6}	4.1×10^{-6}	3.3×10^{-6}	4.3×10^{-6}
3	4.4×10^{-6}	5.9×10^{-6}	7.2×10^{-6}	4.9×10^{-6}	6.7×10^{-6}	5.5×10^{-6}	7.3×10^{-6}
4	6.0×10^{-6}	8.4×10^{-6}	1.0×10^{-5}	7.1×10^{-6}	9.5×10^{-6}	7.7×10^{-6}	1.0×10^{-6}
5	7.5×10^{-6}	1.1×10^{-5}	1.5×10^{-5}	9.6×10^{-6}	1.3×10^{-5}	1.0×10^{-5}	1.4×10^{-6}

Amperometric measurement of H_2O_2 in 0.5% (v/v) Triton X-100/PBS in stirred batch experiments employing electrodes modified with 5, 10 and 15 printed layers of Triton/PBS showed almost the same enhancement of catalytic activity, which all were moderately more catalytic than those observed for 0 to 5 mM H_2O_2 using unmodified electrode in 0.5% (v/v) Triton X-100/PBS solution (Table 5.12 and Fig. 5.33). Electrodes modified with five layers may have illustrated a slight improvement over 10 and 15 layers. In conclusion, since there were not any significant different between 5, 10 and 15 layers modification, and based on the fact that fewer layers requires less preparation time and materials, electrodes modified

with 5 layers of Triton/PBS were used for the quantitative measurement of TC in delipidated serum.

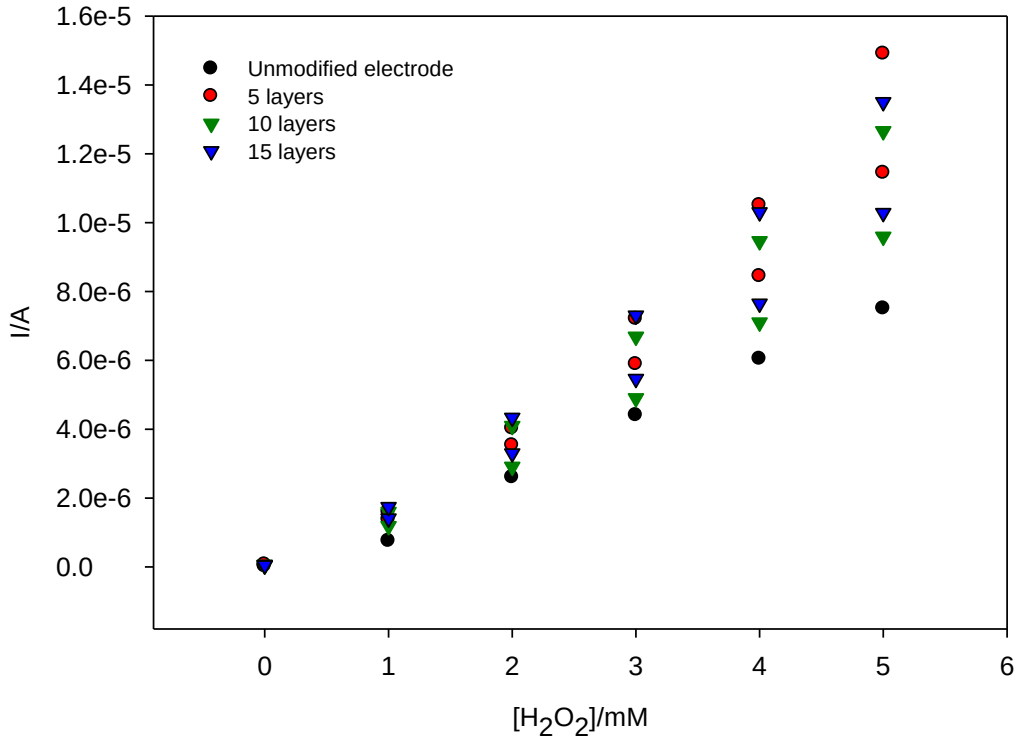

Fig. 5.33. Amperometric measurement of H_2O_2 in 0.5% (v/v) Triton X-100/PBS in stirred batch cell at -0.1 V vs. Ag/AgCl using unmodified electrodes, and electrodes modified with 5, 10 and 15 inkjet-printed layers of Triton/PBS.

5.3.2.1 Amperometric measurement of TC in delipidated serum

The application of electrodes modified with Triton/PBS as a TC biosensor was assessed. The amperometric response of encapsulated electrodes to 8 µL TC in delipidated serum in the presence of 0.5% (v/v) Triton X-100 in combination with optimised enzyme concentrations (Section 5.3.1) was evaluated (Table 5.13 and Fig. 5.34).

The modified electrodes showed a slope of 2.24×10^{-8} A/mM for TC measurement up to 10 mM which was 1.2-fold lower compared to that observed using DBSA/KCl modified electrodes (2.82×10^{-8} A/mM). Triton/PBS modified electrodes showed better linearity (r^2 = 0.984, n=3) up to 10 mM TC, as compared to DBSA/KCl (r^2= 0.978 to 9 mM; 0.953 to 11 mM TC). Almost the same background current was observed for 2 to 10 mM TC prepared in delipidated serum confirming that using delipidated serum may provide more suitable sample matrix. Average RSD observed for three repeats measurement was 10.8 %. Kinoshita *et al.,* (1998) also

reported the use of their own HDL-C platform (a peroxidase-entrapped and ferrocene-embeded carbon paste electrode) for TC measurement by replacing only 0.5% (v/v) Triton X-100 as a non-specific surfactant. However, no detailed analytical data was reported for TC measurements. To summarise, the biosensor platform developed for the measurement of HDL-C was adapted for the measurement of TC by modifying the electrodes with 0.5% (v/v) Triton X-100/PBS. The biosensor was found to be suitable for the amperometric measurement of TC in delipidated serum in the presence of optimised assay reagents which again employed 0.5% (v/v) Triton X-100 in combination with ChEs and ChOx. Analysis of clinical samples using the TC biosensor

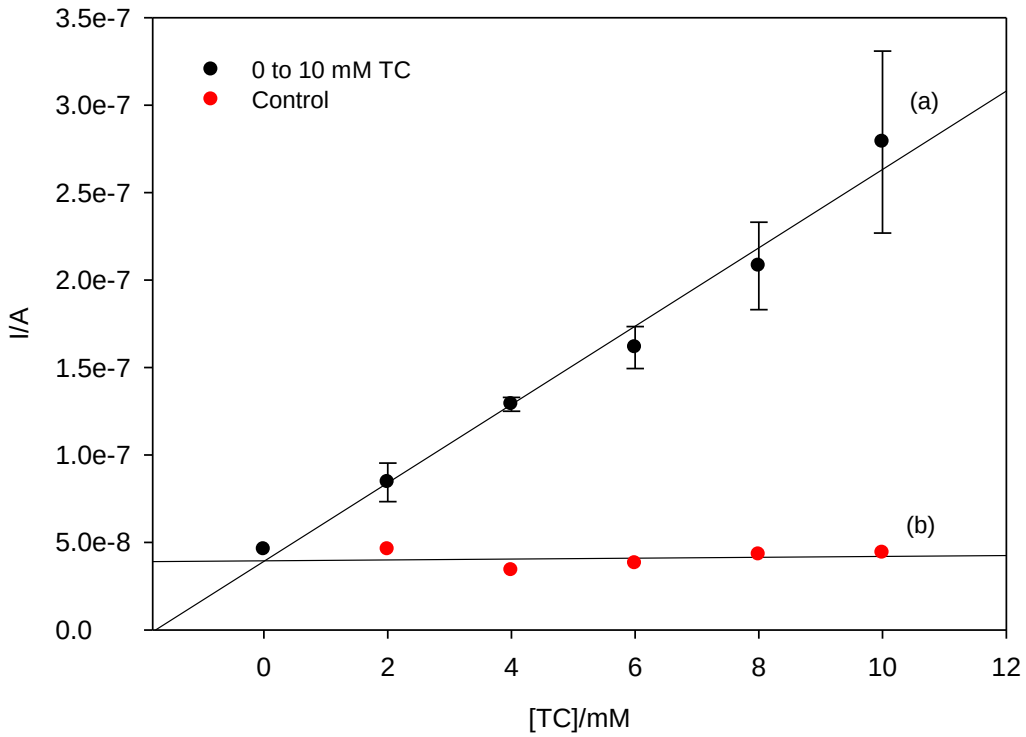

Fig. 5.34. Biosensor response to total cholesterol in delipidated serum at -0.1 V (vs Ag/AgCl) after 420 s. a) modified electrode using externally mixed reagents containing 0.5% (v/v) Triton X-100, 156 U/mL ChEs and 60 U/mL ChOx; (slope=2.24×10^{-8} A/mM, r^2=0.984 and RSD=10.8%; n=3); b) controls in the absence of ChEs and ChOx.

Table 5.13. Detailed analytical data of amperometric measurement of TC using the optimised TC biosensor.

TC/ mM	Average (I/A)	SD	RSD (%)	Control
0	-	--		4.60×10^{-8}
2	8.44×10^{-8}	1.10×10^{-8}	13.0	4.60×10^{-8}
4	1.29×10^{-7}	3.96×10^{-9}	3.1	3.40×10^{-8}
6	1.61×10^{-7}	1.20×10^{-8}	7.4	3.80×10^{-8}
8	2.08×10^{-7}	2.50×10^{-8}	12.0	4.30×10^{-8}
10	2.79×10^{-7}	5.20×10^{-8}	18.7	4.40×10^{-8}
Average RSD			10.8	-

5.3.3 Analysis of clinical samples using the TC biosensor

The developed TC biosensor was used to measure TC in serum samples. Two types of samples were used as mentioned earlier (Section 5.2.3, Table 5.8). Since keeping the samples in the freezer did not show any significant change in TC serum concentration, the TC concentration in all the samples was taken from the certificate of analysis which accompanied the samples (Ignatius *et al.,* 2009).

Samples 1, 3, 9, 10 and 11 with TC concentrations of 1.47, 4.23, 6.34, 7.50 and 8.96 mM, respectively were used as calibrators and the remaining samples in Table 5.14 were used for clinical sample measurements (Fig. 5.35). Serum-based calibrators were also employed for spectrophotometric measurement of TC by Nakamura *et al.,* (2006), to provide a comparable calibrator matrix to the clinical samples. The calibration curve yielded a slope of 2.34×10^{-8} A/mM which was slightly lower than that observed previously using just LDL-C in delipidated serum as calibrators with a linearity (r^2) of 0.98 (n=2) which was very similar to that observed in 5.3.2.1 showing that variability in the serum matrix including different concentrations of triglycerides and glucose for each clinical sample did not show any significant impact on electrodes response. The TC concentrations of 11 samples (2, 4 to 8 and 18 to 22) were then measured amperometrically using the biosensor and their TC values were then determined by interpolation on the linear region of the calibration curve. Each sample was only measured twice due to sample limitations. The results are shown in Table 5.14 and correlated against certified value reported by Alere using Cholestech LDX in Fig. 5.36 A.

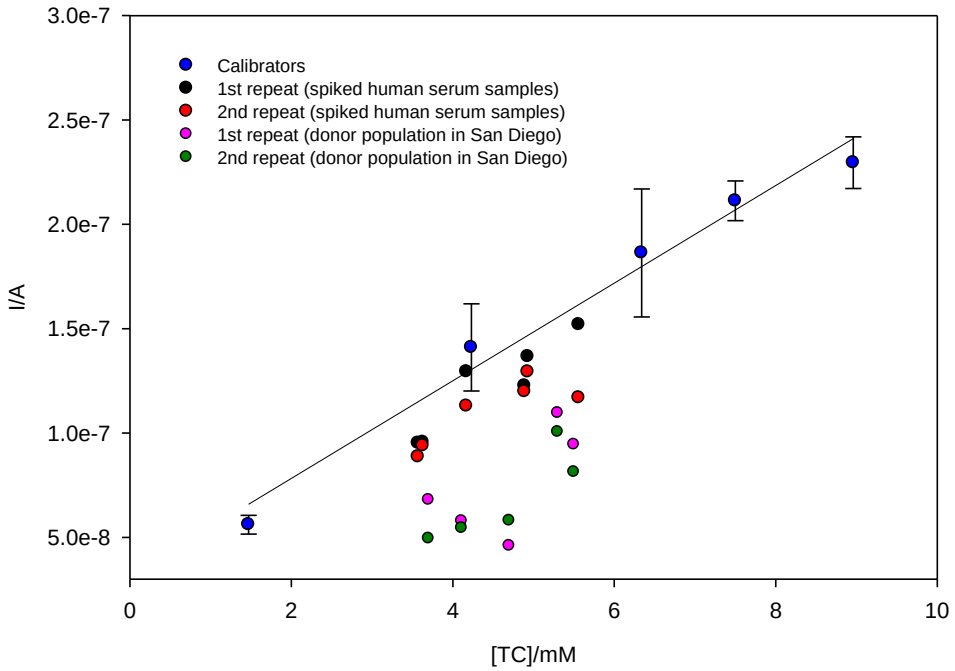

Fig. 5.35. TC biosensor responses to clinical samples at -0.1 V vs. AgAgCl, 420 s after 240 s incubation at room temperature, TC concentrations of 1.47, 4.23, 6.34, 7.50 and 8.96 were used as calibrators while the remaining were used for sample measurement which were either duplicate measurements of spiked human serum samples or the San Diego donor population.

Table 5.14. TC concentrations calculated using TC biosensor compared to the results taken from certificate of analysis reported by Alere. Samples 2, 4-8 are spiked human serum with the same sample matrix as those used for calibration curve and the samples 18-22 are real samples from donor population in San Diego.

samples ID	TC/mM	TC sensor	
		1st repeat	2nd repeat
2	3.57	3.06	2.79
7	3.63	3.08	3.01
6	4.17	4.51	3.81
5	4.89	4.22	4.10
4	4.93	4.81	4.50
8	5.56	5.46	3.98
21	3.70	1.92	1.14
19	4.11	1.49	1.35
20	4.70	1.00	1.50
22	5.30	3.67	3.29
18	5.50	3.04	2.48

Samples 2, 4 to 8 had the same matrix as the calibrators showing the deviation of less than 1 mM in 92% of the measurement. Samples collected by donor population in San Diego (18 to 22) showed a significant deviation which was

between 2 to 3 mM which might be due to the matrix effect which may not be very similar to those used for making a calibration curve (Helm, Jalukse and Leito, 2010). The correlation had a slope of 1.07 and a Pearson correlation coefficient of 0.87.

CardioChek PA and Cholestech LDX, the two POCT systems that support the total cholesterol measurement in NHS health check, showed a slope of 0.72 and 0.95 and pearson correlation coefficient of 0.86 and 0.95 respectively (Batki *et al.,* 1995; Batki *et al.,* 2005; Patel *et al.,* 2011). A luminol electrochemiluminescence-based biosensor in conjunction with ChEs in solution for TC was also reported which was capable of measuring TC in human sera within 900 s with the lower TC observed in normal serum compared to the Pathological serum samples (Marquette, Ravaud and Blum, 2000).

Fig. 5.36 B demonstrates the Bland–Altman plot of the developed TC biosensor against the certified values which calculates the mean difference between two methods of measurement was within mean ±2*SD limit indicating that no significant bias was observed between the two methods across the measured range (Bland and Altman, 1999). The mean difference of the method representing the estimated bias was found to be 0.92 mM with SD of approx. 1.34 mM showing an approximate deviation of 3 mM or less which is significant across the measurement range. Since average bias for TC measurement according to NECP guideline was reported to be less than 3% (0.16 mM), the estimated bias according to this method was 5.8-fold greater. This greater deviation mostly related to un-manipulated ‘off the clot’ serum samples from a donor population in San Diego. The biosensor indicated slightly lower total concentrations for some of the clinical samples collected from the donor population in San Diego which might be due to either incompatibility of the calibrator matrix with donor samples or some unknown reasons such as the effect of other unknown serum components present in donor populations (Helm, Jalukse and Leito, 2010).

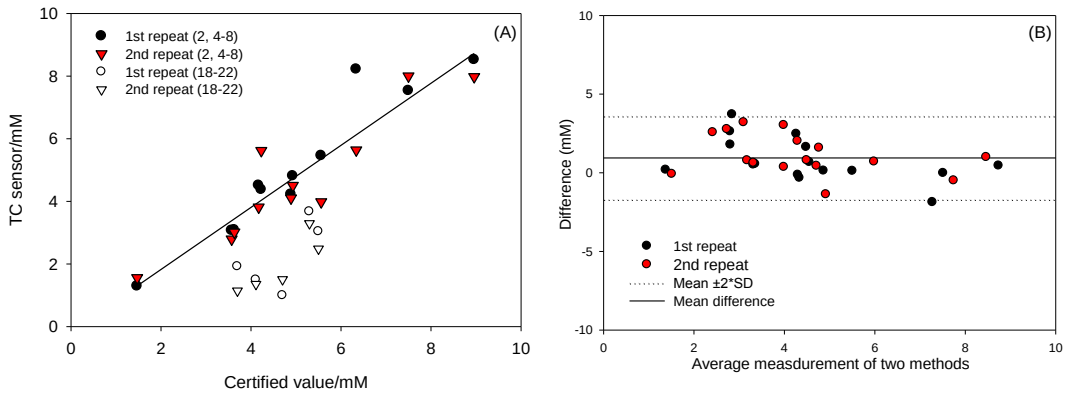

Fig. 5.36. Correlation of total cholesterol in serum measured by the biosensor and certified value (slope= 1.07, r=0.87). Inset: plot of differences versus average with 95% limits of agreement

Although there are many reports for TC measurement using biosensors (Arya, Datta and Malhotra, 2008), there has only been limited reporting of TC biosensors employing measurement in serum.

Dey and Raj (2014) reported a TC biosensor developed by integrating ChOx and ChEs using reduced graphene oxide-dendritic Pd nanoparticle (rGO-nPd) hybrid material without any redox mediator as a platform for H_2O_2 detection which was successfully applied for the measurement of two human serum samples after 10-fold dilution using PBS to minimise the sample matrix effect. Their biosensor was in very close agreement with clinical data (RSD=3.45 and 1.51 for two samples), However, the assay required a 10-fold dilution which would not be suitable for direct TC serum measurement.

Urmila *et al.,* (2011) reported the application of immobilized ChEs and ChOx on to a gold electrode modified with electrode multiwalled carbon nanotubes (MWCNTs) uniformly dispersed in a Nafion matrix using a layer by layer technique to measure TC in real samples. A dilution factor of 30-fold was used to make the final sample concentration fall within the biosensor linear range limit (0.080–0.950mM) (Urmila *et al.,* 2011). Although it was found that the result taken from the biosensor was in good agreement with the enzymatic cholesterol assay estimation kit (CHOD-PAP method), once again applying a dilution factor of 30-fold would make it unsuitable for direct measurement of TC in serum.

A disposable amperometric TC biosensor was also developed containing a sensing electrode and a reference electrode which were in contact with an integrated

reagent layer formed by coating a working ink containing ChEs, cholesterol dehydrogenase, coenzyme, redox mediator, surfactant and stabilizer. Cholesteryl acetate was used as a substrate. The biosensor response was linear up to 12.95 mM with minimum detection limit of 1.3 mM. No significant interfererences were found for the measurement of 5 mM cholesteryl acetate in the presence of ascorbic acid, glucose, uric acid, EDTA, acetone and bilirubin and acetaminophen at their physiological or therapeutic levels. Once again there was no report on the application of biosensor to the direct measurement of TC in serum samples (Fang, 2011).

In summary, the TC biosensor was used to measure TC in delipidated serum with good linearity across the diagnostically relevant range. It was applied to the measurement of TC in clinical serum samples. Although measurement of TC was more straight forward than HDL-C measurement due to the fact that there was no requirement for differentiation between different lipoproteins, challenges remain in the precision and accuracy of the developed biosensor.

5.4 Conclusion

To sum up, while most of the spectrophotometric assay protocols using externally mixed reagents and sample with a typical minimum 100-fold dilution of sample with assay reagents, the biosensor using the modified electrodes developed in this research employed only a two-fold dilution as the ultimate aim of this thesis is to develop a fully integrated TC biosensor. Therefore, an amperometric assay based on optimised HDL-C assay reagents as mentioned in Chapter 3 and using DBSA/KCl modified silver electrodes was developed which was used for the quantitative measurement of H_2O_2 and cholesteryl acetate in PBS and for HDL-C in both PBS and delipidated serum. The same strategy was also applied to the measurement of TC in delipidated serum using the same electrodes. A study on the possibility of replacing the DBSA/KCl modification of the electrodes with either Emulgen B-66/PBS for HDL-C and Triton X-100/PBS for TC demonstrated that both exhibited acceptable enhancement in catalysis of the silver electrodes for H_2O_2.

Two types of biosensors were developed which were based on the external mixing of assay reagents (buffer, surfactants and enzymes) with the sample prior to their measurement using the modified electrodes. Biosensors employing modification

and measurement in 6% (w/v) Emulgen B-66/PBS with 39 U/mL ChEs and 23 U/mL ChOx were applied to the measurement of HDL-C (0 to 4 mM) in delipidated serum. The biosensor yielded a slope of 3.32×10^{-8} A/mM with a linearity (r^2) of 0.999 and average RSD of 9.5% (n=5).

Biosensors employing modification and measurement in 0.5% (v/v) Triton X-100/PBS with 156 U/mL ChEs and 60 U/mL ChoX were used for the measurement TC (0 to 10 mM) in delipidated serum. The biosensor had a sensitivity of 2.24×10^{-8} A/mM with linearity (r^2) up to 10 mM of 0.984 and average RSD of 10.8% (n=3).

Both biosensors were employed for the measurement of HDL-C and TC in clinical samples with calibration curves prepared using calibrators provided by Alere to provide a comparable sample matrix. The correlation between the HDL-C sensor and the Abcam assay kit had a slope of 0.87 and a Pearson correlation coefficient of 0.76 (n=13) (Fig. 5.24 and Table 5.9) while the correlation for TC measurement had a slope of 1.07 and a Pearson correlation coefficient of 0.87. However, significant deviation was observed for amperometric measurement of TC in donor samples which were un-manipulated 'off the clot' serum samples from a donor population in San Diego calculated by interpolation on the linear region of the calibration curve which was prepared using calibrators which were spiked human serum samples. Considering that there are still challenges in real sample measurement and method correlation with other standard methods, the next step was to fabricate printed integrated biosensors that could more effectively measure TC and HDL-C which will be discussed in next chapter.

References

Allain, C.C., Poon, L.S., Chan, C.S.G., Richmond, W. and Fu, P.C. (1974) Enzymatic determination of serum total cholesterol. *Clinical Chemistry*. 20 (4), pp. 470-475.

Arranz-Peña, M.L. (1998) Comparison of two homogeneous assays with a precipitation method and an ultracentrifugation method for the measurement of HDL-cholesterol. *Clinical Chemistry*. 44 (12), pp. 2499.

Arya, S.K., Datta, M. and Malhotra, B.D. (2008) Recent advances in cholesterol biosensor. *Biosensors and Bioelectronics* . 23 (7), pp. 1083-1100.

Bairaktari, E.M., Elisaf, A., Katsaraki, V., Tsimihodimos, A.D., Tselepis, K., Siamopoulos, C. and Tsolas, O. (1999) Homogeneous HDL-Cholesterol Assay Versus Ultracentrifugation/Dextran Sulfate-Mg2+ Precipitation and Dextran Sulfate-Mg2+ Precipitation in Healthy Population and in Hemodialysis Patients. *Clinical Biochemistry*. 32 (5), pp. 339-346.

Batki, A.D., Holder, R., Hughes, L., Nayyar, P. and Thorpe, G.H.G., (2005) *Polymer Technology Systems CardioCheck PA Lipid Monitoring System.* Report number: 05051.NHS purchasing and supply agency, Centre for evidence-based purchasing evaluation.

Batki, A.D., Thomason, H.L., Holder, R. and Thorpe, G.H.G., (1995) *Cholestech LDX Lipid Analyser*. Report number: MDA/95/23.Medical device agency evaluation.

Bland, J.M. and Altman, D.G. (1999) Measuring agreement in method comparison studies. *Statistical Methods in Medical Research.* 8, pp. 135-160.

Cobbaert, C., Zwang, L., Ceriotti, F., Modenese, A., Cremer, P., Herrmann, W., Hoss, G., Jarausch, J. and Nauck, M. (1998) Reference standardization and triglyceride interference of a new homogeneous HDL-cholesterol assay compared with a former chemical precipitation assay. *Clinical Chemistry*. 44 (4), pp. 779-789.

Contois, J.H., (2012) *A Critical Review of LDL Cholesterol and HDL Cholesterol Measurement*. Sun Diagnostics, LLC.

Copeland, R.A. (2000) *ENZYMES: A Practical Introduction to Structure, Mechanism and Data Analysis*. Second edition ed. New York: Wiley-VCH, Inc.

Deacon, A.C. and Dawson, P.J.G. (1979) Enzymic Assay of Total Cholesterol Involving Chemical or Enzymic Hydrolysis-A Comparison of Methods. *Clinical Chemistry*. 25 (6), pp. 976-984.

Dey, R.S. and Raj, C.R. (2014) Enzyme-integrated cholesterol biosensing scaffold based on in situ synthesized reduced graphene oxide and dendritic Pd nanostructure. *Biosensors and Bioelectronics*]. 62 (0), pp. 357-364.

Eastoe, J. (2010) Surfactant Aggregation and Adsorption at Interfaces In: Cosgrove, T., ed. (2010) *Colloid Science: Principles, Methods and Applications*. John Wiley & Sons, pp. 61.

Fang, C. (2011) A disposable amperometric biosensor for determining total cholesterol in whole blood. *Sensors and Actuators B: Chemical*. 155 (2), pp. 545-550.

Foster, R., Cassidy, J. and O'Donoghue, E. (2000) Electrochemical Diagnostic Strip Device for Total Cholesterol and Its Subfractions. *Electroanalysis.* 12 (9), pp. 716-721.

Gonzalez-Macia, L., Smyth, M.R. and Killard, A.J. (2011) Enhanced electrochemical reduction of hydrogen peroxide at metallic electrodes modified with surfactant and salt. *Electrochimica Acta*. 58, pp. 562-570.

Gonzalez-Macia, L., Smyth, M.R. and Killard, A.J. (2012a) Evaluation of a silver-based electrocatalyst for the determination of hydrogen peroxide formed via enzymatic oxidation. *Talanta*. 99, pp. 989-996.

Gonzalez-Macia, L., Smyth, M.R. and Killard, A.J. (2012b) A Printed Electrocatalyst for Hydrogen Peroxide Reduction. *Electroanalysis*. 24 (3), pp. 609-614.

Grennan, K., Killard, A.J. and Smyth, M.R. (2001) Physical Characterizations of a Screen-Printed Electrode for Use in an Amperometric Biosensor System. *Electroanalysis*. 13 (8), pp. 745-750.

Harris, N., Galpchian, V., Thomas, J., Iannotti, E., Law, T. and Rifai, N. (1997) Three generations of high-density lipoprotein cholesterol assays compared with ultracentrifugation/dextran sulfate-Mg2+ method. *Clinical Chemistry*. 43 (5), pp. 816-823.

Helm, I., Jalukse, L. and Leito, I. (2010) Measurement Uncertainty Estimation in Amperometric Sensors: A Tutorial Review. *Sensors*. 10, pp. 4430-4455.

Hutchings, I.M. and Martin, G.D. (2013) *Inkjet Technology for Digital Fabrication*. 1st ed. UK: John Wiley & sons Ltd.

Ignatius, M., Emeka, N., Ebele, I., Chinelo, M., Fidelis, E. and Sila, U. (2009) The Effect of Sample Storage on Total Cholesterol and Hdl-cholesterol Assays. *Current Research Journal of Biological Sciences*. 1 (2), pp. 1-5.

Jeroschewski, P., Steuckart, C. and Kulhl, M. (1996) An Amperometric Microsensor for the Determination of H2S in Aquatic Environments. *Analytical Chemistry*. 68, pp. 4351-4357.

Kinoshita, H., Torimura, M., Kano, K. and Ikeda, T. (1998) Amperometric determination of high-density lipoprotein cholesterol using polyethylene glycol-modified enzymes and a peroxidase-entrapped electrode. *Annals of Clinical Biochemistry*. 35 pp. 739-744.

Kondo, A. (1999) Dynamic reaction in a homogeneous HDL-cholesterol assay visualized by electron microscopy. *Clinical Chemistry*. 45 (11), pp. 1974.

Lackner, K.J. and Schmitz, G. (1998) β-VLDL of patients with type III hyperlipoproteinemia interferes with homogeneous determination of HDL-

cholesterol based on polyethylene glycol-modified enzymes. *Clinical Chemistry*. 44 pp .2546-2548.

Magdassi, S., ed. (2008) *The Chemistry of Inkjet Ink*. Singapore: World scientific.

Marquette, C.A., Ravaud, S. and Blum, L.J. (2000) Luminol electrochemiluminescence-based biosensor for total cholesterol determination in natural samples. *Analytical Letters*. 133 (9), pp. 1779-1796.

Miller, W.G., Myers, G.L., Sakurabayashi, I., Bachmann, L.M., Caudill, S.P., Dziekonski, A., Selvin, E., Kimberly, M., Korzun, W.J., Leary, E.T., Nakajima, K., Nakamura, M., Nilsson, G., Shamburek, R.D., Vetrovec, G.W., Warnick, G.R. and Remaley, A.T. (2010) Seven Direct Methods for Measuring HDL and LDL Cholesterol Compared with Ultracentrifugation Reference Measurement Procedures. *Clinical Chemistry*. 56 (6), pp. 977-986.

Murphy, L., Burrows, C., Bayly, S., Wilkinson, K. and Harris, J. (2009) *HDL Cholesterol Sensor using Selective Surfactant* Anon. US20090130696 A1.

Nakamura, M., Kayamori, Y., Sato, S. and Shimamoto, T. (2006) Lipid's standardization results of Japanese manufacturers by US cholesterol reference method laboratory network certification protocols and reagents' specificity and performance. In: Kramer, M.A., ed. (2006) *Focus on Cholesterol Research*. New York: Nova biomedical book, .

Nakamura, M., Taniguchi, Y., Yamamoto, M. and Manabe, M. (2009) *Method of Pretreatment of Sample for Quantitating Cholesterol and Method for Quantitating Cholesterol in Specific Lipoproteins.*Daiichi Pure Chemicals Co., Ltd. 20090226945.

Owiredu, W.K.B.A., Teye, E.K. and Quaye, L. (2013) Proficiency testing of total serum cholesterol assay by the ATAC 8000® random access chemistry auto analyzer at the Komfo Anokye teaching hospital. *Journal of Medical and Biomedical Sciences*. 2 (1), pp. 22-29.

Panz, V.R., Raal, F.J., Paiker, J., Immelman, R. and Miles, H. (2005) Performance of the CardioChek PA and Cholestech LDX point-of-care analysers compared to clinical diagnostic laboratory methods for the measurement of lipids. *Cardiovascular Journal of South Africa*. 16, pp. 112-117.

Patel, J.V., Thorpe, G.H., Springer, L. and Hughes, E.A. (2011) Accuracy and precision of point-of-care testing for serum cholesterol, triglycerides and HDL cholesterol. *Atherosclerosis*. 218 (2), pp. e8-e9.

Rifai, N., Warnick, R. and Dominiczak, M.H., eds. (2001) *Handbook of Lipoprotein Testing*. Washington: AACC Press.

Segrest, J.P., Jones, M.K., Loof, H.D. and Dashti, N. (2001) Structure of apolipoprotein B-100 in low density lipoproteins. *Journal of Lipid Research*. 42, pp. 1346-1367.

Tekin, E., Smith, P.J. and Schubert, U.S. (2008) Inkjet printing as a deposition and patterning tool for polymers and inorganic particles . *Soft Matter*. 4, pp. 703-713.

Tipton, K.F. (1992) *Principles of Enzyme Assay and Kinetic Studies*. Oxford, UK: .

Urmila, S., Madhuri, D., Barbora, A., Borthakur, L., Verma, M., Bora, A. and Goswami, U. (2011) Multiwalled carbon nanotube-based bi-enzyme electrode for total cholesterol estimation in human serum *Journal of Experimental Nanoscience,* 6 (1), pp. 84-95.

Valentine Charlton-Menys (2007) The robustness of the Roche 2nd generation homogenous HDL cholesterol (PEGME) method: Assessment of the effect of serum sample storage for up to 8 years at -80 °C. *Clinica Chimica Acta*. 382 (1-2), pp. 142-144.

Warnick, G.R. (1995) National Cholesterol Education Program recommendations for measurement of high-density lipoprotein cholesterol: executive summary. The National Cholesterol Education Program Working Group on Lipoprotein Measurement. *Clinical Chemistry*. 41 (10), pp. 1427.

Yamamoto, S., Yamamoto, M. and Nakanishi, K. (2011) *Method of Measuring Lipid in Specific Lipoprotein.*Daiichi Chemical Co., L. US 2011/0091917.

Zhang, X., Ju, H. and Wang, J. (2008) *Electrochemical Sensors, Biosensors and their Biomedical Applications*. first ed. USA: Elsevier.

Chapter 6

Development of integrated HDL-C and TC biosensors

6.1 Introduction

Monitoring HDL-C and TC using point of care testing provides several advantages compared to clinical laboratory testing including ease of use, patient accessibility, low cost and instant results. However, currently there are just two certified point of care cholesterol monitoring devices available in the UK which are capable of measuring TC and HDL-C which may be used in the doctor's office, clinic or home. As was discussed in Chapter 3, most of the cholesterol point of care testing devices are based on Trinder reaction for the optical determination of TC (Taylor, 2004). However, in the last ten years, biosensors have gained much more attention in the development of TC point of care devices (Arya, Datta and Malhotra, 2008).

In order to develop a disposable biosensor suitable for POCT, an integrated biosensor ideally needs to be fabricated by deposition of assay reagents on the sensor in a way that results in the controlled, reproducible interaction of assay components in both time and space, which is achieved by using assay reagents that result in uniform and stable enzyme activity and assay components diffusion rates, etc. Thus, the ability of the method to re-solubilise the deposited reagent and provide reproducible and stable responses is also crucial. Although there are many reports of the measurement of TC using amperometric biosensors (Arya *et al.,* 2008), there are just few reports of the amperometric determination of HDL-C (Foster, Cassidy and O'Donoghue, 2000; Kinoshita *et al.,* 1998; Murphy *et al.,* 2009). An important step in the fabrication of biosensors is finding simple and suitable techniques to deposit the assay reagents in a reproducible way. Among deposition techniques, inkjet-printing is one such technique capable of the controlled deposition of small droplets of reagents onto a substrate in a simple, rapid and reproducible manner, which is suitable for the mass production of biosensors (Komuro *et al.,* 2013; Tekin, Smith and Schubert, 2008). In addition, graphical software can be used for printing different pattern depending on the electrode design.

In this chapter, the effect of direct deposition of assay reagents using inkjet-printing technology on the amperometric behaviour of the resulting integrated biosensor while measuring HDL-C and H_2O_2 was investigated. Enzymes are biocatalysts in which their activities may differ once deposited compared to those in solution due to irreversible protein misfolding which may occur when water molecules are removed from the protein following immobilisation (Bommarius and Payeb, 2013).

The mode of deposition may also alter their stability, activity or selectivity, and so changes in assay reagent (ink) formulation may be required to improve the enzymes activity and stability as well as ink characteristic for its suitability to be inkjet-printed and immobilised (Bommarius and Payeb, 2013; Komuro *et al.,* 2013; Rodrigues *et al.,* 2013). In order to stabilize enzymes, there are several ways such as immobilisation, medium engineering (formulation), protein engineering. Among them, a simple enzyme immobilisation technique may be employed to prevent removal of water molecules and unfolding during the deposition step. Immobilisation techniques may be reversible such as adsorption, ionic binding, affinity binding, chelation or metal binding and disulphide bonding or irreversible such as the formation of covalent bond, entrapment, micro-encapsulation and cross-linking (Brena and Batista-Viera, 2006). Based on the fact that enzymes may possess a net positive or negative charge depending on their isoelectric point and the pH of the solution, using ionic interactions by introducing an oppositely charged mediator may increase the rigidity of the active site (Bommarius and Payeb, 2013; Hanefeld, Cao and Magner, 2013). Some optimisation of the assay reagent formulation to make it suitable for deposition and immobilisation in this manner was performed here, initially for the development of an integrated HDL-C biosensor. Understanding of these new assay conditions was then applied to the fabrication of the TC integrated biosensor. In addition to these sensor design issues, diffusional barriers are often used to limit the diffusion rate of substrates, effectively diluting the sample before measurement, However, in this research a direct measurement approach was taken to eliminate the need for diffusional barriers, as this approach had been previously shown to be successful for the colorimetric measurement of HDL-C (Tamura *et al.,* 2009).

6.2 Development of an integrated HDL-C biosensor

It was shown in Chapter 5 that Emulgen B-66/PBS modified electrodes were suitable for the amperometric measurement of H_2O_2 following the enzymatic reaction of HDL-C using externally mixed assay reagents. However, to develop a point of care biosensor, the next step was to remove the assay reagent addition step through their deposition on the electrode surface in a reproducible and stable manner. Therefore, the first attempt was to apply assay reagent formulated in Chapter 3 and 4 and used in Chapter 5, directly onto the electrode surface to evaluate its effect on the amperometric measurement of HDL-C.

6.2.1 Inkjet-printed deposition of HDL-C assay reagents on Emulgen B-66/PBS modified electrodes

Initial experiments using 3 µL of assay reagent deposited using drop-coating onto the sensor working electrodes resulted in the formation of a surface film after being kept at room temperature for 2 hrs to dry which can be seen in Fig. 6.1

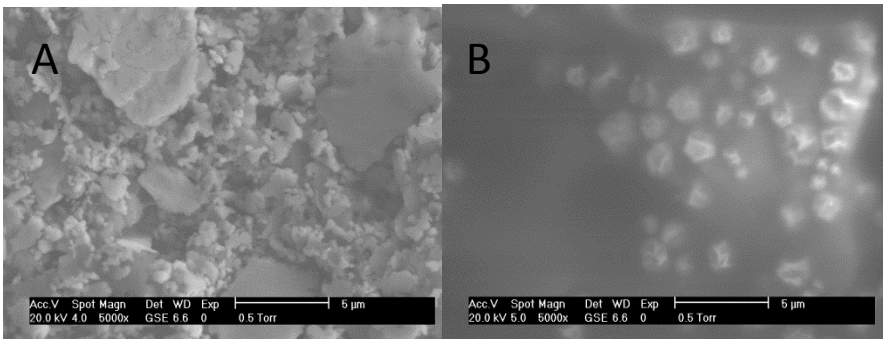

Fig. 6.1. SEM images of modified electrodes: A before drop-coating of assay reagent and B after deposition. Acceleration voltage of 20 keV. (5000x magnification). using GSE detector.

Consequently, the experiments were performed to evaluate the effect of the inkjet-printed deposition of assay reagents on the amperometric measurement of HDL-C. Therefore, ChOx (122 U) and ChEs (261 U) were dissolved in 2 mL 6% (w/v) Emulgen B-66/PBS solution to yield final concentrations of 130.5 U/mL for ChEs and 62 U/mL for ChOx and this was inkjet printed onto a modified electrode. First, modified electrodes were prepared by inkjet printing of five layers of 6% (w/v) Emulgen B-66/PBS on a single silver electrode (3.2 × 3.2 mm) and then employed

for HDL-C determination unless otherwise stated. All the modified electrodes were dried for at least 10 min and washed prior to the assay reagent deposition step.

6.2.1.1 Optimisation of print layers

The number of layers of printed reagent was investigated. Initially, 10 layers of assay reagent with the total volume of assay reagent of 2.38 µL were inkjet-printed. This was calculated using a droplet volume of 9.2×10^{-6} µL which was experimentally obtained using drop volume calibration feature and 3.2 × 3.2 mm pattern representing 25,921 droplets. A single silver electrode was used for deposition of assay reagent (Fig. 2.6 A). This was then used following encapsulation to measure 8 µL of 0.5 to 4 mM HDL-C in delipidated serum. The cathodic current at -0.1 V (vs Ag/AgCl) was measured at 360 s and 420 s following 240 s incubation at room temperature. A minimum of two repeats were performed at each concentration.

Fig. 6.2 shows that in general by increasing the concentration of HDL-C, the current increased proportionately. However, some variability was observed in the case of some of the encapsulated biosensors which might be due to some variability caused by the silver electrodes and their modification characteristics as well as the homogeneity of final solution after re-solubilisation and enzymatic reaction. The current measured at 360 s and 420 s showed almost the same signal demonstrating that measurement could be performed at either of these two time points. Further assays were measured after 420 s.

A comparison between the amperometric measurement of HDL-C using externally mixed reagents performed in Chapter 5 (Section 5.2.2) and the integrated biosensor shown here showed that the sensitivity decreased from 3.32×10^{-8} A/mM to 1.76×10^{-8} A/mM, with a loss of linearity (r^2) from 0.999 to 0.745 which may be due to rates of re-solubilisation, changes in rates of diffusion or effects on enzyme activity following deposition. As can be seen in Table 6.1, the percentage error increased by 2.2-fold using the integrated biosensor, as compared to externally added reagent which could also be due to loss of enzyme activity over time or an inability to solubilise the deposited reagent in a reproducible manner as well as homogeneity of the final solution following storage at room temperature after deposition. Variability from the modified silver electrodes may also contribute to this.

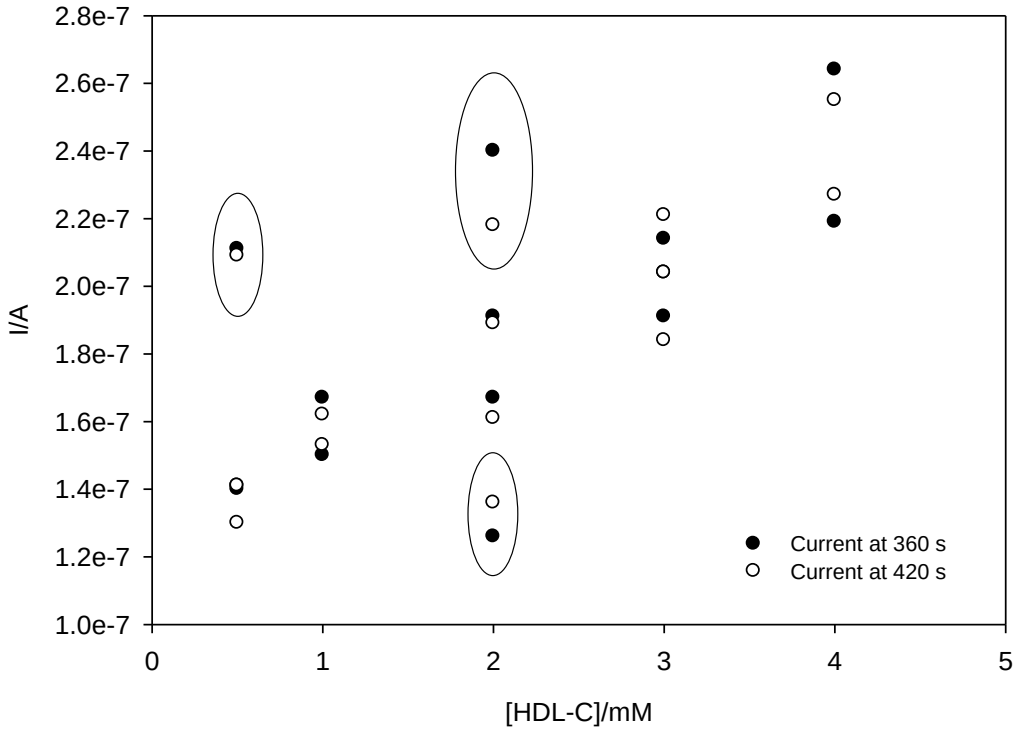

Fig. 6.2. Chronoamperometric measurement (-0.1 V vs Ag/AgCl) of HDL-C using encapsulated HDL-C biosensors prepared using 10 layers of assay reagent inkjet-printed on Emulgen/PBS modified silver electrodes. The current was measured at 420 s after 240 s incubation; (slope=1.76×10^{-8}A/mM, r^2=0.745).

Table 6.1. Detailed analytical data for HDL-C measurement following inkjet-printed of 10 layers of assay reagent on Emulgen/PBS modified silver electrodes.

HDL-C/ mM	Average current at 420 s (A)	SD	RSD (%)
0.5	1.61×10^{-7}	4.39×10^{-8}	27.3
1	1.90×10^{-7}	5.70×10^{-8}	30.0
2	1.87×10^{-7}	3.99×10^{-8}	21.3
3	1.90×10^{-7}	3.12×10^{-8}	16.4
4	2.41×10^{-7}	1.98×10^{-8}	8.2
Average RSD			20.6

As was shown, by deposition of assay reagents using inkjet printing, analytical parameters such as linearity, sensitivity and reproducibility were altered significantly. This may be due to not only loss of enzyme activity, but also some changes in kinetics of the reaction taking place inside the encapsulated biosensor including the concentration verses time profiles of reaction products across and throughout the sample chamber during the measurement, the speed of reagent

dissolution inside the sample chamber and the resulting rates of formation of H_2O_2 and its concentration distribution and rate of diffusion to the surface, in the presence of all the assay and matrix components. The kinetics of the sensor are complex and are driven by multiple processes including fluid dynamics and convection of the sample filling the chamber, sample chamber geometry, reproducibility and surface properties, the rates of resolubilisation and rehydration of the enzymes and their concentration profiles as they diffuse into the sample, the kinetics of the enzymes as they interact with the multi-step pathway and the final kinetics of the formation and distribution of the generated H_2O_2, followed by its catalysis at the electrode surface. The result of this complexity is ultimately manifested in the quality of the chronoamperograms of the sensor. Minute variations in the properties of the electrodes that impact these processes introduce variability in response and the sensor reproducibility ultimately defines the robustness of the technique and how well it compares with other sensor systems. While removal of a biological molecule such as HRP is mostly considered advantageous due to the inherent issues related to the use of biological molecules, this must be counter-balanced by the fact that HRP is a highly efficient and well-behaved enzyme, and that the electrocatalytic modification may, itself, introduce variability into the assay system.

The first step in order to improve the analytical parameter of the method was to evaluate the number of prints considering that the deposition step may reduce the enzyme activity, causing a decrease in the sensitivity of the method. Therefore, the same experiments as mentioned above was performed but this time by inkjet-printing of 20 layers (4.77 µL) on single modified electrode representing double the enzyme concentrations compared to the previous experiment. This was done to see if double enzyme concentration will improve the sensitivity of the integrated biosensor (Fig. 6.3).

Although some variability could still be seen in sensors fabricated using 20 layers, a slight increase in sensitivity and linearity was observed in comparison with 10 layers suggesting that the increase in enzyme concentration improved the analytical performance of the biosensor. However the percentage error also increased to 32.9% for 20 printed layers compared to 20.6% observed for 10 layers (Table 6.2). This might be expected as by increasing the printed layers, the systematic error may increase resulting in the increased percentage error of the measurement. Moreover, when the sample is introduced into encapsulated

electrodes by capillary force, the deposited assay reagents must be dissolved in order to initiate the enzymatic reaction. Since the assay reagent was so far only deposited on the working electrode area, this may have had an affect on the homogeneity of the assay solution which may have led to the poor reproducibility of the integrated biosensor.

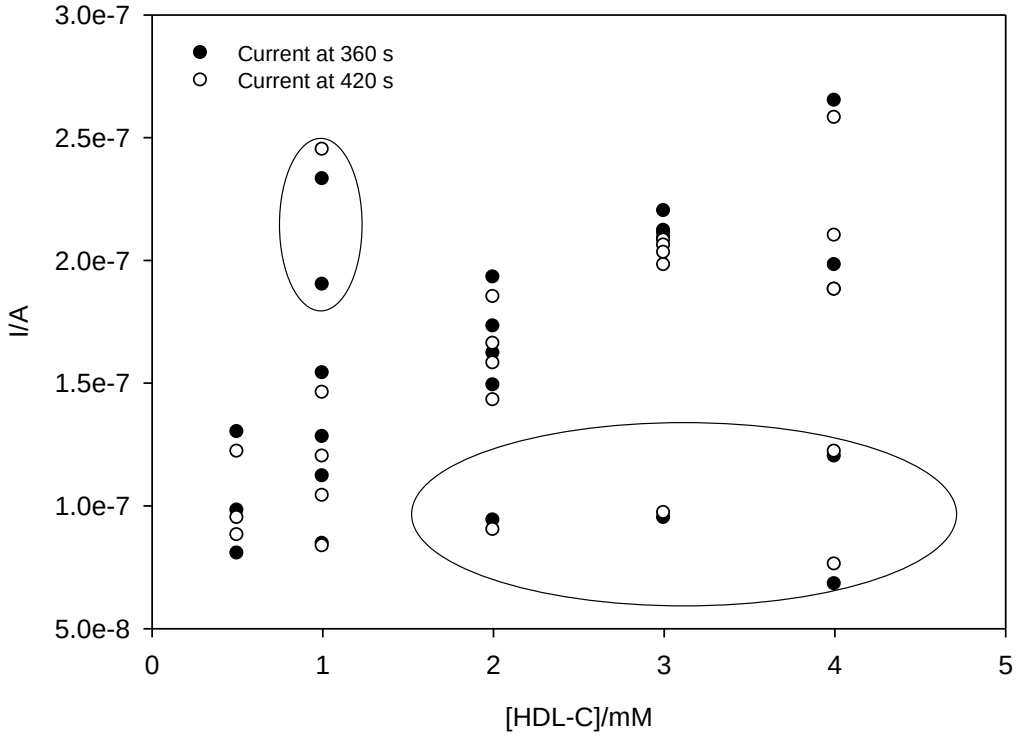

Fig. 6.3. Chronoamperometric measurement (-0.1 V vs Ag/AgCl) of HDL-C using encapsulated HDL-C biosensors using 20 layers of inkjet-printed assay reagent deposited on a single Emulgen b-66/PBS modified silver electrode, after 240 s incubation at room temperature, (slope=1.86×10^{-8}A/mM, r^2=0.782).

Table 6.2. Detailed analytical data of amperometric measurement (-0.1 V vs Ag/AgCl) of HDL-C using 20 layers of assay reagent deposited on single modified silver electrode (n ≥3).

HDL-C/ mM	Average current at 420 s (A)	SD	RSD (%)
0.5	1.05×10^{-7}	2.40×10^{-8}	22.9
1	1.40×10^{-7}	6.31×10^{-8}	45.2
2	1.46×10^{-7}	4.10×10^{-8}	28.1
3	1.82×10^{-7}	4.79×10^{-8}	26.3
4	1.71×10^{-7}	7.21×10^{-8}	42.2
Average RSD			32.9

6.2.1.2 Optimisation of the printed area

Since increasing the number of assay reagent layers on a single electrode from 10 to 20 showed some improvement in assay sensitivity and linearity but no improvement in reproducibility, the next experiment was performed using 10 layers of assay reagent inkjet-printing on the two silver electrodes (Fig. 2.6 B) to assess if any improvement in method reproducibility would be achieved by depositing the assay reagent in two sample chamber locations instead of one while the same concentration of deposited reagent was used (2.4.7). This may help to improve the product concentration/time profile across and throughout the sample chamber during the measurement as well as the speed of reagent dissolution inside the sample chamber. In addition this may improve the rate of H_2O_2 formation and its even diffusion to the surface resulting in better homogeneity of the final solution. The final total volume of assay reagent deposited was 4.77 µL which was very similar to that deposited using 20 layers on a single electrode and was double that of 10 layers deposited on a single electrode. The encapsulated biosensors were then used to measure HDL-C in delipidated serum. The results are shown in Fig. 6.4 and Table 6.3.

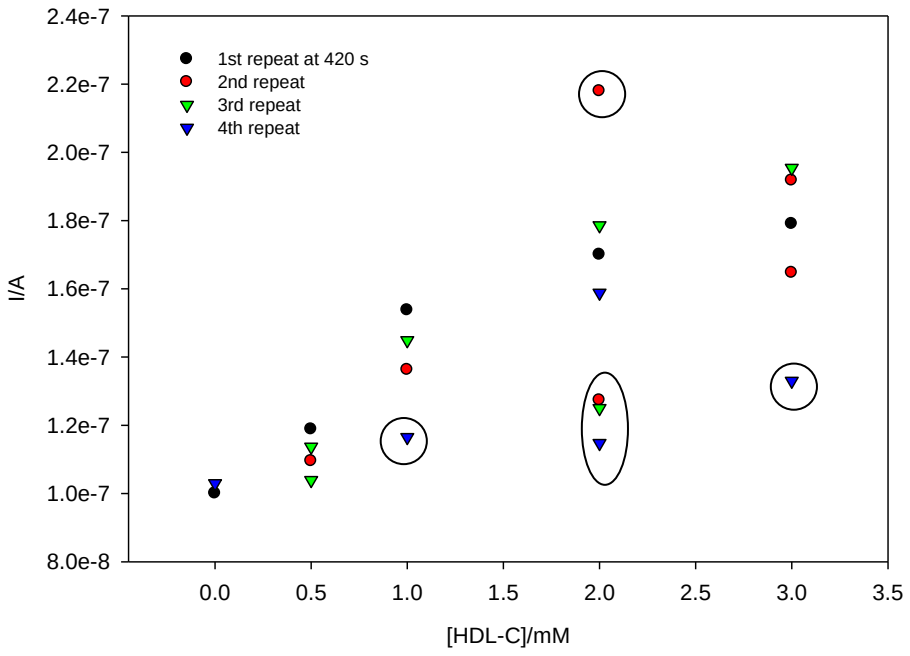

Fig. 6.4. Chronoamperometric measurement (-0.1 V vs Ag/AgCl) of HDL-C using encapsulated HDL-C biosensors using 10 layers of inkjet-printed assay reagent deposited on both silver electrodes, after 240 s incubation at room temperature, (slope=2.42×10^{-8}A/mM, r^2=0.958).

Table 6.3. Detailed analytical data of amperometric measurement of HDL-C using 10 layers of inkjet-printed assay reagents deposited on both silver electrodes (n ≥ 3).

HDL-C /mM	Average current at 420 s (A)	SD	RSD (%)
0.5	1.11×10^{-7}	6.32×10^{-9}	5.7
1	1.38×10^{-7}	1.59×10^{-8}	11.6
2	1.56×10^{-7}	3.66×10^{-8}	23.5
3	1.73×10^{-7}	2.53×10^{-8}	14.6
Average RSD			13.9

To assess whether this variability was due to the inhomogeneous diffusion of the released H_2O_2 following the introduction of the HDL-C using capillary force into the encapsulated biosensors, the same experiment was performed, with HDL-C substituted with H_2O_2 in delipidated serum (Fig. 6.5 and Table 6.5). Evaluation with H_2O_2 eliminates any variability caused by the enzymatic reaction steps and provides a sample solution with better homogeneity which requires only dissolution of the deposited assay reagents. Improved sensitivity (3.62×10^{-8} A/mM) was observed for the measurement of H_2O_2 compared to the measurement of HDL-C (2.42×10^{-8}A/mM), which may result from a range of causes such as the kinetics of the enzymatic reactions and rates of diffusion when measuring HDL-C compared to H_2O_2. Although no significant changes in linearity and reproducibility of the method were observed for the measurement of H_2O_2 compared to HDL-C measurement, the linearity and reproducibility of the externally mixed experiment for HDL-C measurement (r^2=0.999, RSD=9.5%; n=5) (Section 5.2.2) were better than the integrated biosensor used for amperometric measurement of H_2O_2 (r^2=0.956, RSD=12.6%; n=3) which might be due to some inhomogeneity caused following re-solubilisation of the deposited assay components by the H_2O_2 sample.

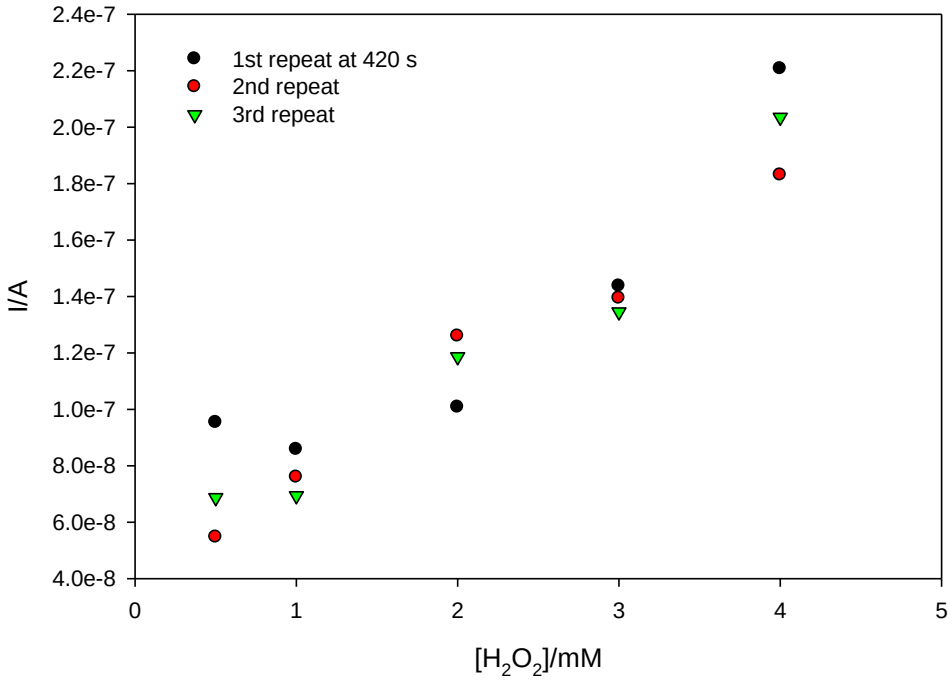

Fig. 6.5. Chronoamperometric measurement (-0.1 V vs Ag/AgCl) of H_2O_2 using encapsulated HDL-C biosensors using 10 layers of inkjet-printed assay reagent deposited on both silver electrode, after 240 s incubation at room temperature, (slope=3.62×10^{-8}A/mM, r^2=0.956).

Table 6.4. Detailed analytical data of amperometric measurement (-0.1 V vs Ag/AgCl) of H_2O_2 using 10 layers of inkjet-printed assay reagents deposited on both silver electrodes, n=3).

H_2O_2 /mM	Average current at 420 s (A)	SD	RSD %
0.5	7.29×10^{-8}	2.06×10^{-8}	28.3
1	7.70×10^{-8}	8.26×10^{-9}	10.7
2	1.15×10^{-7}	1.30×10^{-8}	11.3
3	1.39×10^{-7}	4.58×10^{-8}	3.3
4	2.02×10^{-7}	1.89×10^{-8}	9.3
Average RSD			12.6

In order to further improve assay reagent homogeneity, assay reagent was next inkjet-printed across an area of 15 × 8 mm (Fig. 2.6), instead of just onto the silver working electrodes as was shown in Chapter 2. This may help accessibility of introduced HDL-C sample to assay reagent following being introduced by capillary force in a homogeny and reproducible way. As was mentioned before, 10 layers of assay reagent deposited on silver electrodes represented 4.77 µL of assay reagent being deposited in total. By increasing the print area to 15 × 8 mm, the number of prints was reduced from 10 to two layers of assay reagent containing ChEs (261 U) and ChOx (122.2 U) prepared in 2 mL of 6% (w/v) Emulgen B-66/PBS. The use of

two layers on a 15 × 8 mm electrode surface represented 5.54 µL of assay reagent which was similar in volume to the previous two electrode print (4.77 µL) presenting 1.16-fold increase in enzyme concentration. Amperometric measurement of HDL-C using the developed sensors is shown in Fig. 6.6 and Table 6.5. As can be seen, the biosensors yielded a sensitivity of 2.06×10^{-8}A/mM and linearity (r^2) of 0.952 for four repeat measurements with an average RSD of 11.5% demonstrating a slight improvement in reproducibility of the integrated biosensors compared to those with printing on two electrodes (12.6%). Again little improvement in sensitivity or linearity was achieved.

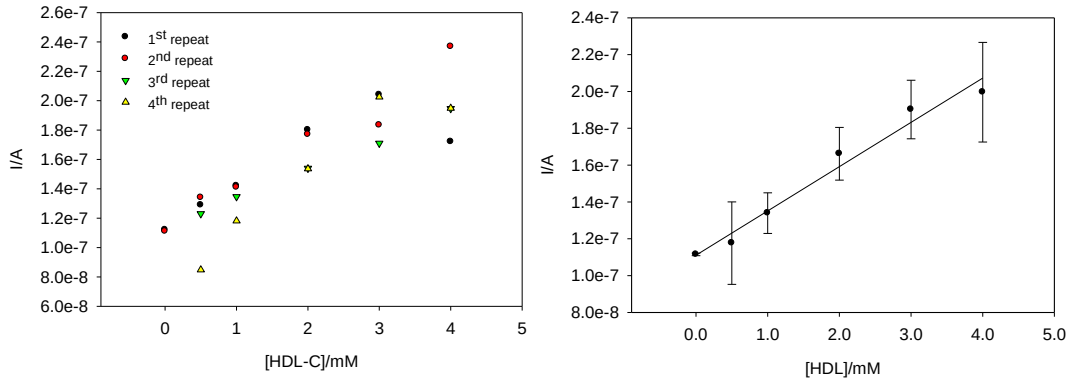

Fig. 6.6. Chronoamperometric measurement (-0.1 V vs Ag/AgCl) of HDL-C using encapsulated HDL-C biosensors using 2 layers of assay reagent inkjet-printed on 15 × 8 mm of Emulgen/PBS modified electrode, after 240 s incubation at room temperature, (slope=2.06×10^{-8}A/mM, r^2=0.952, n=4).

Table 6.5. Detailed analytical data of amperometric measurement (-0.1 V vs Ag/AgCl) of HDL-C using two layers of assay reagents on 15 × 8 mm of Emulgen/PBS modified electrode (n=4).

HDL-C /mM	Average current at 420 s (A)	SD	RSD %
0.5	1.18×10^{-7}	2.24×10^{-8}	19.0
1	1.34×10^{-7}	1.10×10^{-8}	8.2
2	1.66×10^{-7}	1.43×10^{-8}	8.6
3	1.90×10^{-7}	1.59×10^{-8}	8.4
4	2.00×10^{-7}	2.70×10^{-8}	13.5
Average RSD			11.5

To summarise, some of the main challenges in the development of the integrated biosensor was the variability observed in some of the encapsulated biosensors due to the silver electrodes and their modification characteristics as well as the homogeneity of the final solution after re-solubilisation and enzymatic reaction and therefore reproducibility of the integrated system. The rate of re-solubilisation and consequently the rates of diffusion of H_2O_2 and enzyme activity following deposition were also other ramification for development of integrated cholesterol sensor. Improved reproducibility was obtained following enzyme deposition using the pattern shown in Fig. 2.6C. However, further improvement in the linearity, sensitivity and reproducibility of the biosensor were still required. Since the amperometric measurement of H_2O_2 using the integrated biosensor showed similar sensitivity to that observed for HDL-C measurement when assay reagent was added externally (Section 5.2.2), and considering factors such as enzyme activity, reagent dissolution and sample filling characteristics which are all important factors in improving the analytical performance of an integrated biosensor, the catalytic activity of the enzymes was probably affected by the deposition and immobilisation processes which might be through different mechanisms such as reducing enzyme dynamic properties as well as accessibility of the substrate to enzymes active sites due to changes in their structure after immobilisation (Secundo, 2013). Further studies were performed, now employing deposition across the entire dual electrode cell to see whether the selectivity and stability was affected following deposition of assay reagent.

6.2.1.3 Measurement of the selectivity of the integrated biosensor for the measurement of HDL-C over LDL-C

It was shown in Chapter 5 that the Emulgen/PBS modified biosensor was capable of selectively measuring HDL-C over LDL-C using externally added reagents. To assess whether this remained the case using an integrated biosensor design, amperometric measurement of LDL-C (1 and 2 mM), HDL-C (2 mM) and combinations of LDL-C and HDL-C were performed using the encapsulated integrated biosensor. Two layers (total 5.54 µL) of assay reagent containing ChEs (261 U) and ChOx (122.2 U) prepared in 2 mL of 6% (w/v) Emulgen B-66/PBS inkjet-printed on a 15 × 8 mm electrode surface were used to prepare the integrated biosensor. Delipidated serum was used for control measurements.

As can be seen in Fig. 6.7, almost the same current response was observed for the amperometric measurement of 1 mM HDL-C in the presence or absence of 1 mM LDL-C, and which was half the current observed for 2 mM HDL-C after removing the background signal. LDL-C at 1 and 2 mM showed almost the same current as that observed for delipidated serum, confirming that the selectivity of the integrated biosensors toward HDL-C over LDL-C was not reduced in comparison the the externally added sensor approach.

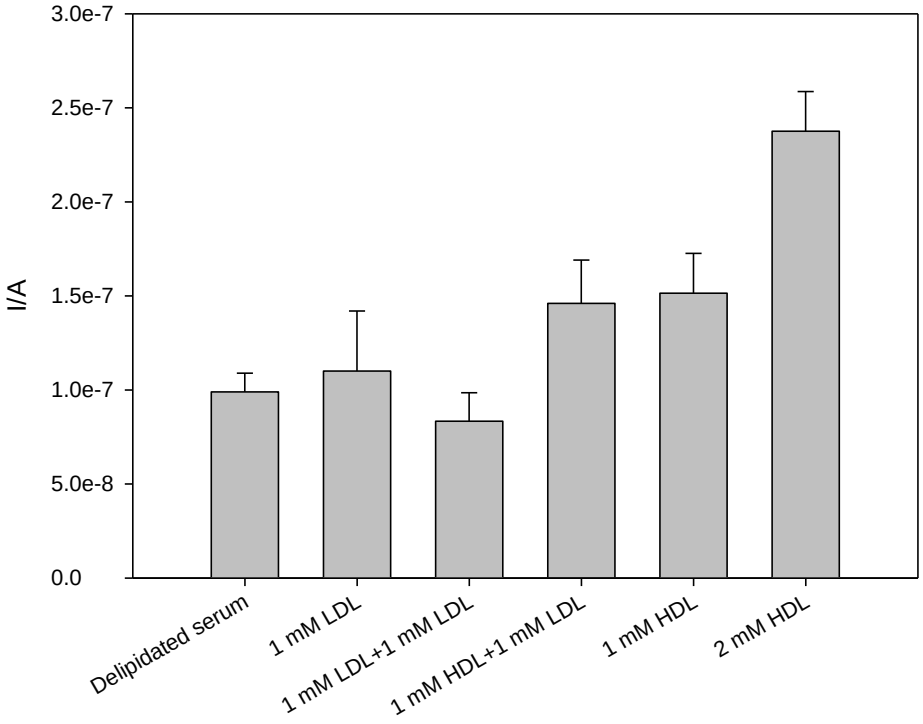

Fig. 6.7. Chronoamperometric measurement (-0.1 V vs Ag/AgCl) of LDL-C, HDL-C and combination of HDL-C and LDl-C using using two layers of assay reagent inkjet-printed on 15 × 8 mm of Emulgen/PBS modified electrode, after 240 s incubation at room temperature (t=420 s).

Table 6.6. Detailed analytical data of amperometric measurement (-0.1 V vs Ag/AgCl) corresponding to selectivity measurement of HDL-C over LDL-C at 420 using 2 layers assay reagents on 15×8 mm of Emulgen/PBS modified electrode (n=3).

Substrate	Average current at 420 s (A)	SD	RSD %
Delipidated serum	9.89×10^{-8}	9.98×10^{-9}	10.1
1 mM LDL	1.10×10^{-7}	3.19×10^{-8}	29.0
1 mM LDL+1mM LDL	8.33×10^{-8}	1.52×10^{-8}	18.2
1 mM HDL+1 mM LDL	1.46×10^{-7}	2.30×10^{-8}	15.8
1 mM HDL-C	1.51×10^{-7}	2.11×10^{-8}	14.0
2 mM HDL	1.71×10^{-7}	2.39×10^{-8}	13.9

6.2.2 Evaluation of biosensor stability

The next step was then to assess the biosensor stability over time after inkjet-printing of assay reagent. To do this, four sets of encapsulated biosensors were prepared using a single inkjet printed layer of assay reagent printed on 15 × 8 mm electrode surface. Three sets were tested immediately following fabrication, while the fourth was tested five hours later. Fig. 6.8 demonstrates a clear decrease in biosensor response following storage for five hours.

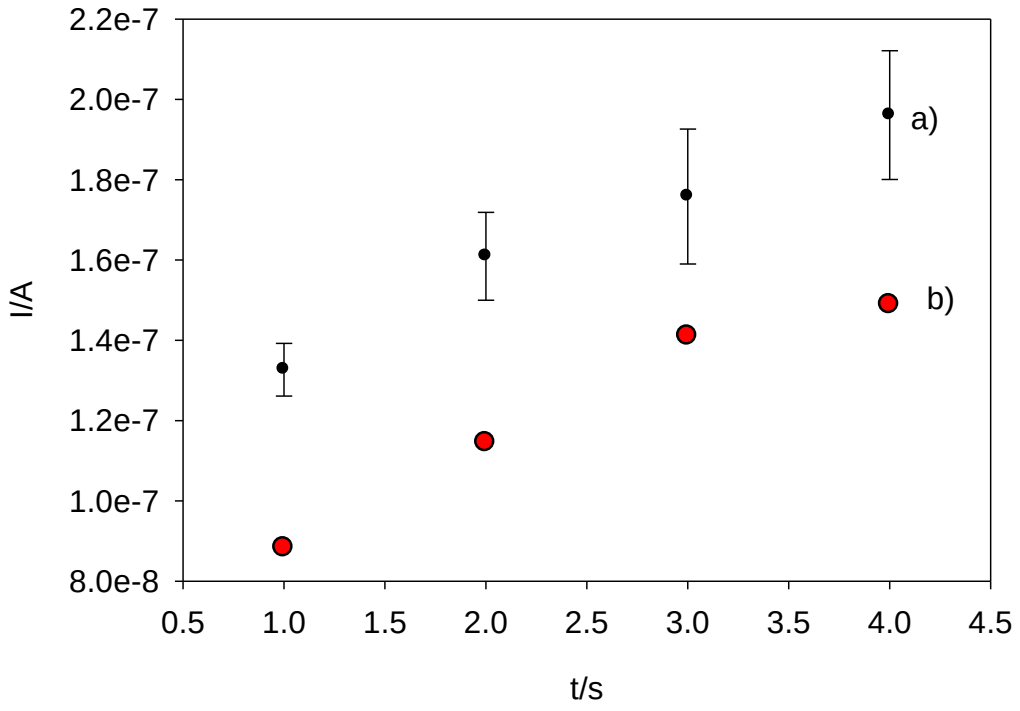

Fig. 6.8. Chronoamperometric measurement of HDL-C using encapsulated HDL-C biosensors inkjet-printed using one layer of assay reagent 15×8 mm^2 electrode surface after 240 s incubation at room temperature: a) 3 sets performed after printing (slope: 2.05×10^{-8}A/mM, r^2=0.984, n=3), b) set 4 performed 5 hrs after printing (slope: 2.08×10^{-8}A/mM, r^2=0.954).

The average current response of 1.96×10^{-7}, 1.76×10^{-7}, 1.61×10^{-7} and 1.33×10^{-7} A was observed for 4, 3, 2 and 1 mM HDL-C using three set of electrodes used after fabrication while these were 1.49×10^{-7}, 1.41×10^{-7}, 1.14×10^{-7} and 8.82×10^{-8} A for biosensors used five hours after fabrication showing 1.32, 1.25, 1.41 and 1.5-fold decrease in current intensity respectively. This could be due to some changes in surface modification related to the presence of enzymes deposited on the Emulgen/PBS modified electrode surface over time (Gonzalez-Macia, Smyth and

Killard, 2012) as well as reduction in enzyme activity due to the removal of water of hydration over time.

Since this experiment did not provide adequate information regarding the stability of biosensor, the following experiment was performed a week after printing by comparing the amperometric measurement of HDL-C performed on the same day of the preparation of the integrated biosensors with those performed after a week kept in desiccator at room temperature. As can be seen in Fig. 6.9, the current response of 8.93×10^{-8}, 1.57×10^{-7} and 2.03×10^{-7} A were observed for 0.5, 2.0 and 4.0 mM HDL-C using biosensors printed and used in the same day while this was between 5.3 $\times10^{-8}$ and 9.08×10^{-8} A for all HDL-C concentrations using biosensors kept at room temperature for a week showing almost the same current response for different concentrations of HDL-C.

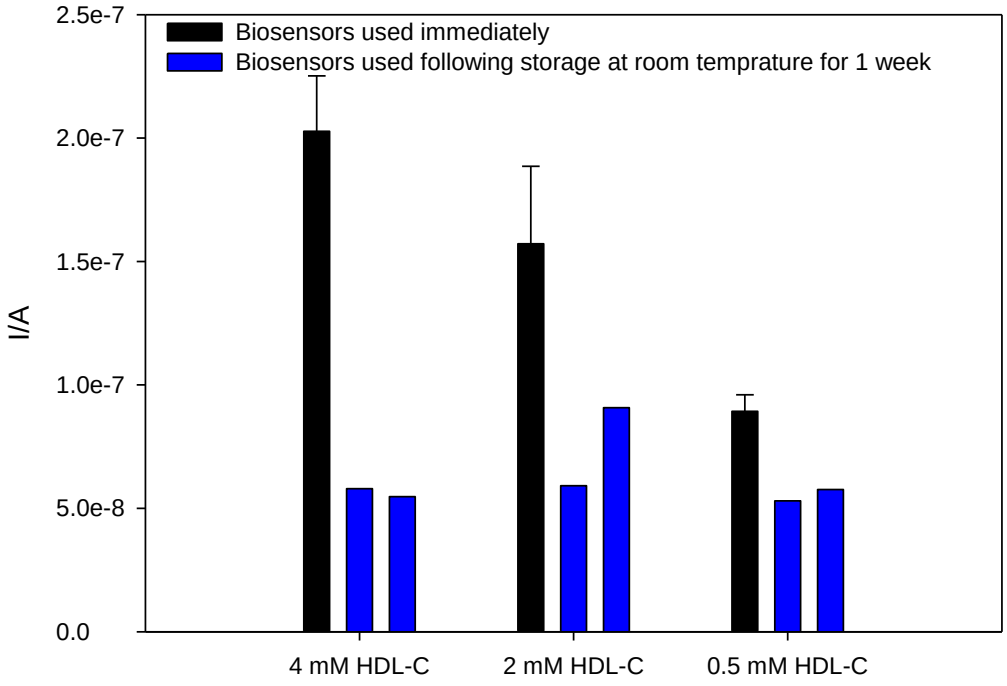

Fig. 6.9. Amperometric measurement of HDL-C (-0.1 V vs Ag/AgCl) using the developed biosensor used on the day of fabrication (black) and after one week in a desiccator at room temperature.

The catalytic activity of enzymes depends on the correct position and geometry of specific amino acids responsible for the enzyme's action towards a given substrate. Deposition, followed by removal of water molecule over time when stored at ambient temperature may alter the enzyme's structure and consequently affect its catalytic activity by reducing substrate accessibility to the enzyme active site, loss

of enzyme dynamic properties and alteration of conformational integrity (Secundo, 2013; Tsou, 1993). Enzyme conformation plays a crucial role in enzyme catalytic activity, which may change at low temperatures and temperatures above its unfolding temperature due to dehydration (Rodrigues *et al.,* 2013; Secundo, 2013). It has been shown that the addition of reagents containing a sugar, PEG or albumin into ink formulations before deposition appears to protect the enzyme from the negative effects of organic solvents as well as influencing the positive disruption of water molecules in the enzyme and protecting them against the shear produced during droplet ejection during inkjet-printing (Hanefeld, Cao and Magner, 2013).

As was mentioned above, although the selectivity of the integrated biosensor was not affected by the deposition and immobilisation processes, the biosensor demonstrated poor stability when stored at room temperature following deposition and drying step. Since protected enzymes are expected to show less alteration in their structure compared to the free enzymes once deposited (Hanefeld, Cao and Magner, 2013; Rodrigues *et al.,* 2013), it was necessary to re-formulate the ink composition in a way to protect the enzymes before deposition. The influence of storage temperature on integrated biosensor stability was also assessed.

6.2.3 Optimisation of biosensor stability

Inkjet printing has been reported to be suitable in overcoming issues such as speed limitation and reagent waste in comparison to traditional reagent deposition techniques such as screen printing and drop deposition (Delaney, Smith and Schubert, 2009; Hutchings and Martin, 2013). However, developing a robust formulation for the assay reagents to make them suitable for printing and minimise the impact on enzyme structure and activity has been shown to be challenging (Jospeh, 2012; Magdassi, 2008). An important consideration in drop-on-demand inkjet printing is whether the printable ink formula can be ejected from the nozzle in a reproducible and stable manner (Delaney, Smith and Schubert, 2009). Ink physical properties such as viscosity and surface tension are important. It has been shown that the viscosity of the ink does not change in the presence of enzymes at the typical concentrations used (Hutchings and Martin, 2013; Jospeh, 2012). An important issue was whether features of the printing process such as the shear produced during droplet ejection damages the protein structure. The rapid and intense pressure pulse set up by piezoelectric printers to eject a drop may result in denaturation of enzymes, the effect of which can be reduced by the addition of

stabilisers such as sugars which are polar compound helping enzyme to preserve its hydration state while it could also protect enzymes from shear stress produced by inkjet-printing (Hutchings and Martin, 2013). The precise mechanism by which some enzyme denatured in the absence of stabilizers through inkjet-printing is not fully understood (Delaney, Smith and Schubert, 2009).

Some of the important factors in developing an ink formulation for inkjet printing enzymes are as follows (Jospeh, 2012):

- It was reported that most enzymes were capable of tolerating the shear produced by the cartridge head without losing their activity during printing procedure.
- It is important that the ink formulation containing enzymes stabilises the enzyme structure to protect them from un-folding due to the drying step and storage condition.
- There should be no cracking or flaking once the reagent film is formed.
- Surface tension was also found to be important as when it is too high it does not allow proper mixing of the printed reagent droplets and when it is too low it results in the spreading of the reagent film as well as leakage from the nozzle.
- The surfactant present in the ink is also important as ionic surfactants may damage enzyme activity due to their amphiphilic characteristics. However, surfactants may decrease the surface tension and are found to be compatible with the enzyme/mediator system
- Polymers used in the ink formulation may have an effect on the homogeneity of the reagent layer (Iyer and Ananthanarayan, 2008).

Therefore, an optimal ink formulation may contain ingredients such as a plasticiser like ethylene glycol, a polymer to act as a film former, rheological modifiers such as polyvinylpyrrolidone (PVP) (Tamura *et al.,* 2009), a surfactant such as Triton X-100, a buffer such as phosphate buffer, enzymes and an ionic strength modifier such as KCl or NaCl (Jospeh, 2012).

As was shown in the previous section, direct inkjet-deposition of the developed assay reagent did not show sensitivity, reproducibility or linearity comparable to that observed for externally added reagent. In addition, the integrated biosensor did not show good shelf-life when stored at room temperature. This might have been expected as enzymes can be unstable in aqueous solution at room temperature in the absence of stabilizers and are typically required to be stored in a frozen state or liquid at a minimum of -20°C. Protecting enzymes while present in solution and prior to deposition may prevent them from denaturation after inkjet-print deposition. By changing the storage condition and storing the biosensor at 2-8°C, the shelf-life and operational stability of the integrated biosensor might be improved (Gibson, 1999). When proteins (e.g., enzymes) unfold or denature, their structure changes which may effect in the arrangement of its active site resulting in loss of activity. Several approaches such as introducing some additives to the ink formula, use of solvents and water modifiers such as sugars and polyhydric alcohols and protein engineering techniques have been used for the protecting of enzymes. The deposition process in conjunction with the optimised ink formulation and storage condition may lead to a more stable integrated biosensor (Rosier, Cruz and Wilkosz, 2001). One enzyme stabilisation technique is based on the electrostatic assembly of oppositely charged polyelectrolytes (Gibson, 1999). Enzymes are polyelectrolytes where their charge is dependent on their isoelectric point (IP) and the solution pH (Kang *et al.,* 2014). ChEs from *Pseudomonas* sp. and ChOx have isoelectric points of less than 6, which is below the assay reagent pH of 6.8, resulting in the enzymes being negatively charged which can be used as polyanion under these experimental conditions (Schuurmans Stekhoven, Gorissen and Flik, 2008). Enzymes are thus able to make an electrostatic bond with a polycation film without any complex chemical reactions leading to strong and rigid enzyme structure which is linked to enzyme activity (Ram *et al.,* 2001; Rodrigues *et al.,* 2013). In this work, poly(dimethyldiallyammonium chloride) (PDA) was used as a hydrophilic polycation to prepare an enzyme-polyelectrolyte surface. PDA has been reported as a suitable polycation for use in the assembly of enzyme electrodes using the layer-by-layer deposition technique (Eguílaz *et al.,* 2011; Kang *et al.,* 2014).

As was mentioned above, the other component which is important in the formation of a homogeneous reagent film is a polymer. Several polymers such as Natrosol 250 LR, polyvinylpyrrolidone (PVP) K25 and K30 and Aquazol 50 have been investigated as film formers for inkjet deposition of reagents. Among them, both

PVP K25 and K30 were found to be suitable film formers, capable of being inkjet-printed. Both possess good solubility in all conventional solvents and have adhesive power with good affinity to hydrophilic and hydrophobic surfaces (Jospeh, 2012).

Kang *et al.,* (2014) reported the use of 1% (w/v) PDA for layer-by-layer assembly of enzyme electrodes and 0.5% (w/v) PVP K30 was reported to be suitable and reliable for inkjet-printing (Jospeh, 2012). Studies were performed here to optimise the PDA and PVP K30 concentrations to make them suitable for combination with the modified silver electrodes with minimal impact on electrocatalysis. In order to optimise concentration, solution phase experiments were performed using Emulgen/PBS modified silver electrodes and the electrode responses to 4 mM H_2O_2 in 6% (w/v) Emulgen B-66 was assessed. Polymers were assessed individually and in combination. Cyclic voltammograms (Fig. 6.10) demonstrated a slight decrease in non-Faradic current in the presence of 1% (w/v) PDA, showing a slight negative effect on the Emulgen B-66/PBS surface modification. No other interferences were observed in the presence of PDA at these applied potentials.

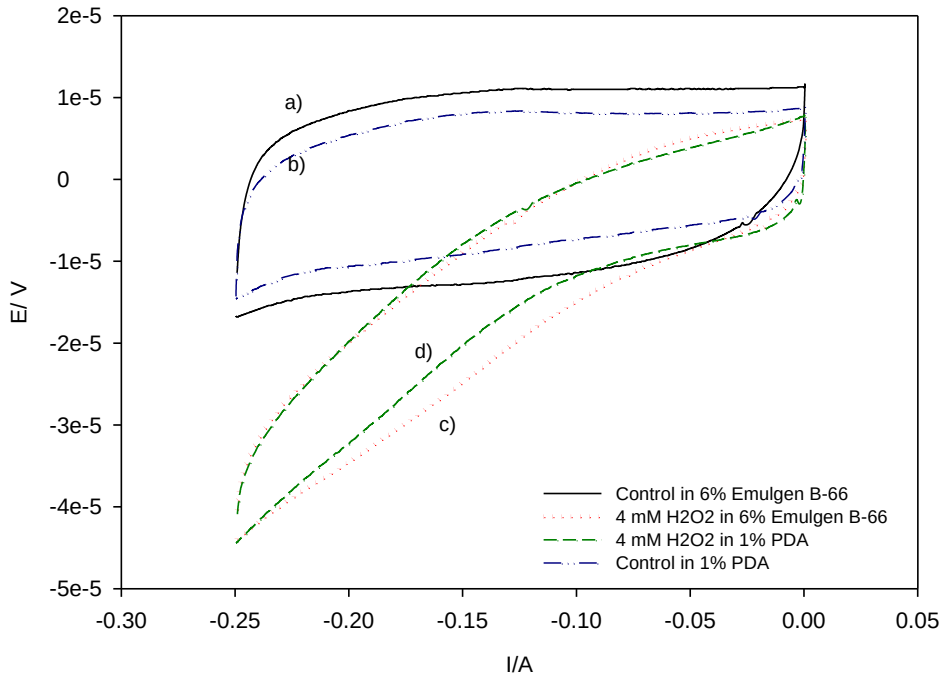

Fig. 6.10. Cyclic voltamogram of modified electrodes in a) 6% (w/v) Emulgen B-66, b) 1% (w/v) PDA, c) 4 mM H_2O_2 in 6% (w/v) Emulgen B-66 and d) 4 mM H_2O_2 in 1% (w/v) PDA, delipidated serum was used for control experiments.

Following this, the effect of PDA (0.1 to 1.0% (w/v) prepared using 0.1 M PBS pH 6.8) on the amperometric measurement of 4 mM H_2O_2 on Emulgen B-66/PBS

modified electrodes in un-stirred batch solution was evaluated. As is shown in Fig. 6.11, the current observed was elevated in the presence of 0.1, 0.2 and 0.3 % (w/v) PDA, showing some positive effect on catalytic activity of the modified electrode. However, further increases in PDA concentration led to significant decreases in current. This might be due to some disruption of the Emulgen/PBS surface structures at higher concentrations. A concentration of 0.2% (w/v) PDA was used for any further formulation.

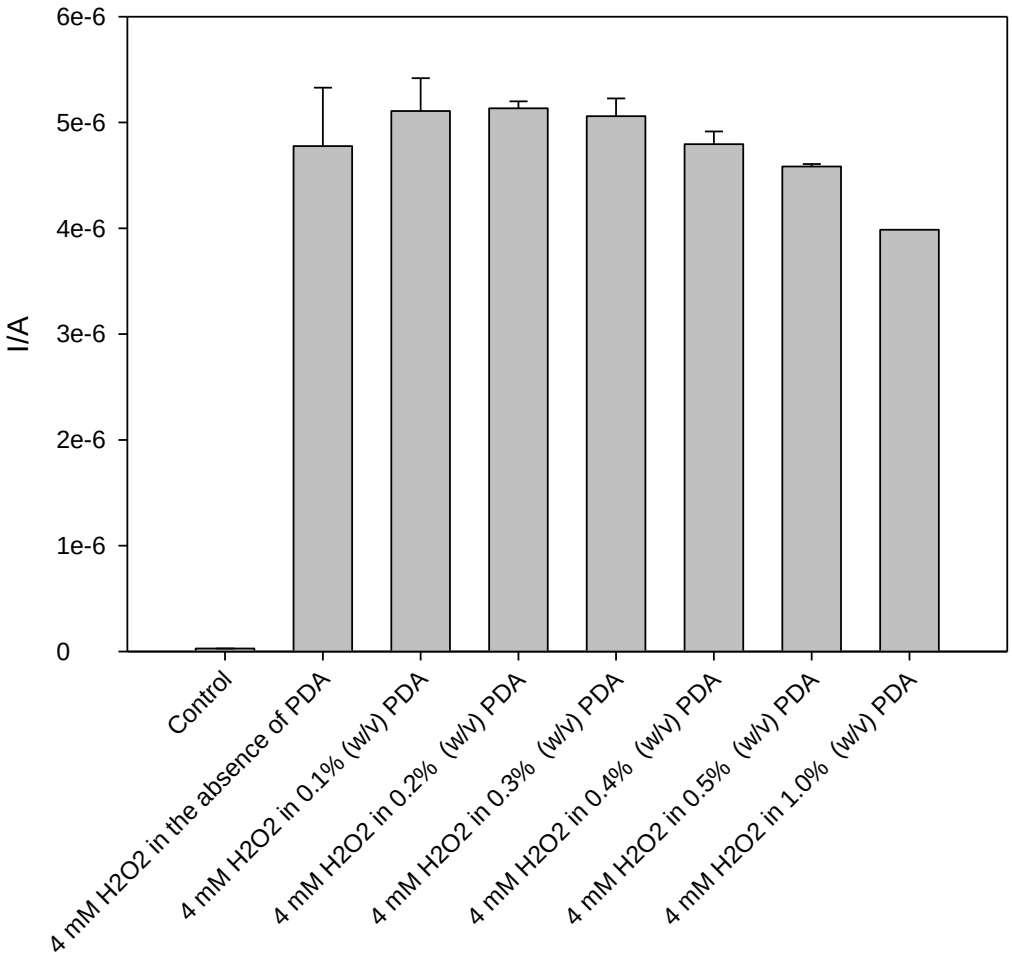

Fig. 6.11. Effect of PDA concentration on chronoamperometric measurement (-0.1 V vs Ag/AgCl) of 4 mM H_2O_2 in the presence of different concentrations of PDA using modified electrodes in non-stirred batch solution, delipidated serum was used for control experiments.

After looking at the effect of PDA on the catalysis of the modified electrode towards H_2O_2 measurement, the next step was to evaluate the effect of PVP in a similar way. Fig. 6.12 demonstrates cyclic voltammograms of modified electrodes in the presence and the absence of 1% (w/v) PVP. As can be seen, a 1.6-fold decrease in non-Faradic current was observed in the presence of 1% (w/v) PVP K30, showing a

negative effect on surface modification. The current observed for 4 mM H_2O_2 measurement at -0.1 V (vs Ag/AgCl) also decreased by approx. 1.3-fold in the presence of increasing concentrations of PVP which might be due to some diffusional effect. PVP is a polymer used in drug formulation in pharmaceutical industries which is capable of solubilizing poorly water-soluble drugs in a way very similar to surfactants by exploiting the large dipole moment of its side group which can interact with any dipole present in the solution which may lead to some change in critical micelle point of present surfactant (Reintjes, 2011).

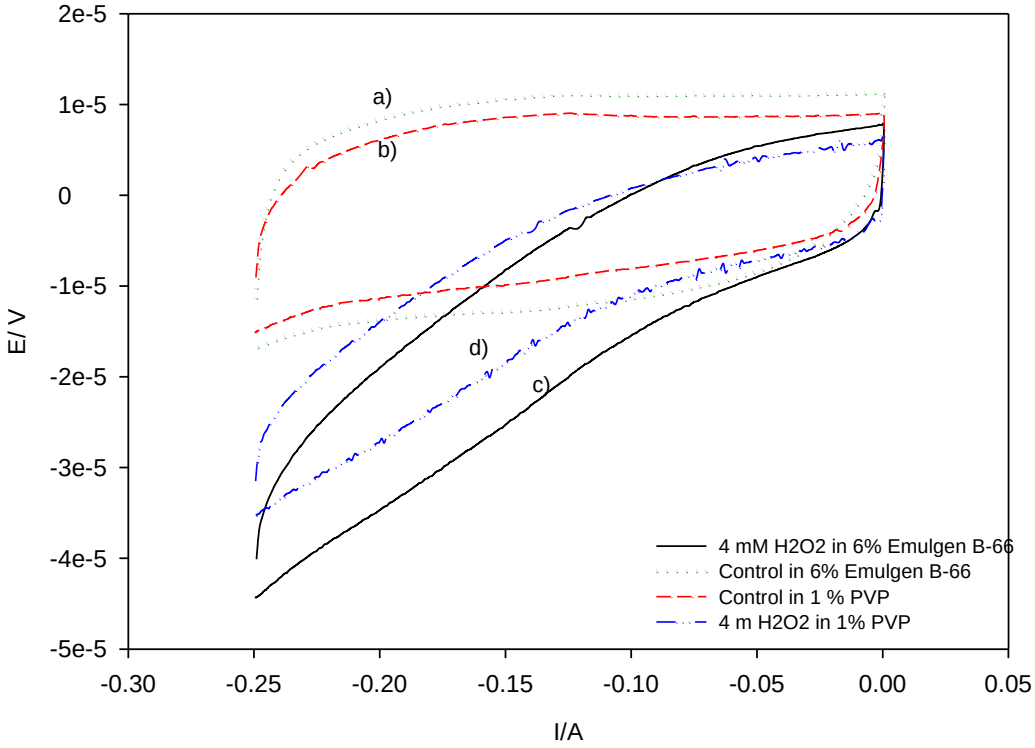

Fig. 6.12. Cyclic voltamogram of modified electrodes in a) 6% (w/v) Emulgen B-66, b) 0.1% (w/v) PVP, c) 4 mM H_2O_2 in 6% (w/v) Emulgen B-66 and d) 4 mM H_2O_2 in 0.1% (w/v) PVP, Delipidated serum was used for control experiments.

Fig. 6.13 shows the amperometric measurement of 4 mM H_2O_2 in PVP from 0 to 1.0%. The current observed decreased with increasing PVP concentration, showing some negative effect on catalytic activity of the modified electrode. This might be due to some disruption of the Emulgen/PBS surface structures at higher concentrations or maybe changes in the rates of diffusion. Since, 0.1 and 0.2 % (w/v) PVP showed comparable amperometric responses, 0.2% (w/v) was used for future experiments. Since 0.2 % (w/v) PDA showed a slight positive effect on surface modification while 0.2% (w/v) PVP showed some negative effect, the effect of both PVP and PDA (0.2% (w/v)) on the amperometric measurement of 4 mM H_2O_2 was assessed (Fig. 6.14). As can be seen, a smoother amperometric

response, with almost the same current response was achieved at 420 s in the presence combined 0.2% (w/v) PVP and PDA.

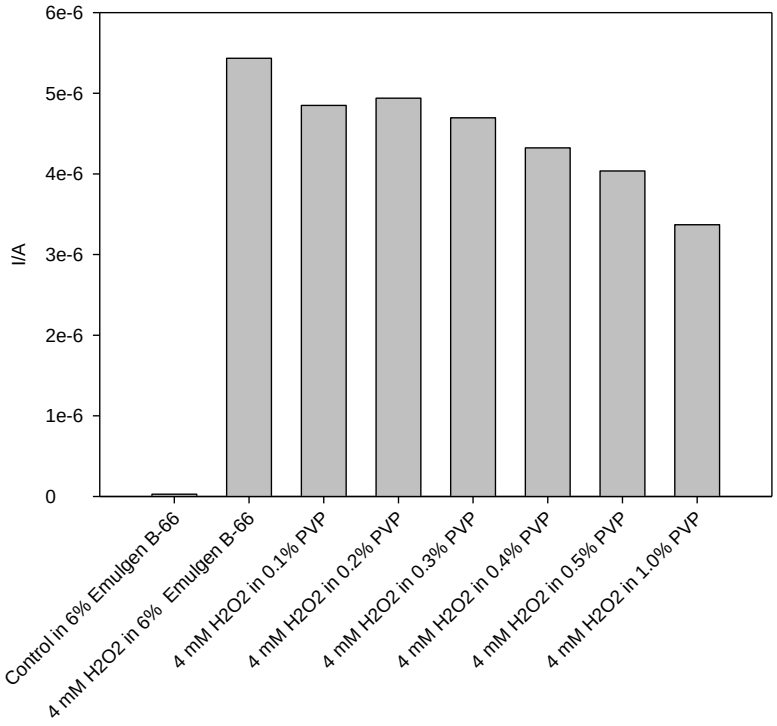

Fig. 6.13. Effect of PVP concentration on chronoamperometric measurement (-0.1 V vs Ag/AgCl) of 4 mM H_2O_2 in the presence of different concentrations of PVP using modified electrodes in non-stirred batch solution, delipidated serum was used for control experiments.

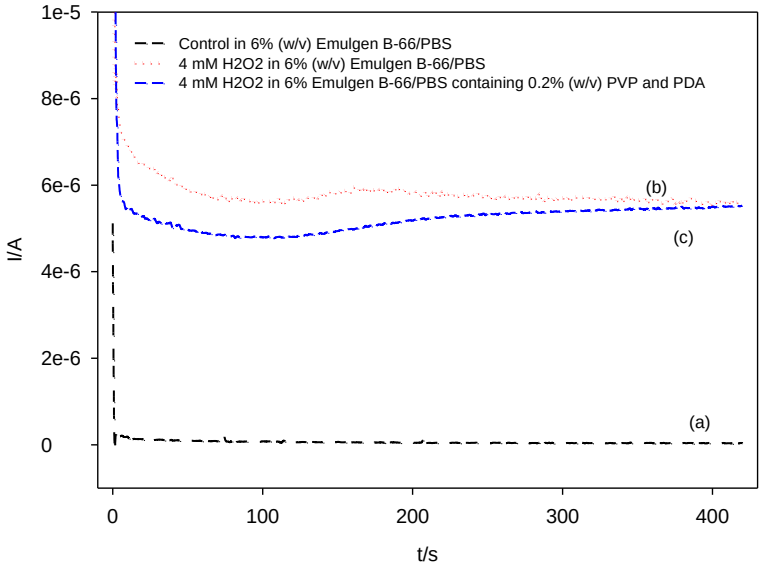

Fig. 6.14. Amperometric measurement of H_2O_2 (4 mM) in the absence (b) and the presence (c) of combined 0.2% (w/v) PVP and PDA in 6% (w/v) Emulgen B-66/PBS; Delipidated serum used for control measurement (a) in the absence of combined 0.2% (w/v) PVP and PDA.

To summarise, the combined addition of 0.2% (w/v) PVP and 0.2% (w/v) PDA in 6% (w/v) Emulgen B-66/PBS were shown to have only a slight reduction on electrode catalysis for the amperometric measurement of H_2O_2. Therefore, this formulation was used for the preparation of assay reagents. The suitability of the final assay reagent formulation following inkjet-print deposition on the electrode surface was evaluated followed by assessment of its ability to measure HDL-C.

ChEs (261 U) and ChOx (122.2 U) were dissolved in 2 mL of 6% (w/v) Emulgen B-66 containing 0.2% (w/v) PVP and 0.2% (w/v) PDA with a final pH of 6.8. Two layers of the final solution were then inkjet-printed onto 15×8 mm of electrode surface. All the electrodes had been modified previously using Emulgen B-66/PBS. Approx. 8 µL of HDL-C sample was introduced by capillary force into the encapsulated biosensors and amperometric measurement was performed following two incubation periods of 240 s and 360 s. Measurement after 360 s incubation was performed to assess if any increase in incubation time would lead to improved response, as further increases in incubation time may increase the amount of H_2O_2 released. Fig. 6.15 shows the amperometric response of the integrated biosensor to HDL-C in delipidated serum on the same day of sensor fabrication.

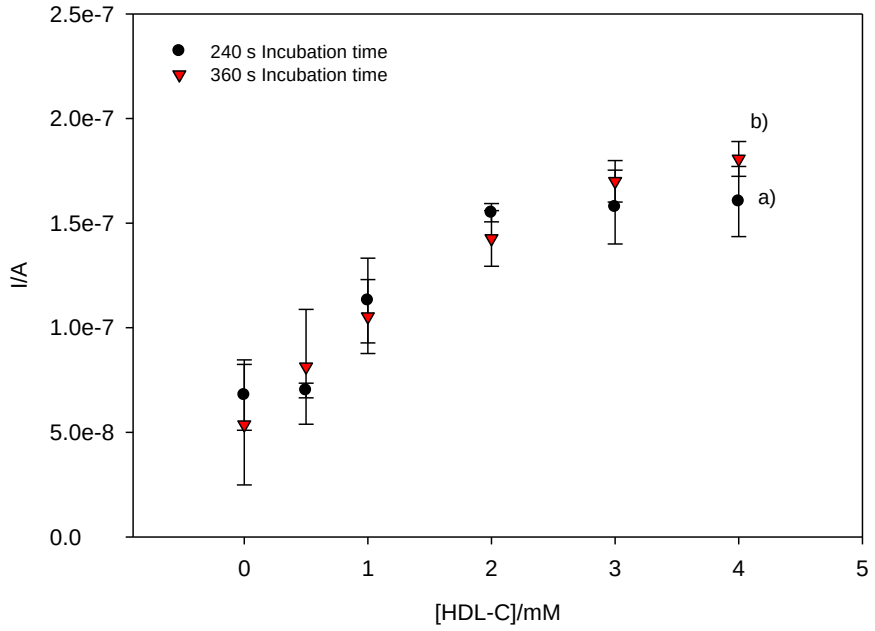

Fig. 6.15. Amperometric measurement (-0.1 V vs Ag/AgCl) of HDL-C in delipidated serum using the integrated biosensor: a) after 240 s incubation (slope=2.57×10^{-8} A/mM and r² = 0.825, n=3) and b) after 360 s Incubation (slope=3.20×10^{-8} A/mM and r² = 0.952, n=3).

The biosensor showed only marginally improved sensitivity and linearity (slope=3.20×10^{-8} A/mM and r^2=0.952) after 360 s incubation compared to 240 s incubation (slope=2.57×10^{-8} A/mM and r^2=0.825) showing that the enzymes may still limit the enzymatic reaction. The amperometric results for HDL-C (0 to 4 mM) using two layers of assay reagent inkjet-printed on modified electrodes in the absence of PVP and PDA had a sensitivity of 2.06×10^{-8}A/mM with linearity (r^2) of 0.952 for n=4 (RSD=11.5%) measurements (Fig. 6.6 and Table 6.5). A comparison of HDL-C measurement after 240 s incubation in the presence and absence of PDA and PVP illustrated an improvement in the sensitivity and reproducibility of the method (Table 6.7 vs Table 6.5) showing improved reproducibility, stability and loss of enzymes activity. However, linearity may be further improved by an increase in enzyme concentration.

Table 6.7. Detailed analytical data of amperometric measurement (-0.1 V vs Ag/AgCl) of HDL-C in delipidated serum using the integrated biosensor (n=3).

HDL-C /mM	Average current at 420 s (A)	SD	RSD %
0.5	7.00×10^{-8}	3.46×10^{-9}	4.9
1	1.13×10^{-7}	2.02×10^{-8}	17.9
2	1.55×10^{-7}	4.36×10^{-9}	2.8
3	1.58×10^{-7}	1.76×10^{-8}	11.2
4	1.60×10^{-7}	1.67×10^{-9}	10.4
Average RSD			9.5

It was shown previously in Section 6.2.2 that integrated enzymes developed using inkjet-print deposition of assay reagent showed reduction in enzyme activity even within a day of preparation, while showing almost no activity after being kept at room temperature for one week. Since enzymes stabilised before deposition may show less alteration in their structure compared to free enzymes following deposition, future evaluation was performed to assess if the new formulation containing PVP and PDA could provide better biosensor stability. Therefore two sets of biosensors employing the inkjet-printed assay reagent formula detailed earlier in this section were stored at 2-8°C for two days in a desiccator and measurement of HDL-C at 420 s was compared with responses from biosensors used on the day of fabrication (Fig. 6.16). Control experiments were performed using enzymes-less assay reagents. As can be seen, better sensitivity and linearity (slope= 3.32×10^{-8}, r^2 = 0.849) was observed for biosensors stored at 2-8°C compared with those used on the same day of fabrication (slope= 2.57×10^{-8}, r^2 =

0.825) showing that enzymes may need some time to stabilise themselves after being deposited on the modified electrodes. This was also reported by Pemberton *et al.,* 2013 that the reliable response of the microfabricated glucose biosensor was obtained when used fresh without any drying step at room temperature before use or when stored immediately at 4 °C. The other explanation for the increase in sensitivity may be due to increases in enzyme activity, rates of diffusion, changes in the kinetics of the reaction as well as changes in surface modification following contact with the solution containing Emulgen B-66/PBS/PVP/PDA. Both PDA and Emulgen B-66 were shown to positively effect on the catalysis while measuring H_2O_2.

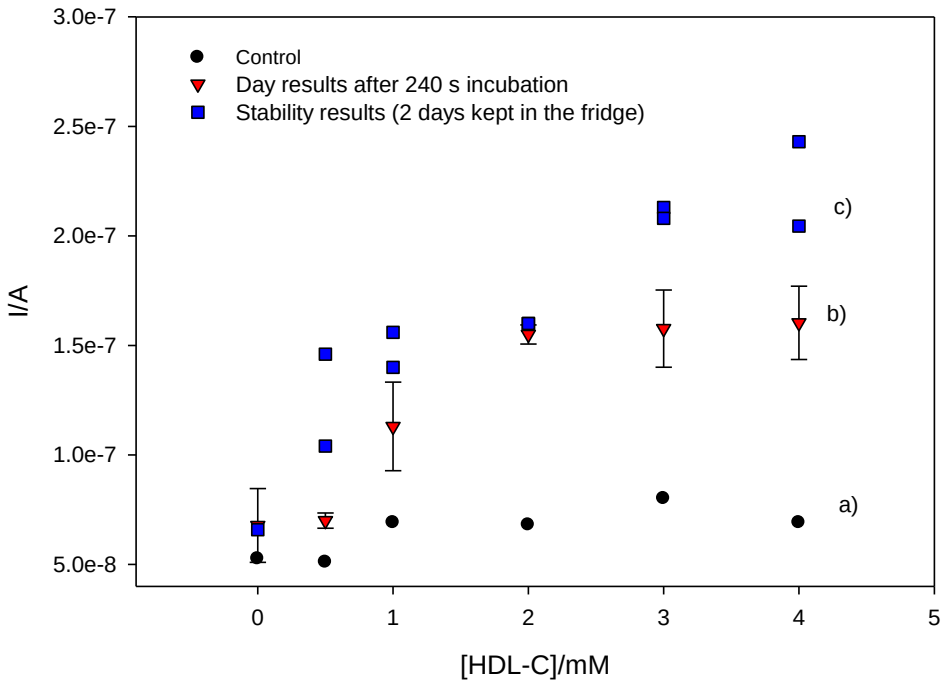

Fig. 6.16. Amperometric measurement (-0.1 V vs Ag/agCl) of HDL-C at 420 s using the integrated biosensor after 240 s incubation: a) control experiment in the absence of the enzymes), b) response from biosensors tested on the same day as fabrication (slope= 2.57×10^{-8}, r^2 = 0.825, (n=3)) and c) after two days storage at 2-8 °C (slope= 3.32×10^{-8}, r^2 = 0.849, (n=2))

While the stability and sensitivity of the integrated biosensors was significantly improved in the presence of PDA and PVP, the integrated biosensor had not yet demonstrated adequate linearity across the clinical range. In order to assess whether by increasing the number of inkjet-printed layers and consequently increasing the assay reagent concentration, the linearity of the method would be improved, four layers of assay reagent formulation (Section 6.2.3) were inkjet-printed on Emulgen B-66/PBS modified electrodes. On this occasion, biosensors

were kept at 2-8°C in a sealed container in the presence of silica gel and were tested after two days and 20 days storage (Fig. 6.17 and Table 6.7).

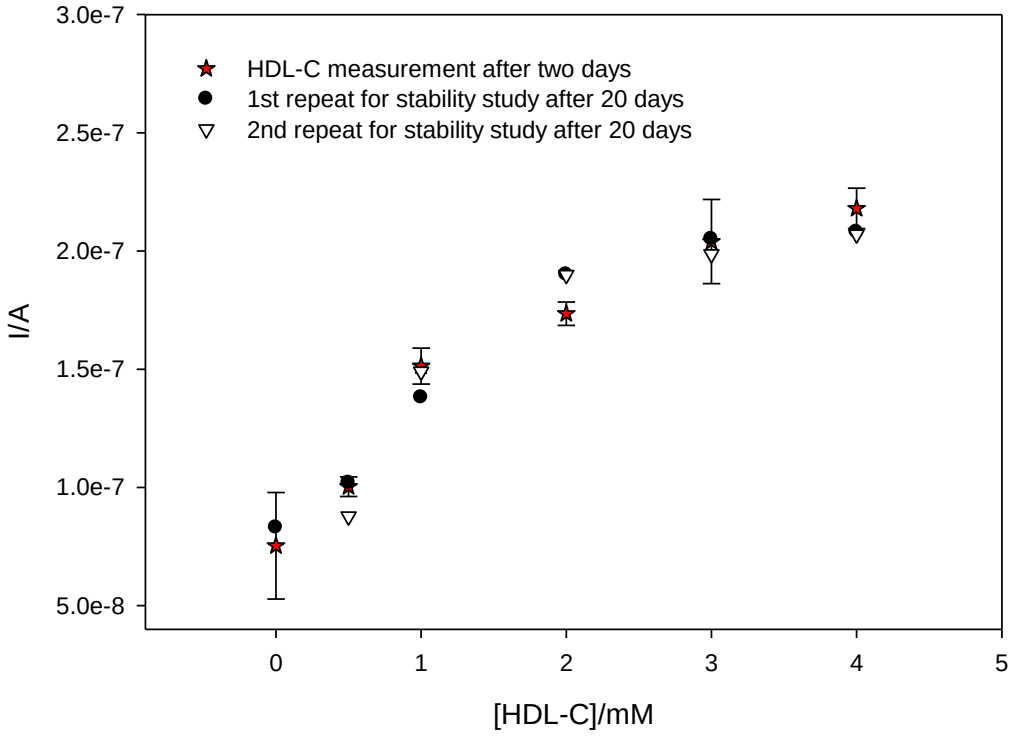

Fig. 6.17. Amperometric measurement of HDL-C at -0.1 V vs Ag/AgCl using integrated biosensors with four printed reagent layers following: a) two and; b) 20 days storage at 2-8 °C. a) slope=3.52×10^{-8} and r^2=0.92, n=4. b) slope=3.33×10^{-8} and r^2=0.87, n=2.

As can be seen, the amperometric response from biosensors with four printed layers showed a sensitivity of 3.52×10^{-8} with linearity (r^2) of 0.92 after two days storage, showing an increase in linearity and sensitivity compared to those performed using two layers (slope= 2.57×10^{-8}, r^2=0.825; n=3). The biosensors also exhibited very similar current signals after 20 days with just a slight decrease in sensitivity and linearity, suggesting that the new formulation containing PVP and PDA did bring about an improvement in biosensor performance by improving enzyme stability, re-solubilisation and consequently better homogeneity. A comparison between these results and those performed in the absence of PDA and PVP at room temperature, illustrates a significant improvement in biosensor stability with storage at 2-8°C (Pemberton *et al.,* 2013) in combination with the stabilizers.

Table 6.8. Detailed analytical data of amperometric measurement of HDL-C at -0.1 V vs Ag/AgCl using integrated biosensors with four printed reagent layers following two and 20 days storage at 2-8 °C.

	Two days kept at 2-8°C			20 days kept at 2-8°C	
HDL-C /mM	**Average current at 420 s (I/A)**	**SD**	**RSD (%)**	**1st repeat Average current at 420 s (I/A)**	**2nd repeat Average current at 420 s (I/A)**
0	7.53×10^{-8}	2.25×10^{-8}	29.9	8.30×10^{-8}	-
0.5	1.00×10^{-7}	4.16×10^{-9}	4.1	1.02×10^{-7}	8.77×10^{-8}
1	1.51×10^{-7}	7.57×10^{-9}	5.0	1.38×10^{-7}	1.49×10^{-7}
2	1.74×10^{-7}	4.95×10^{-9}	2.9	1.90×10^{-7}	1.90×10^{-7}
3	2.04×10^{-7}	1.78×10^{-9}	8.7	2.05×10^{-7}	1.99×10^{-7}
4	2.18×10^{-7}	8.54×10^{-9}	3.9	2.08×10^{-7}	2.07×10^{-7}
Average RSD			9.1		

A similar experiment was performed, this time with biosensors stored in a desiccator at room temperature, with measurement at one day and 20 days following fabrication (Fig. 6.18).

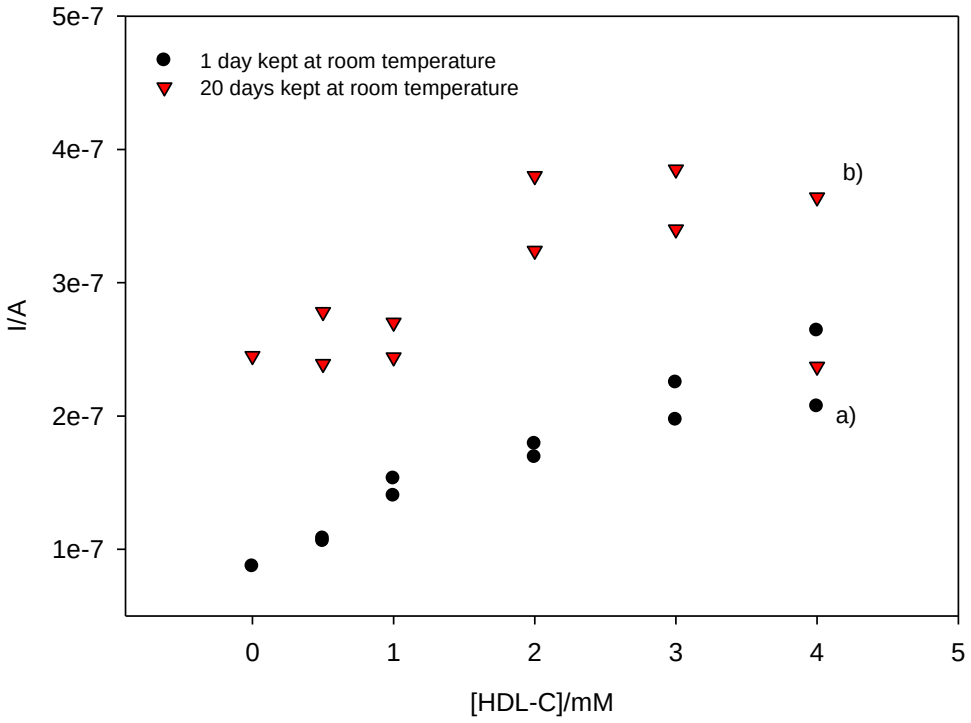

Fig. 6.18. Amperometric measurement of HDL-C at -0.1 V vs Ag/AgCl using integrated biosensors with four printed reagent layers following: a) one (slop=3.47×10^{-8}, r^2=0.95, n=2) and; b) 20 days storage at room temperature.

As can be seen in Fig. 6.18, the sensitivity of 3.47×10^{-8} and linearity (r^2) of 0.95 for two repeat measurements using biosensors fabricated with four layers assay reagent and stored for one day at room temperature were observed which

demonstrated much better sensitivity and linearity when compared with those reported in Section 6.2.2 which were in the absence of PVP and PDA at room temperature, confirming that the addition of PDA and PVP were suitable for stabilising enzymes after deposition. However, after 20 days storage, although sensors still exhibited some activity, there had been a significant decrease in analytical performance. However, after 20 days, enzymes still showed some activity, while in the absence of PDA and PVP almost no activity was observed after a week of storage at room temperature.

In summary, a combination of 0.2 % (w/v) PDA and PVP was able to significantly improve enzyme stability. This may be due to some electrostatic bonding with enzyme to maintain structural integrity as well as keeping them hydrated in order to prevent denaturation and make them suitable to be re-solubilised in the presence of substrate to produce a solution with better homogeneity. Consequently, the reproducibility of the biosensors was improved due to more consistency in assay reagent deposition, reagent rehydration and re-solubilisation in a more consistent manner which will be assessed in the following sections. Although the analytical performance of the integrated biosensor was improved using four printed layers, it still suffered from lack of linearity (r^2=0.92) when compared to externally mixed experiments (r^2=0.999).

As was mentioned in Chapter 5, a two-fold dilution factor was used when reagent was externally added to the substrate. In other words, while 0.5 to 4 mM HDL-C were introduced as substrate, the final concentrations of HDL-C in solution after addition of assay reagent were from 0.25 to 2 mM. However, due to removal of the dilution step with reagent integration, the integrated biosensor must actually measure 0.5 to 4 mM, which is effectively twice the concentration measured by the externally mixed reagent approach. This range was very similar to the TC final concentration measured by externally addition of reagent which was discussed in Chapter 5. Considering the dilution factor of 2, the final concentration of TC measured using externally added reagent was up to 5 mM. Therefore, the next step was to assess whether increasing the enzyme concentration in the assay reagent would further improve the analytical performance of the integrated biosensor Increasing the number of printed layers may increase the systematic error and consequently the reproducibility of the integrated biosensor, As a result, increasing enzyme concentration was preferred over increasing the number of layers. Therefore, ChOx (218 U/mL) and ChEs (633 U/mL) were prepared in 6% (w/v)

Emulgen B-66/PBS containing 0.2% (w/v) PVP and PDA and was used to fabricate HDL-C integrated biosensor. Sensitivity, linearity and reproducibility of them were evaluated. Control experiment was performed using integrated biosensor having four layers enzymes-less assay reagent.

Fig. 6.19 demonstrates the measurement of HDL-C using the biosensor at the elevated enzyme concentrations. The biosensor showed an acceptable sensitivity of 3.33×10^{-8} with linearity (r^2) of 0.993 and reproducibility of 10.3% (n=4) for HDL-C measurement (0.5 to 4 mM). These results were comparable with those observed for HDL-C measurement using externally mixed reagents as presented in Chapter 5 (slope=3.32×10^{-8}, r^2=0.999, RSD=9.5% n=5). However, the integrated biosensor was not able to differentiate between 0 and 0.5 mM HDL-C resulting in an LOD of 0.5 mM (Table 6.9).

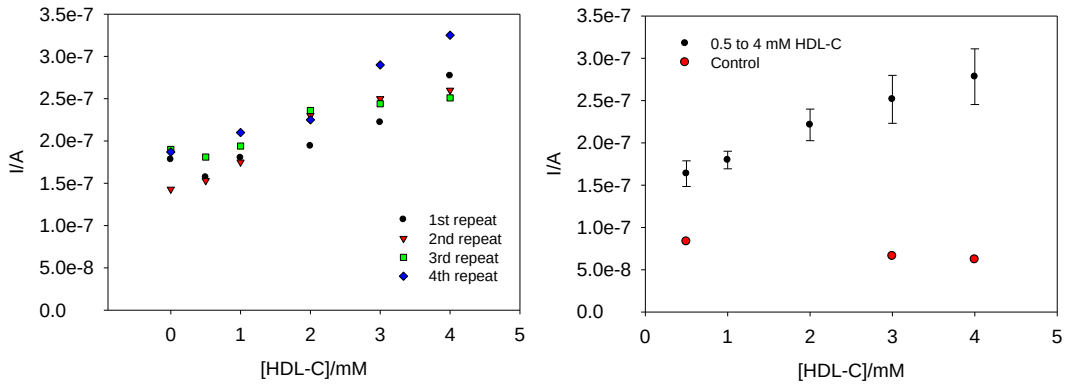

Fig. 6.19. Amperometric measurement of HDL-C (0.5-4 mM) at -0.1 V vs Ag/AgCl using integrated biosensors with four printed reagent layers containing ChOx (218 U/mL) and ChEs (633 U/mL) in 6% (w/v) Emulgen B-66/PBS and 0.2% (w/v) PVP and PDA (slop=3.33×10^{-8}, r^2=0.993 and RSD=10.3%; n=4), Enzymes-less assay reagent inkjet-print deposited on modified electrode surface was used for control measurement.

As a result of a further elevation in enzyme concentration, linearity (r^2) increased from 0.92 to 0.99 which was very similar to that observed using externally mixed reagents and was within the acceptable criteria for HDL-C measurement according to NECP guideline ($r^2\geq0.975$) (CRMLN, 2002). Although the reproducibility of the integrated biosensor was very similar to the one based on externally added reagents, it did not meet the CRMLN criteria which were less than 5%. In order to improve reproducibility, the next step was to evaluate whether removal of the electrode modification step, with the potential accompanying reduction in systematic error would improve the precision of the integrated biosensor.

Table 6.9. Detailed analytical data of amperometric measurement of HDL-C at -0.1 V vs Ag/AgCl using integrated biosensors with four printed reagent layers containing ChOx (218 U/mL) and ChEs (633 U/mL) in 6% (w/v) Emulgen B-66/PBS and 0.2% (w/v) PVP and PDA.

HDL-C/ mM	I/A (average at 420 s)	S/B	SD	RSD (%)
0	1.75×10^{-7}	-	2.16×10^{-8}	12.4
0.5	1.64×10^{-7}	0.9	1.51×10^{-8}	9.3
1	1.80×10^{-7}	1.0	1.03×10^{-8}	5.8
2	2.21×10^{-7}	1.3	1.87×10^{-8}	8.5
3	2.52×10^{-7}	1.4	2.83×10^{-8}	11.3
4	2.78×10^{-7}	1.6	3.30×10^{-8}	11.9
Average RSD				**10.3**

6.2.4 Assessment of biosensor performance in the absence of electrode modification

As was discussed in Chapter 5, inkjet printing of five layers of 6% (w/v) Emulgen B-66/PBS onto silver paste electrodes was successfully used as a means of modifying the silver paste electrodes to achieve enhanced electrocatalytic reduction of H_2O_2. The modified electrodes were then employed as a platform for the amperometric measurement of HDL-C by external addition of assay reagents and for the fabrication of the integrated HDL-C biosensor. Although this step was shown to increase the catalytic activity of silver electrode towards H_2O_2, the additional processing step required may contribute to the irreproducibility of the integrated biosensor. Since the assay reagents also contain surfactant and salt it was thought possible that sufficient catalysis could be achieved without this additional preparation step. Therefore, four layers of assay reagent were inkjet-printed on the electrode surface without any prior modification. All biosensors were stored in a sealed container at 2-8 °C for two days and then used for measurement of HDL-C (Fig. 6.20 and

Table 6.10). As can be seen, the background current observed at -0.1 V (vs Ag/AgCl) was 1.04×10^{-7} A which was 1.7-fold lower than that observed on modified electrodes which may be due to a reduction in formation of surface structures on the electrode. However, a significant improvement was observed in sensitivity and reproducibility (slope=4.55×10^{-8}, RSD=6.6%; n=3) of these biosensors compared to

those reported using Emulgen/PBS modified electrodes (slope=3.33×10^{-8}, RSD=10.3%; n=4).

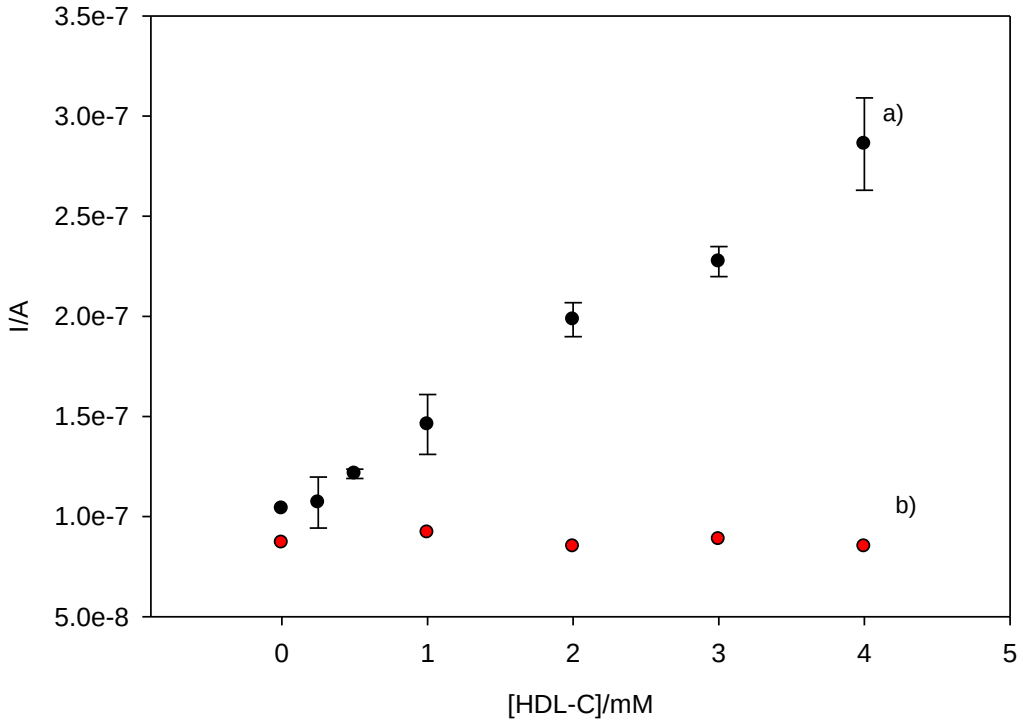

Fig. 6.20. a) Measurement of HDL-C using unmodified silver paste electrodes (slope=4.55×10^{-8}, r^2=0.993, RSD=6.6%; n=3); b) control in the absence of enzymes.

Table 6.10. Detailed analytical data of amperometric measurement (-0.1 vs Ag/AgCl) of HDL-C using unmodified silver paste electrodes (n=3).

HDL-C / mM	I/A (average at 420 s)	S/B	SD	RSD (%)
0	1.04×10^{-7}	-	-	-
0.25	1.07×10^{-7}	1.0	1.27×10^{-8}	11.9
0.5	1.21×10^{-7}	1.2	2.31×10^{-9}	1.9
1	1.46×10^{-7}	1.4	1.49×10^{-8}	10.2
2	1.98×10^{-7}	1.9	8.50×10^{-9}	4.3
3	2.27×10^{-7}	2.2	7.51×10^{-8}	3.3
4	2.86×10^{-7}	2.8	2.31×10^{-8}	8.1
Average RSD				**6.6**

Significant improvement was observed in the signal to background (S/B) of the integrated biosensor used without prior modification. This may be due to the smaller and more stable background currents as demonstrated in the control experiments, while the current signal for 4 mM HDL-C was almost the same using either modified or unmodified biosensors. The reproducibility of the method was

also improved which could be attributed to the removal of a variable process step. Although the linearity of the method did not change, the LOD went from from 0.5 mM to 0.25 mM which is important for the calculation of non-HDL-C.

In summary, an integrated HDL-C biosensor was developed with acceptable assay sensitivity of 4.55×10^{-8} and good linearity (r^2) of 0.993 with the ability to detect HDL-C in the range of 0.25 to 4 mM. The average RSD of the method for three measurements was calculated to be 6.6% (1.90-11.9%). This results were comparable with those reported in Chapter 5 using externally mixed assay reagents (slope=3.32×10^{-8}, r^2=0.999, RSD=9.5 %; n=5) with a slight improvement in sensitivity and reproducibility. However, the linearity and S/B were found to be slightly better when assay reagents were mixed externally. Although the integrated HDL-C biosensor showed good linearity as well as acceptable sensitivity, the method precision was still outside those set by NECP guidelines (≤5.0%) (Bishop, Fody and Schoeff, 2013). However, this target could be achieved by the implementation of tight engineering controls in large scale manufacture.

Although there are many reports of the amperometric measurement of cholesterol (TC or FC) using various immobilisation techniques (Arya, Datta and Malhotra, 2008; Ruecha, Siangproh and Chailapakul, 2011; Ruecha *et al.,* 2014; Shin and Kameoka, 2012; Türkarslan, Kayahan and Toppare, 2009), there are few reports of their application to the measurement of cholesterol in serum (Dey and Raj, 2014; Fang, 2011; Foster, Cassidy and O'Donoghue, 2000; Hooda *et al.,* 2009; Manjunatha *et al.,* 2012; Ruecha *et al.,* 2014; Urmila *et al.,* 2011).

Foster *et al.,* (2000), reported a diagnostic device for HDL-C (0.76 – 2.3 mM) and TC (2.81-13.0 mM) based on the electrochemical measurement of H_2O_2 released after enzymatic reaction of cholesterol leading to peroxidase oxidation of potassium ferrocyanide. The device had a detection zone containing the reagent necessary for enzymatic reaction and ferrocyanide as an electrochemical mediator allowing electron transfer between the H_2O_2 and the electrode. Potassium ferrocyanide was oxidised in the presence of H_2O_2 followed by its reduction at the electrode and producing current. The detection zone was in contact with electrodes and received the cholesterol sample from one end by capillary action. A 20-fold dilution of serum sample was required for effective measurement using the device and the current was measured at -0.2 V (vs Ag/AgCl) at 3.5 min following 7.5 min incubation time (total measurement time of 11 min). The method also required sample pre-

treatment for HDL-C measurement including a precipitation step using PTA and $MgCl_2$ followed by 10 min centrifugation at 10,000 rpm or filtering before introduction of the supernatant to the device. A comparison between the method development in this research and that reported by Foster *et al.,* (2000) demonstrates that the method developed in this research might be more suitable to be used as diagnostic device as there is no need for any sample dilution or pre-treatment before introducing the serum sample to the device (Foster, Cassidy and O'Donoghue, 2000). However, challenges remain due to the variability of screen printed electrodes which has also been reported by Foster *et al.,* (2000).

6.3 Development of an integrated TC biosensor

As was mentioned in Chapter 1, use of non-HDL-C for decision-making in treatment of atherosclerotic cardiovascular risk in adults (ACRAD) was introduced by NCEP (Adult Treatment Panel III (ATP III), 2002; Boekholdt *et al.,* 2012; Stone *et al.,* 2014). While non-HDL-C is calculated by subtraction of the HDL-C value from the TC value, to develop a diagnostic device capable of measuring non-HDL-C, it was important to also develop an integrated TC biosensor using the equivalent platform as that developed in Chapter 5. It was shown in Chapter 5 that a modified silver electrode using Triton X-100/PBS was capable of measuring TC (0 to 10 mM) using external reagent addition. The next step was to then develop an integrated TC biosensor through the inkjet-print deposition of a TC assay reagent.

6.3.1 Effect of PVP and PDA on the amperometric measurement of TC using externally addition of assay reagent

As was mentioned in Section 6.2, an ink formulation was developed containing PVP and PDA (0.2% (w/v)) which was suitable for the fabrication of the HDL-C integrated biosensor. The same ink formulation as mentioned in Section6.2.3 was again prepared, but this time in 0.5% (v/v) Triton X-100/PBS instead of Emulgen B-66/PBS. The effect of PDA and PVP on the amperometric measurement of TC was first investigated using external reagent addition before moving to the fabrication of the integrated biosensor. Fig. 6.21 illustrates the amperometric responses of Triton X-100/PBS modified electrodes to TC. 20 µL of assay reagent containing final concentrations of 60 U/mL ChOx and 156 U/mL ChEs prepared in 0.5% (v/v) Triton X-100/PBS/0.2% (w/v) (PVP and PDA) was mixed with 20 µL TC as substrate. Assay reagent was used immediately following preparation (1st repeat) or for a minimum of one hour (2nd-4th repeats) (Fig. 6.22 and Table 6.11).

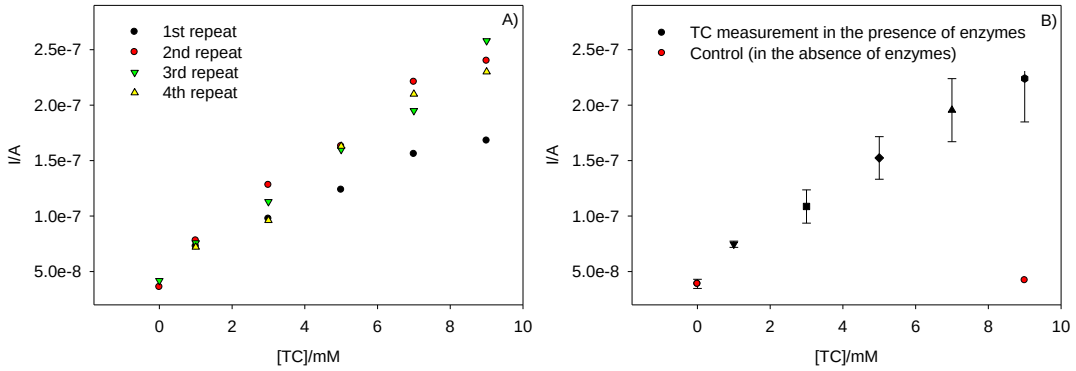

Fig. 6.21. Biosensor response to TC in delipidated serum at -0.1 V (vs Ag/AgCl) after 420 s using ChOx (60 U/mL) and ChEs (156 U/mL) prepared in 0.5% (v/v) Triton X-100/PBS containing 0.2% (w/v) PVP and PDA which was externally added and incubated for 240 s; A: 1st repeat performed immediately following assay reagent preparation; 2nd-4th repeats performed one hour after preparation. Slope=2.04×10^{-8} , r^2=0.992 and RSD=12.1%, n=4; B: three repeats measurement (slope=2.25×10^{-8} , r^2=0.994 and RSD=7.0%, n=3).

Table 6.11. Detailed analytical data of amperometric measurement (-0.1 V vs Ag/AgCl) of TC using Triton/PBS modified electrode by external addition of assay reagents.

	N=4			N=3		
TC/mM	**I/A (average at 420 s)**	**SD**	**RSD (%)**	**I/A (average at 420 s)**	**SD**	**RSD (%)**
0	3.89E-08	4.10×10^{-9}	10.5	3.89×10^{-8}	4.10×10^{-9}	10.5
1	7.46E-08	2.79×10^{-8}	3.7	7.53×10^{-8}	3.04×10^{-9}	4.0
3	1.09E-07	1.50×10^{-8}	13.8	1.12×10^{-7}	1.60×10^{-8}	14.3
5	1.52E-07	1.92×10^{-8}	12.6	1.62×10^{-7}	1.73×10^{-9}	1.1
7	1.96E-07	2.84×10^{-8}	14.5	2.09×10^{-7}	1.31×10^{-8}	6.3
9	2.24E-07	3.91×10^{-8}	17.5	2.43×10^{-7}	1.42×10^{-8}	5.8
	Average RSD		12.1	**Average RSD**		7.0

As can be seen in Fig. 6.21 A, the results using the first set of modified electrodes which were obtained immediately following preparation of assay reagent showed significant deviation from the later repeats, which might be due to the fact that the enzymes may need some time to stabilise and settle their activity following the addition of PDA and PVP. This was also observed in Section 6.2.3 following the immediate deposition of stabilised enzymes. The calibration curve using all four sets of measurement showed a slope of 2.04×10^{-8} A/mM, linearity (r^2) of 0.992 and RSD of 12.1% (Fig. 6.21 B). However, by removing the first set of replicates, the modified electrodes showed a sensitivity 2.25×10^{-8} A/mM, linearity (r^2) of 0.994 and RSD of 7.0% showing a slight increase in sensitivity of the method and a significant improvement in method reproducibility (Table 6.11).

A comparison of the amperometric measurement of TC with and without PDA and PVP following external reagent addition is shown in Fig. 6.22, taking data from Chapter 5 (Section 5.3.2.1).

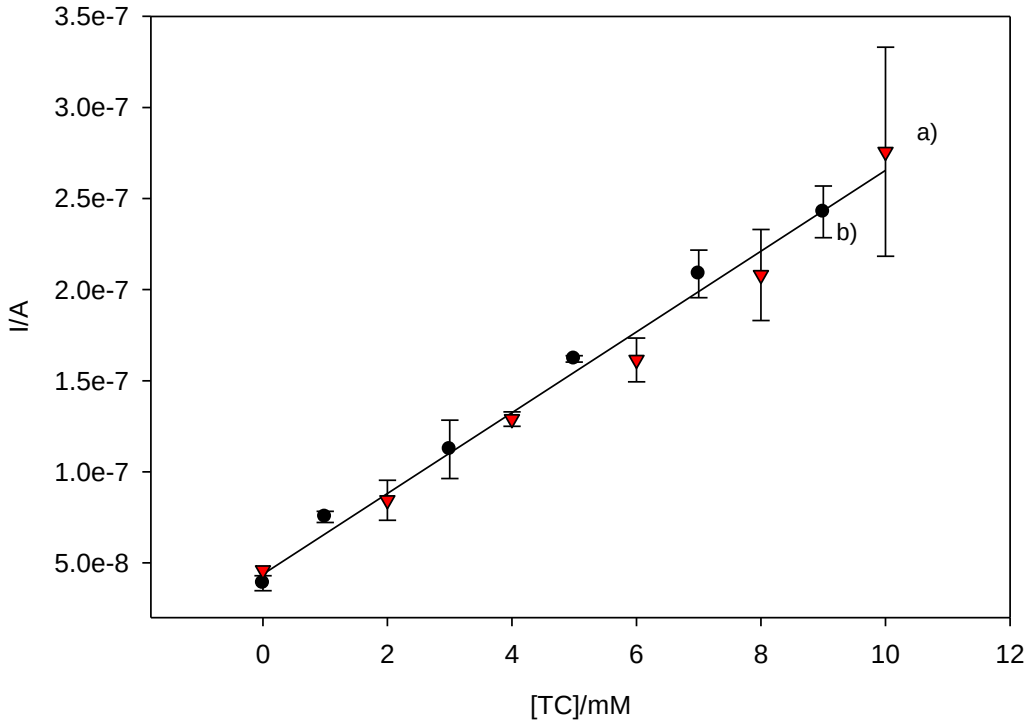

Fig. 6.22. Biosensor response to TC in delipidated serum at -0.1 V (vs Ag/AgCl) after 420 s using externally addition of assay reagent a) (triangles) in the absence of PVP and PDA (slope=2.24×10^{-8} A/mM, r^2 = 0.984 and RSD=10.8%; n=3) b) (circles) in the presence of stabilizers (slope=2.25×10^{-8} A/mM, r^2 = 0.994 and RSD=7.0%; n=3).

A sensitivity of 2.25×10^{-8} A/mM was observed following the addition of stabilizers which was very similar to that observed previously in their absence (2.24×10^{-8} A/mM) confirming that no significant effect was observed on the modified electrode response in the presence of stabilizers which was also shown previously in Section 6.2.3. Linearity was slightly improved (0.994 vs 0.984) with significant improvement in reproducibility of the method for three repeat measurements following the addition of stabilizers with RSDs of 7.0% and 10.8%, respectively.

6.3.2 Inkjet-print deposition of TC assay reagent on modified silver electrodes

As was shown previously in Section 6.2, due to the effects of the deposition and immobilisation methods on the analytical performance of the integrated HDL-C

biosensor which caused issues such as enzyme activity, stability, reagent solubility, assay homogeneity, etc, the integrated biosensor required optimisation of its reagent formulation to minimise these effects. In the previous section, the effect of stabilizers on the amperometric measurement of TC using the encapsulated modified electrodes was evaluated using externally added reagents demonstrating improved reproducibility and linearity. In this section, the effect of inkjet-print deposition of assay reagent on the amperometric measurement of TC was assessed. Therefore, ChOx (329.8 U) and ChEs (913.5 U) in 1.5 mL 0.25% (v/v) Triton X-100/PBS containing 0.2% (w/v) PVP and PDA was prepared. Two or four layers of the formulation was inkjet-printed on the electrodes which had previously been modified with Triton X-100/PBS according to Section6.2.3. The integrated biosensors were stored in a sealed container at 2-8 °C for 24 h and used for the amperometric measurement of TC. The inter-relationships between enzyme concentration, incubation time and print layers on the assay response were evaluated. A concentration of 0.25% (v/v) Triton X-100 was chosen as concentrations above 0.5% have been shown to have a negative effect on enzymatic reaction of cholesterol which was demonstrated in Chapter 3.

Measurement of TC using the integrated biosensors using two and four printed layers is shown in Fig. 6.23. Measurements using two print layers were performed with 240 s and 420 s incubation in order to assess the effect of incubation time which may be limiting the response due to enzyme activity and other kinetic processes. Increasing the incubation time was shown to increase both the sensitivity and linearity of the method, confirming such limiting conditions. At four printe layers with 240 s incubation, this gave a sensitivity of 2.139×10^{-8} A/mM and linearity (r^2) of 0.819, which was 1.6-fold greater than that observed for two layers after 240 s incubation (slope of 1.31×10^{-8} A/mM and (r^2) of 0.772). Since increasing either incubation time or layer number led to better sensitivity and linearity, the next step was to increase the enzymes concentrations to twice the initial concentrations and evaluate the effect. This was done to prevent enzymes from limiting the enzymatic reaction.

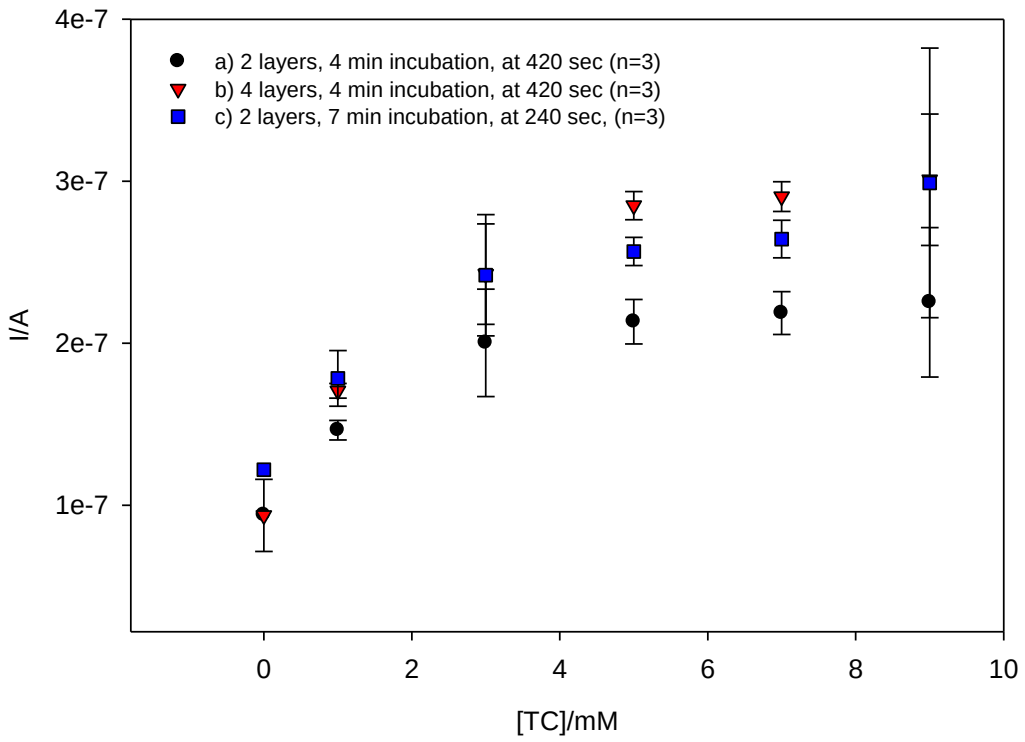

Fig. 6.23. Amperometric measurement of TC using the integrated biosensor (-0.1V vs Ag/AgCl) at 420 s using: two printed reagent layers a) after 240 s incubation (slope=1.31×10^{-8} A/mM and r^2=0.772) and c) after 420 s incubation (slope=1.73×10^{-8} A/mM and r^2=0.864) and also b) using four reagent layers after 240 s incubation (slope=2.139×10^{-8} A/mM and r^2=0.864).

Therefore, four layers of assay reagent containing ChEs (1566 U) and ChOx (582 U) prepared in 1.5 mL of 0.25% (v/v) Triton X-100 containing 0.2% (w/v) PVP and PDA inkjet-printed onto modified electrodes. As can be seen in Fig. 6.24, the integrated biosensor showed a sensitivity of 1.42×10^{-8} A/mM with linearity (r^2) of 0.949 and RSD=11.6% (n=3), the sensitivity of which was 1.6-fold lower than that observed for externally added reagent in the presence of stabilizers, while the imprecision was similar (slope=2.24×10^{-8} A/mM, r^2=0.984 and RSD=10.8%; n=3). These differences may again be due to some variability in screen printed electrodes as well as the enzymatic reaction characteristic especially at higher concentrations. To sum up, four layers of higher enzymes concentrations was considered optimal and was used for any further investigations.

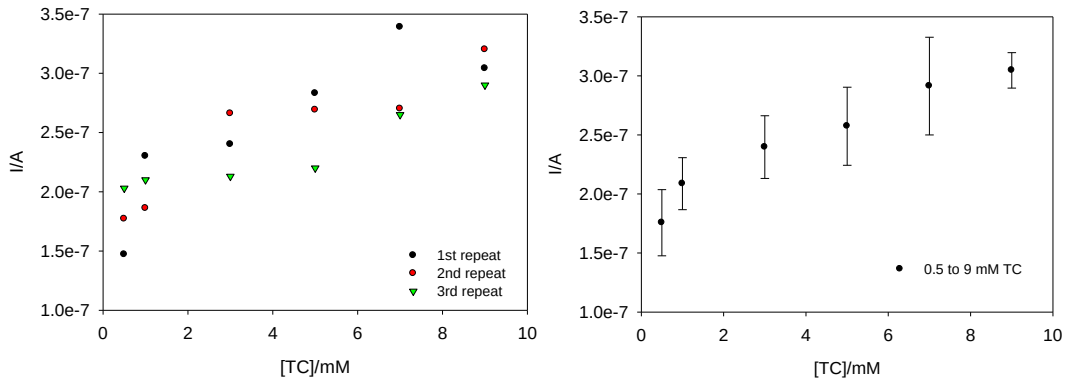

Fig. 6.24. Amperometric measurement of TC (-0.1 V vs Ag/AgCl) using the integrated biosensor containing ChEs (1566 U) and ChOx (582 U) in 1.5 mL of 0.25% (v/v) Triton X-100 containing 0.2% (w/v) PVP and PDA after 240 s incubation at 420 s (slope=1.415×10^{-8} A/mM and r^2=0.949, RSD=11.6% n=3).

Table 6.12. Detailed analytical data of amperometric measurement of TC using integrated biosensor previously modified using 0.5% Triton X-100/PBS (n=3).

TC/ mM	I/A Average at 420 s	SD	RSD (%)
0.5	1.76×10^{-7}	2.80×10^{-8}	16.0
1	2.09×10^{-7}	2.20×10^{-8}	10.6
3	2.40×10^{-7}	2.65×10^{-8}	11.1
5	2.57×10^{-7}	3.31×10^{-8}	12.9
7	2.91×10^{-7}	4.14×10^{-8}	14.2
9	3.05×10^{-7}	1.50×10^{-8}	4.9
Average RSD			11.6

6.3.3 Assessment of TC biosensor performance in the absence of electrode modification

Having evaluated the impact of enzyme concentration, print layer number and incubation time on the response of the integrated TC biosensor, it was then assessed whether it was possible to remove the electrode modification step in a manner analogous to that demonstrated for HDL-C (Section 6.2.4). As is shown in Fig. 6.25, huge variability was observed at 0.5 and 1 mM TC which may be due to the reliability of the fabrication process. However, in general better linearity and reproducibility (r^2=0.982 and RSD=9.5%) were observed for integrated biosensors using the unmodified electrodes which is believed to be due to the elimination of a fabrication step. On the other hand, the sensitivity decreased by 1.4-fold compared to modified electrodes, which might also be expected as there may be a trade off between the magnitude and reproducibility of the catalytic modification.

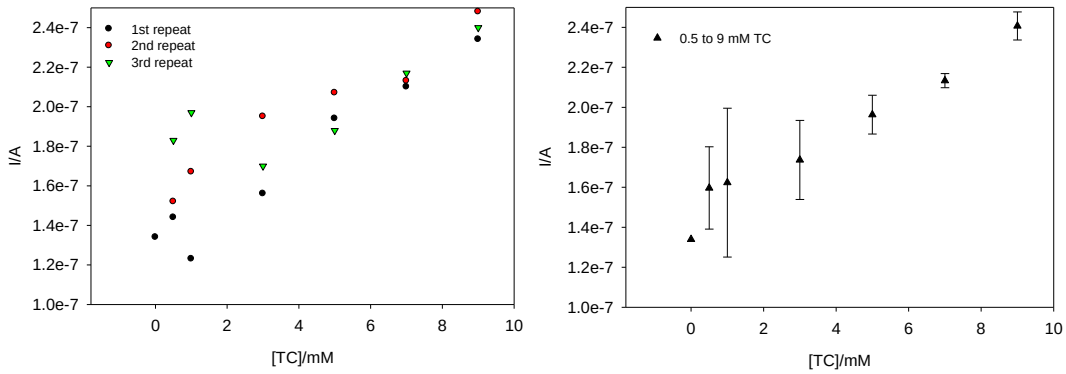

Fig. 6.25. Measurement of TC using unmodified silver paste electrodes (slope=9.38×10^{-9}A/mM and r^2 of 0.982, RSD=9.5%; n=3).

Table 6.13. Detailed analytical data of amperometric measurement of TC using unmodified silver paste electrodes (n=3).

TC/mM	I/A Average at 420 s	S/B	SD	RSD (%)
0	1.34×10^{-7}	-	-	-
0.5	1.60×10^{-7}	1.19	2.06×10^{-8}	12.9
1	1.62×10^{-7}	1.20	3.72×10^{-8}	22.9
3	1.74×10^{-7}	1.30	1.98×10^{-8}	11.4
5	1.96×10^{-7}	1.46	9.71×10^{-9}	4.9
7	2.13×10^{-7}	1.59	3.51×10^{-9}	1.6
9	2.41×10^{-7}	1.80	7.02×10^{-9}	2.9
Average RSD				9.5

The integrated TC biosensor was further optimised with respect to the amperometric measurement time. In order to improve the S/B and consequently sensitivity of the biosensors, the benefit of measurement as soon as current had reached a steady state response (approx. 200 s) was assessed. A comparison between the calibration curves obtained at 200 s and 420 s (Fig. 6.26) reveals that the current sensitivity was 1.4-fold greater when measured at 200 s compared to that measured at 420 s, also with better linearity. It has been reported that the onset of a steady state current can be achieved more rapidly by limiting the expansion of diffusion layers using working and auxiliary electrode in close opposition. Using thinner cells may also help to achieve steady state as well as better sensitivity (Foster, Cassidy and O'Donoghue, 2000).

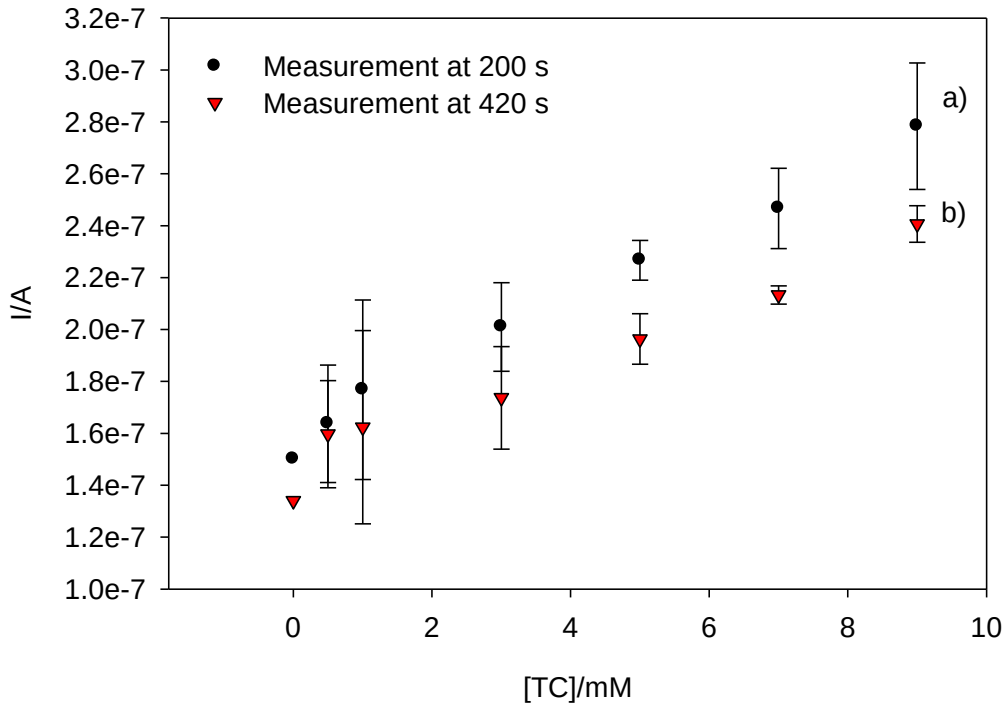

Fig. 6.26. Amperometric measurement of TC using integrated biosensors with unmodified silver paste electrodes (-0.1 V vs Ag/AgCl) at a) 200 s (slope=1.34×10^{-8}, r^2=0.99 and RSD=10% (n=3)) and b) 420 s (slope=9.38×10^{-9}A/mM and r^2 of 0.982, RSD=9.5%; n=3).

While better sensitivity and linearity was observed at 200 s, the integrated biosensor failed to provide a precision of less than the 3%, as required for TC measurement as per NECP guidelines (Bishop, Fody and Schoeff, 2013). Further investigation would be required to improve the sensitivity of the integrated TC biosensor by optimising the concentrations of surfactant in a way that does not interfere with enzymatic reaction as well as finding a way to provide less variable screen printed electrodes. Once again, implementation of rigorous engineering controls would also improve sensor reproducibility.

Recently, a disposable TC biosensor was fabricated using a screen printed three electrode configuration (Fang, 2011). The reagent ink containing ChEs and cholesterol dehydrogenase, combined with nicotinamide adenine dinucleotide (NAD) as coenzyme, and 1,10-phenanthroline-5,6-dione (PD) as the electron transfer mediator in the presence of buffer, binder, filler and enzyme stabilizers was also screen printed onto the surface of the working and reference electrodes to form an integrated reagent layer. The integrated biosensors were then used for amperometric measurement of 1.3 to 13 mM cholesteryl acetate prepared in Triton

X-100/PBS. The current was measured after 38 s at a fixed potential of 300 mV vs Ag/AgCl. The biosensor was stable for up to 100 days and was reported to satisfactorily measure blood TC. However, no detailed analytical data was reported

Manjunatha, *et al.,* (2012) reported the use of functionalized graphene (FG) modified graphite electrodes with good electrocatalytic activity towards H_2O_2 as a platform for the amperometric measurement of TC (0.05-0.3 mM) and FC (0.05-0.35 mM). ChEs and ChOx enzymes were covalently co-immobilised onto the modified electrode and cholesterol palmitate was used as a substrate for ChEs. The integrated biosensor was applied to the amperometric measurement of TC in serum which required a minimum 15-fold dilution to allow its concentration fall within the linear range of the sensor (0.05-0.3 mM), which is not ideal for point of care testing.

Hooda, *et al.,* (2009) reported the fabrication of a cholesterol biosensor based on the incorporation of HRP into a carbon paste screen printed electrode and immobilised ChEs and ChOx using glutaraldehyde onto the surface of a polyvinyl chloride (PVC) 15 mL container to form an electrochemical cell while HRP was added into the carbon paste working electrode. Although the system showed good correlation (r=0.9916) when compared with Bayer's Enzo Kit at 45°, such a system using 15 mL and minimum 200 µL serum sample would not be suitable for development of disposable diagnostic device. However, the experiment showed suitability of using PVC as platform for enzymes immobilisation which might be suitable for future development of biosensors.

6.4 Conclusions

Integrated biosensors for the measurement of HDL-C and TC were developed and optimised and showed an acceptable linear regression of 0.993 (0.25 to 4 mM) and 0.982 (0.5 to 9 mM), respectively which were in agreement with the recommended method linear regression (r^2) reported by the NCEP working group for both HDL-C and TC analyses which is more than 0.975 within their clinical range.

The final HDL-C integrated biosensor showed an acceptable sensitivity of 4.55×10^{-8} A/mM with an average RSD of 6.6% (n=3). These results showed good agreement with the results obtained by the biosensors using external addition of assay reagents (Section 5.2.2; slope=3.32×10^{-8}, RSD=9.5%, n=5). Although better precision was observed using the integrated biosensor, this was still greater than

the precision expected as per NECP criteria for HDL-C measurement which is less than 5%.

The final TC integrated biosensor yielded a sensitivity of 9.38×10^{-9}A/mM with an average RSD of 9.5% (n=3). These results also showed good agreement with the results obtained by using external reagent addition (Section 5.2.2; slope=2.24×10^{-8} A/mM, and average RSD=10.8%; n=3). However, the TC integrated biosensor also failed to provide acceptable precision of less than 3% as per NECP guidelines for TC measurement. The integrated biosensors were stable for twenty days when stored at 2-8 °C.

References

Adult Treatment Panel III (ATP III) (2002) Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. 2012(2/23/2012).

Arya, S.K., Datta, M. and Malhotra, B.D. (2008) Recent advances in cholesterol biosensor. *Biosensors and Bioelectronics*. 23 (7), pp. 1083-1100.

Bishop, M.L., Fody, E.P. and Schoeff, L.E., eds. (2013) *Clinical Chemistry: Principles, Techniques, and Correlations*. seventh ed. USA: Lippincott Williams & Wilkins.

Boekholdt, S.M., Arsenault, B.J., Mora, S., Pedersen, T.R., LaRosa, J.C., Nestel, P.J., Simes, R.J., Durrington, P., Hitman, G.A., Welch, K.M., DeMicco, D.A., Zwinderman, A.H., Clearfield, M.B., Downs, J.R., Tonkin, A.M., Colhoun, H.M., Gotto, J.r., Ridker, P.M. and Kastelein, J.J. (2012) Association of LDL cholesterol, non-HDL cholesterol, and apolipoprotein B levels with risk of cardiovascular events among patients treated with statins: a meta-analysis. *JAMA*. 307, pp. 1302-1309.

Bommarius, A.S. and Payeb, M.F. (2013) Stabilizing biocatalysts. *Chemical Society Review*. 42, pp. 6534-6565.

Brena, B.M. and Batista-Viera, F. (2006) Immobilization of enzymes. In: Guisan, J.M., ed. (2006) *Immobilization of Enzymes and Cells*. Springer, pp. 15-30.

CRMLN, , (2002) *HDL Cholesterol Certification Protocol for Manufacturers*. Report number: CRMLN HDL cholesterol protocol.Cholesterol Reference Method Laboratory Network (CRMLN).

Delaney, J.T., Smith, P.J. and Schubert, U.S. (2009) Inkjet printing of proteins. *Soft Matter*. 5, pp. 4866-4877.

Dey, R.S. and Raj, C.R. (2014) Enzyme-integrated cholesterol biosensing scaffold based on in situ synthesized reduced graphene oxide and dendritic Pd nanostructure. *Biosensors and Bioelectronics*. 62 (0), pp. 357-364.

Eguílaz, M., Serafín, V., Agüí, L., Yáñez-Sedeño, P. and Pingarrón, J.M. (2011) An electrochemical immunosensor for testosterone using gold nanoparticles – carbon nanotubes composite electrodes. *Electroanalysis*. 23, pp. 169-176.

Fang, C. (2011) A disposable amperometric biosensor for determining total cholesterol in whole blood. *Sensors and Actuators B: Chemical*. 155 (2), pp. 545-550.

Foster, R., Cassidy, J. and O'Donoghue, E. (2000) Electrochemical Diagnostic Strip Device for Total Cholesterol and Its Subfractions. *Electroanalysis*. 12 (9), pp. 716-721.

Gibson, T.D. (1999) Biosensors: The Stability Problem. *Analusis*. 27 (7), pp. 630-638.

Gonzalez-Macia, L., Smyth, M.R. and Killard, A.J. (2012) Evaluation of a silver-based electrocatalyst for the determination of hydrogen peroxide formed via enzymatic oxidation. *Talanta*. 99, pp. 989-996.

Hanefeld, U., Cao, L.Q. and Magner, E. (2013) Enzyme immobilisation: fundamental and application. *Chemical Society Review*. 42, pp. 6211-6212.

Hooda, V., Gahlaut, A., Kumar, H. and Pundir, C.S. (2009) Biosensor based on enzyme coupled PVC reaction cell for electrochemical measurement of serum total cholesterol. *Sensors and Actuators B: Chemical*. 136 (1), pp. 235-241.

Hutchings, I.M. and Martin, G.D. (2013) *Inkjet Technology for Digital Fabrication*. 1st ed. UK: John Wiley & sons Ltd.

Iyer, P.V. and Ananthanarayan, L. (2008) Enzyme stability and stabilization—Aqueous and non-aqueous environment. *Process Biochemistry*. 43 (10), pp. 1019-1032.

Jospeh, A.D. (2012) *Inkjet Reagent Deposition for Biosensor Manufacturing* Anon. US 2012/0122197 A1.

Kang, J., Hussain, A.T., Catt, M., Trenell, M., Haggett, B. and Yu, E.H. (2014) Electrochemical detection of non-esterified fatty acid by layer-by-layer assembled enzyme electrode. *Sensors and Actuators B: Chemical*. 190, pp. 435-541.

Kinoshita, H., Torimura, M., Kano, K. and Ikeda, T. (1998) Amperometric determination of high-density lipoprotein cholesterol using polyethylene glycol-modified enzymes and a peroxidase-entrapped electrode. *Annals of Clinical Biochemistry*. 35, pp. 739-744.

Komuro, N., Takaki, S., Suzuki, K. and Citterio, D. (2013) Inkjet printed (bio) chemical sensing device. *Analytical and Bioanalytical Chemistry*. 405 pp.5785-5805.

Kurosaki, Y. and Ogawa, Z. (2009) Reactivity of a detergent, polyoxyethylene derivative, with high density lipoprotein subclass. *Journal of Analytical Bio-Science*. 32 pp. ,434-439.

Magdassi, S., ed. (2008) *The Chemistry of Inkjet Ink*. Singapore: World scientific.

Manjunatha, R., Shivappa Suresh, G., Savio Melo, J., D'Souza, S.F. and Venkatarangaiah Venkatesha, T. (2012) An amperometric bienzymatic cholesterol biosensor based on functionalized graphene modified electrode and its electrocatalytic activity towards total cholesterol determination. *Talanta*. 99 (0), pp. 302-309.

Murphy, L., Burrows, C., Bayly, S., Wilkinson, K. and Harris, J. (2009) *HDL Cholesterol Sensor using Selective Surfactant* Anon. US20090130696 A1.

Pemberton, R.M., Cox, T., Tuffin, R., Sage, I., Drago, G.A., Biddle, N., Griffiths, J., Pittson, R., Johnson, G., Xu, J., Jackson, S.K., Kenna, G., Luxton, R. and Hart, J.P. (2013) Microfabricated glucose biosensor for culture welloperation. *Biosensors and Bioelectronics*. 42 (0), pp. 668-677.

Ram, M.K., Bertoncello, P., Ding, H., Paddeu, S. and Nicolini, C. (2001) Cholesterol biosensors prepared by layer-by-layer technique. *Biosensors & Bioelectronics*. 16, pp. 849-856.

Reintjes, T., (2011) *Solubility Enhancement with BASF Pharma Polymers: Solubilizer Compendium*. BASF SE Pharma ingrefients & services.

Rodrigues, R.C., Ortiz, C., Berenguer-Murcia, A., Torresd, R. and Ferna´ndez-Lafuente, R. (2013) Modifying enzyme activity and selectivity by immobilization. *Chemical Society Review*. 42, pp. 6290-6307.

Rosier, T.A., Cruz, N.B. and Wilkosz, R.K. (2001) *Method and Formulation for Stbilization of Enzymes* Anon. .

Ruecha, N., Siangproh, W. and Chailapakul, O. (2011) A fast and highly sensitive detection of cholesterol using polymer microfluidic devices and amperometric system. *Talanta*. 15 (84), pp. 1323-1328.

Ruecha, N., Rangkupan, R., Rodthongkum, N. and Chailapakul, O. (2014) Novel paper-based cholesterol biosensor using graphene/polyvinylpyrrolidone/polyaniline nanocomposite. *Biosensors and Bioelectronics*. 52 (0), pp. 13-19.

Schuurmans Stekhoven, F.M.A.H., Gorissen, M.H.A.G. and Flik, G. (2008) The isoelectric point, a key to understanding a variety of biochemical problems: a minireview. *Fish Physiology and Biochemistry*. 34, pp. 18.

Secundo, F. (2013) Conformational changes of enzymes upon immobilisation. *Chemical Society Review*. 42 (15), pp. 6250-6261.

Shin, Y.J. and Kameoka, J. (2012) Amperometric cholesterol biosensor using layer-by-layer adsorption technique onto electrospun polyaniline nanofibers. *Journal of Industrial and Engineering Chemistry*. 18 (1), pp. 193-197.

Stone, N.J., Robinson, J., Lichtenstein, A.H., Bairey Merz, C.N., Lloyd-Jones, D.M., Blum, C.B., McBride, P., Eckel, R.H., Schwartz, J.S., Goldberg, A.C., Shero, S.T., Gordon, D., Smith, S.C. and Levy, D. (2014) 2013 ACC/AHA Guideline on the Treatment of Blood Cholesterol to Reduce Atherosclerotic Cardiovascular Risk in Adults. *Journal of the American College of Cardiology*. 62 (25, Part B), pp.2889-2934.

Tamura, H., Nishino, S., Yamaguchi, T. and Hino, K. (2009) *Test Piece for Measureing HDL- Cholesterol,*Daiichi Chemical Co., L. US7575884.

Taylor, J.R. (2004) Cholesterol: Point-of-Care Testing. *The Annals of Pharmacotherapy*. 38 (7), pp. 1252-1257.

Tekin, E., Smith, P.J. and Schubert, U.S. (2008) Inkjet printing as a deposition and patterning tool for polymers and inorganic particles . *Soft Matter*. 4, pp. 703-713.

Tsou, C.L. (1993) Conformational flexibility of enzyme active-sites. *Science*. 262 (5132), pp. 380-381.

Türkarslan, Ö., Kayahan, S.K. and Toppare, L. (2009) A new amperometric cholesterol biosensor based on poly(3,4-ethylenedioxypyrrole). *Sensors and Actuators B: Chemical*. 136 (2), pp. 484-488.

Urmila, S., Madhuri, D., Barbora, A., Borthakur, L., Verma, M., Bora, A. and Goswami, U. (2011) Multiwalled carbon nanotube-based bi-enzyme electrode for total cholesterol estimation in human serum *Journal of Experimental Nanoscience, Vol. 6, Issue 1, Pp. 84-95*. 6 (1), pp. 84-95.

Chapter 7

Overall conclusions and future work

7.1 Overall conclusions

7.1.1 Suitability of using surfactant/salt modified electrodes as a platform for the amperometric measurement of H_2O_2, HDL-C and TC

This study has shown that sensors capable of the direct reduction of H_2O_2 were suitable for employment as a platform for cholesterol measurement with the eradication of one of the enzymatic steps in classical cholesterol determination. Screen-printed, silver paste electrodes modified with a lyotropic layer of DBSA/KCl were successfully employed in the measurement of H_2O_2 generated after the enzymatic reaction of cholesterol with ChOx and ChEs.

The application of a homogeneous HDL-C assay in combination with this electrocatalytic enhancement was assessed. Despite some negative effects caused by some of the assay reagents such as ChOx and components of the sample matrix, the sensor showed acceptable linearity from 0 to 10 mM H_2O_2 with a sensitivity of 3.7×10^{-8} and linearity (r^2) of 0.99 and RSD of 7.2%, which made it suitable for application to the direct determination of HDL-C in serum, based on the end point enzyme assay protocol developed in Chapter 3. The application of other surfactant/salt combinations such as Emulgen B-66/PBS and Triton X-100/PBS for HDL-C and TC measurement, respectively, was then evaluated and all showed comparable sensitivity and linearity for cholesterol measurement using the silver paste electrode platform (Chapter 5). However, the presence of the electrode modification step was found to contribute to the total variability of the resulting biosensors. The modification step could be effectively removed due to the presence of surfactant and salt in the assay reagent mixture, which improved reproducibility at the expense of sensitivity.

7.1.2 Selective measurement of HDL-C and TC

An assay methodology was developed for the differential measurement of both HDL-C and TC at room temperature using a single step homogeneous assay approach and based on the generation of H_2O_2 as the end product of the enzymatic catalysis of cholesterol and cholesterol esters. The assay methodology was initially based on a spectrophotometric measurement approach to allow convenient assay development, but was then adapted to the measurement of HDL-C and TC using an electrochemical transduction methodology. Other key features of the assay was

the minimisation of the sample dilution requirements, so as to ultimately facilitate direct measurement using the electrochemical sensor, and the use of assay reagents that were compatible with the electrochemical sensing principle which was based on the electrocatalytic reduction of H_2O_2 using a modified silver-based screen printed electrode. The spectrophotometric assay developed employed a four-fold sample dilution step and was capable of measuring cholesteryl acetate up to 4 mM. It was found that 6% (w/v) Emulgen B-66 was suitable for the selective measurement of HDL-C over LDL-C through its selective behaviour towards lipoproteins. Triton X-100 (0.5% (w/v)) was successfully used to measure TC in all the lipoproteins regardless of their type. The modified electrodes were capable of measuring both HDL-C (up to 4 mM) and TC (up to 10 mM) by externally addition of assay reagents. The assay reagents were also able to contribute to the electrocatalysis in a manner analogous to the electrode modification layer, which was later employed to simplify the biosensor fabrication approach.

7.1.3 Clinical measurement of HDL-C and TC using HDL-C and TC biosensors

Biosensors for HDL-C and TC were successfully used to measure HDL-C and TC prepared in delipidated serum when the sample was first mixed with the assay reagents before their application to the electrode and which required a two-fold sample dilution. The biosensors were also applied to the measurement of sets of clinical samples. Further improvement in the performance of the biosensor and accompanying assay are required to bring them into sufficient agreement with standard methods. Such lack of agreement may have been mainly due to the effect of the sample matrices on the amperometric measurement methodology using the modified electrodes, as well as the sensitivity of assay methodology itself in the presence of higher concentrations of other assay components such as TG or bilirubin etc (Iizuka *et al.,* 2012; Miller *et al.,* 2010).

7.1.4 Fabrication of integrated HDL-C and TC biosensors

Although the biosensors based on the modified electrodes were capable of measuring HDL-C and TC in delipidated serum with external addition of the assay reagents, fabrication of analogous integrated biosensors capable of measuring small sample volumes presented many additional challenges including the development of the deposition and immobilisation of the assay reagents to result in

stable and reproducible sensors, as well as optimisation of fabrication parameters such as reagent concentration, deposition process parameters, storage conditions and assay methodology. Another key consideration was optimising the assay to allow direct measurement without the requirement for sample dilution.

As was shown in Chapter 6, even a direct deposition, can bring about alterations in enzyme behaviour, and the kinetics of many processes such as concentration/time profiles of assay reagents and substrates and products across and throughout the sample chamber during the measurement process. Additionally, processes such as reagent re-solubilisation and the speed and reproducibility of sample filling will all impact on the analytical performance of the resulting assay. Any changes in enzyme structure may lead to changes in the catalytic activity of the enzymes by either reducing accessibility of the substrate to the enzymes active site or losing the enzymes dynamic properties. Taking into account these factors, development of an integrated HDL-C biosensor was achieved using inkjet-print deposition of an assay reagent formulation containing ChOx and ChEs in 6% (w/v) Emulgen B-66/PBS and 0.2% (w/v) PVP and PDA in order to improve the kinetic of reaction, diffusion rate, reproducibility, sensitivity and linearity as well as stability of the integrated biosensor. However, to improve the linearity of the method, higher concentrations of enzymes were required. Although the final integrated biosensor HDL-C which was fabricated using unmodified electrodes showed comparable sensitivity, linearity and reproducibility with those based on external addition of the reagents, the method still failed to provide acceptable reproducibility and sensitivity to be applied as a diagnostic test device. 0.25% (v/v) Triton X-100 was used instead of Emulgen B-66 for fabrication of integrated TC biosensor as well as greater enzyms concentrations. This sensitivity and reproducibility was found to be poorer for the integrated TC integrated biosensor compared to the sensor based on external reagent addition. ChEs (1,044 U/L) and ChOx (388 U/L) were used for the fabrication of TC integrated biosensor and ChOx (218 U/L) and ChEs (633 U/L) were employed fore for the fabrication of integrated HDL-C biosensor.

To summarise, electrochemical biosensors were developed that were capable of the selective measurement of HDL-C and TC across the analytical range for application in point of care cholesterol screening. Measurements in clinical samples using external addition of assay reagents showed that further imporovement in the measurement approach was required to bring it within acceptable agreement with standard methods and which may be due to as yet unidentified interactions

between the sensor and interfering sample matrix components. In addition, integrated biosensor configurations were also demonstrated for HDL-C and TC measurement in spiked serum samples. While overall reproducibility still lies outside acceptable standards, it is believed that this could be achieved through further biosensor development and production optimisation.

7.2 Future work

7.2.1 Selection and development of an optimal electrochemical sensing platform

Despite the fact that the silver paste electrode platform was capable of the measurement of cholesterol-derived H_2O_2, the signal to background and reproducibility of the modified electrodes were not found to be reach the performance standards required for the clinical measurement of cholesterol due to the variability in and interference of the electrocatalytic process. Employment of a more sensitive and reproducible H_2O_2 sensor which is less prone to sample matrix interference as the measurement platform would be required to achieve this. Recently, a H_2O_2 sensor has been reported based on carbon screen printed electrodes modified using inkjet-print deposition of Prussian blue nanoparticles (PBNPs). The modified electrodes showed an excellent limit of detection of 2×10^{-7} with a linear range of up to 4.5 mM and sensitivity of 7.64×10^{-4} A/mM.cm^2 with excellent reproducibility (RSD) of less than 5% (Cinti *et al.,* 2014). Such analytical characteristics may make this a good platform to be employed for cholesterol measurement, in particular HDL-C measurement due to its excellent sensitivity and limit of detection which is essential for HDL-C measurement which has a narrow clinical range. The application of the PBNPs carbon paste platform could be applied to the selective assay measurement methodologies developed here, as well as for the amperometric measurement H_2O_2 released after enzymatic reaction of LDL-C (Nauck, Warnick and Rifai, 2002) and TG (Fossati and Prencipe, 1982). LDL-C is the main lipoprotein carrying the most blood cholesterol and is known as a primary risk factor for CHD. H_2O_2 released after enzymatic reaction of LDL-C in the presence of its selective surfactant and ChEs and ChOx may be measured using the same strategy considering the effect of its surfactant on modified electrode catalytic activity. Although the Friedewald calculation method is currently the most convenient method which effectively eliminates the need for LDL-C measurement,

fabrication of an integrated LDL-C biosensor could also be considered. Direct measurement may also be more accurate than subtraction for non-HDL-C.

7.2.2 Detailed assessment of the selectivity of the method towards HDL-C subfractions vs non-HDL-C and further study on the mechanism of selectivity

As was discussed in Chapter 1, large HDL_2 and small HDL_3 are the two main fractions of HDL (Kurosaki and Ogawa, 2009; Okada *et al.,* 2001). Okada *et al.,* (2009) reported the recovery of only 76% HDL_2 and 95% of HDL_3 in the presence of 0.9% Emulgen B-66 compared to the standard heparin-$MnCl_2$ method. Only the addition of $MgCl_2$ at 11 mM raised the selectivity towards HDL_3 resulting in a HDL-C concentration similar to the heparin-$MnCl_2$ standard method, confirming the apo E-rich dependency of direct methods as compared to the standard method. Comparison of eight homogeneous assay kits for HDL-C measurement in clinical samples using selective detergents (Kyowa Medex, Sysmex, Kainos and Serotec) and the elimination method (Denaka, Sekisui, Toyobo and Wako) also showed a partial reaction with apo E-rich HDL-C (Iizuka *et al.,* 2012) in most of the methods. Kuroskai and Ogawa (2009) investigated the characteristics of polyoxyethylene derivatives (POED) such as Emulgen B-66 towards HDL-C showing that Emulgen B-66 binds selectively to apolipoprotein-rich HDL-C. This was performed using 0.8% (w/v) Emulgen B-66 showing two separated peaks using gel filtration column with front peak containing more cholesterol than the latter peak.

Despite the fact that there is not yet a clear understanding of which HDL-C subfractions plays the most important role in CAD, recently there have been some reports on the direct measurement of HDL-C subfractions (Ashmaig *et al.,* 2013; Ito *et al.,* 2014). In this study, the evaluation was performed on HDL-C isolated from human serum which yielded similar responses in the presence of specific and non-specific surfactants, while the addition of $MgCl_2$ did not result in any significant difference. However, further investigation into the measurement of HDL-C subfractions may be necessary for any future clinical measurement of HDL-C. In addition, in this study the selectivity was assessed in the presence of LDL-C which represented the most significant source of non-HDL in the circulation. However, VLDL-C also carries some cholesterol, 25% of which may dissolve in 0.9% Emulgen B-66 (Okada *et al.,* 2001). This also needs further investigation for any

future measurement of clinical samples as this may give false positive results in HDL-C measurement.

Further study on the exact mechanism of surfactant action on selective solubilisation of a specific lipoprotein should be performed and the possibility of using LDL-C specific surfactants for the selective measurement of LDL-C should be evaluated.

7.2.3 Clinical measurement of HDL-C and TC

While further clinical studies would be performed to evaluate an alternative H_2O_2 platform which was less sensitive to interferences and variability an extensive study should also be performed using more clinical samples with more detailed information capable of dividing the samples into different groups to provide better understanding of the biosensor functionality in different clinical sample matrices as well as uncertainty estimation of the method as a standard requirement (Eurolab, 2007).

7.2.4 Development of improved integrated HDL-C and TC biosensors

Despite the fact that other researchers have used diffusional barriers such as membranes to effectively dilute the sample, in this research, the direct measurement of cholesterol was chosen in a way that the sample was required to re-solubilise the deposited reagent before commencing the enzymatic reaction. No membranes or other harsh immobilisation techniques were used since the silver modified electrode itself was the basis of H_2O_2 detection, and any small changes in its surface modification may have affected its sensitivity toward H_2O_2. It is believed that using a thinner electrochemical cell with working, counter and reference electrodes closer together may improve the linearity and sensitivity of the method developed in this study (Foster, Cassidy and O'Donoghue, 2000). However by replacing the platform with the PBNP system mentioned earlier, the possibility of using an inkjet-printable membrane to laminate enzyme and stop them from leaking might be greater. To manipulate the permeability of the substrate to the electrode surface, an outer polymeric membrane could be chosen to fabricate a HDL-C or TC sensor based on the sensitivity and linearity required (Maines, Ashworth and Vadgama, 1996; Maines *et al.,* 1996; Maines *et al.,* 2000; Vadgama and Mandler, 2013; Vadgama and Mandler, 2013). Since inkjet-printing gives the possibility of

layer by layer deposition of the reagent, for the fabrication of the integrated biosensor, a detection layer containing PBNPs could be printed first, followed by inkjet-printing of the reagent layers containing ChEs, ChOx and surfactant. The final layer would be an inkjet-printable water-resistant semi-permeable membrane as an outer layer to encapsulate the reagent and manipulate the permeability of the substrate depending on the required linear range. Dip-coated cellulose acetate membranes have been reported to be used to encapsulate biosensors fabricated using inkjet-printing (Setti *et al.,* 2005; Setti *et al.,* 2007; Yun *et al.,* 2011).

Further investigation on improving the stabilisation of enzymes in solution should also be performed considering hydrophilic-hydrophobic interactions of the enzymes with the carrier which can affect on the enzymes dynamic properties as well as its structure using spectrophotometric techniques should be useful (Cantone *et al.,* 2013; Secundo, 2013). Since enzyme stabilisation is an important step in the improvement of integrated biosensor functionality, looking at different modes of enzyme stabilisation such as medium engineering (optimising the formulation) (Bommarius and Payeb, 2013; Cantone *et al.,* 2013) is important. The medium engineering technique is based on stabilisation of proteins relies on formulating suitable buffer conditions which is based on the importance of salt type and its concentration as well as the final solution ionic strength. Bommarius and Paye (2013) in their report ranked some of the most common anions and cations, based on their ability to stabilize or destabilize the enzymes. Among them, phosphate and Mg^{2+} are categorised as strong stabilising ions which can preserve enzymes activity upon the addition. 0.1 M phosphate buffer at pH 6.8 was used for preparation of assay reagent in this report. Some optimisation of buffer concentrations may help to improve sensitivity and linearity of integrated biosensors.

Closing remarks

This thesis has shown that fabrication techniques such as screen printing and inkjet printing can be effectively combined with other processes such as electrocatalysis, selective biochemistries for analysis of cholesterol subfractions and enzymatic assay strategies to create low cost, disposable enzyme-based sensor suitable for the point of care measurement of cholesterols such as HDL-C, TC and non-HDL-C (LDL-C). While these biosensors developed here require further development to make them viable for clinical analysis, significant proof of concept has been demonstrated and routes to its effective implementation have been identified. The

low cost, disposable and printed nature of the sensors also makes them highly compatible with the emerging printed electronics industry, a very important part of which is the development of sensors and diagnostic devices. The printed devices developed here could be effectively integrated with such systems to create new and disruptive healthcare technologies.

References

Ashmaig, M.E., Gupta, S., McConnell, J.P. and Warnick, G.R. (2013) Validation of a novel homogeneous assay for of HDL3-C measurement. *Clinica Chimica Acta*. 425 (0), pp. 37-41.

Bommarius, A.S. and Payeb, M.F. (2013) Stabilizing biocatalysts. *Chemical Society Review*. 42, pp. 6534-6565.

Cantone, C., Ferrario, V., Corici, L., Ebert, C., Fattor, D., Spizzo, P. and Gardossi, L. (2013) Efficient immobilisation of industrial biocatalysts:criteria and constraints for the selection of organic polymeric carriers and immobilisation methods. *Chemical Society Review*. 42, pp. 6262-6276.

Cinti, , Arduini, F., Moscone, D., Palleschi, G. and Killard, A.J. (2014) Development of a Hydrogen Peroxide Sensor Based on Screen-Printed Electrodes Modified with Inkjet-Printed Prussian Blue Nanoparticles. *Sensors, 14* (8), pp. 14222-14234.

Eurolab, (2007) *Measurement Uncertainty Revisited: Alternative Approaches to Uncertainty Evaluation* . Report number: 1/2007.Paris/France: Eurolab.

Fossati, P. and Prencipe, L. (1982) Serum Triglycerides determined colorimetrically with an enzyme that produces Hydrogen Peroxide. *Clinical Chemistry*. 28 (10), pp. 2077-2080.

Foster, R., Cassidy, J. and O'Donoghue, E. (2000) Electrochemical Diagnostic Strip Device for Total Cholesterol and Its Subfractions. *Electroanalysis* . 12 (9), pp. 716-721.

Iizuka, Y., Ishijima, M., Kato, T., Nanmoku, T., Kuwa, K., Kawakami, Y. and Isobe, K. (2012) Clinical sample comparison of eight homogeneous assay kits for high density lipoprotein cholesterol and low density lipoprotein cholesterol. *J Anal Bio-Science*. 35, pp. 168-178.

Ito, Y., Satoh, N., Ishii, T., Kumakura, J. and Hirano, T. (2014) Development of a homogeneous assay for measurement of high-density lipoprotein-subclass cholesterol. *Clinica Chimica Acta*. 427 (0), pp. 86-93.

Kurosaki, Y. and Ogawa, Z. (2009) Reactivity of a detergent, polyoxyethylene derivative, with high density lipoprotein subclass. *Journal of Analytical Bio-Science*. 32, pp. 434-439.

Maines, A., Ashworth, D. and Vadgama, P. (1996) Diffusion restricting outer membranes for greatly extended linearity measurements with glucose oxidase enzyme electrodes. *Analytica Chimica Acta*. 333 (3), pp. 223-231.

Maines, A., Cambiaso, A., Delfino, L., Verreschi, G., Christie, I. and Vadgama, P. (1996) Use of surfactant-modified cellulose acetate for a high-linearity and pH-resistant glucose electrode. *Analytical Communications*. 33 (1), pp. 27-30.

Maines, A., Prodromidis, M.I., Tzouwara-Karayanni, S.M., Karayannis, M.I., Ashworth, D. and Vadgama, P. (2000) An enzyme electrode for extended linearity

citrate measurements based on modified polymeric membranes. *Electroanalysis*. 12 (14), pp. 1118-1123.

Miller, W.G., Myers, G.L., Sakurabayashi, I., Bachmann, L.M., Caudill, S.P., Dziekonski, A., Selvin, E., Kimberly, M., Korzun, W.J., Leary, E.T., Nakajima, K., Nakamura, M., Nilsson, G., Shamburek, R.D., Vetrovec, G.W., Warnick, G.R. and Remaley, A.T. (2010) Seven Direct Methods for Measuring HDL and LDL Cholesterol Compared with Ultracentrifugation Reference Measurement Procedures. *Clinical Chemistry*. 56 (6), pp. 977-986.

Nauck, M., Warnick, G.R. and Rifai, N. (2002) Methods for Measurement of LDL-Cholesterol: A Critical Assessment of Direct Measurement by Homogeneous Assays versus Calculation. *Clinical Chemistry*. 45 (2), pp. 236-254.

Okada, M., Matsui, H., Ito, Y. and Fujiwara, A. (2001) Direct measurement of HDL cholesterol:Method eliminating apolipoprotein E-rich particles. *Journal of Clinical Laboratory Analysis*. 15 (4), pp. 223-229.

Secundo, F. (2013) Conformational changes of enzymes upon immobilisation. *Chemical Society Review*. 42 (15), pp. 6250-6261.

Setti, L., Fraleoni-Morgera, A., Ballarin, B., Filippini, A., Frascaro, D. and Piana, C. (2005) An amperometric glucose biosensor prototype fabricated by thermal inkjet printing. *Biosensors & Bioelectronics*. 20 (10), pp. 2019-2026.

Setti, L., Fraleoni-Morgera, A., Mencarelli, I., Filippini, A., Ballarin, B. and Di Biase, M. (2007) An HRP-based amperometric biosensor fabricated by thermal inkjet printing. *Sensors and Actuators B-Chemical*. 126 (1), pp. 252-257.

Vadgama, P. and Mandler, D. (2013) Characterization of thin films and membranes. *Analytical and Bioanalytical Chemistry*. 405 (5), pp. 1433-1434.

Yun, Y.H., Lee, B.K., Choi, J.S., Kim, S., Yoo, B., Kim, Y.S., Park, K. and Cho, Y.W. (2011) A Glucose Sensor Fabricated by Piezoelectric Inkjet Printing of Conducting Polymers and Bienzymes. *Analytical Sciences*. 27 (4), pp. 375-379.

List of Publications and Presentations

- T. Ahmadraji, L. Gonzalez-macia and T.J Killard, A biosensor for the determination of high density lipoprotein cholesterol employing combined surfactant-derived selectivity and sensitivity enhancements, ***Anal. Methods***, 2014,**6**, 3975-3981.

- T. Ahmadraji and T.J. Killard, The evolution of selective analyses of HDL and LDL cholesterol in clinical and point of care testing, **Anal. Methods**, 2013,5, 3612-3625.

- IOP Institute of Physics (Advanced printing for medical and health application), Development of a printed biosensor for HDL-cholesterol, Swansea University, UK, 24 Apr 2012

- Great Western Electrochemistry Meeting, Development of a biosensor for HDL-cholesterol based on a novel electrocatalyst for hydrogen peroxide reduction, Bath, UK, 18 June 2012..

- Electrochem 2012 (Poster), Development of a biosensor for HDL-Cholesterol based on a novel electrocatalyst for hydrogen peroxide reduction, Trinity College Dublin, Ireland, 2-4 Sep 2012.

- IOP Students conference 2012 (Oral presentation), Development of a printed biosensor for HDL-cholesterol, Institute of Physics, London, UK, 8 Dec 2013.

- CRIB Annual Meeting 2013 (Poster), Development of a biosensor for HDL-cholesterol based on a novel electrocatalyst for hydrogen peroxide reduction, UWE, Bristol, UK, 11 Jan 2013.

- 3rd International Conference on Bio-Sensing Technology (Poster), Printed biosensors for the selective determination of HDL-C, Sitges (near Barcelona), Spain, 12-15 May 2013.

- Great Western Electrochemistry Meeting (Oral presentation), Development of a printed HDL-cholesterol biosensor based on a novel electrocatalyst for hydrogen peroxide reduction, Bath, UK, 3 Jun 2013.

- HLS Postgraduate Research Conference (winner of the best Poster presentation prize), Printed biosensors for the selective determination of HDL-C, UWE, Bristol, UK, 28 Jun 2013.

- Analytical research forum 2013 (Oral presentation), Development of a printed biosensor for selective determination High Density Lipoprotein Cholesterol (HDL-C), GlaxoSmithKline & the University of Hertfordshire, UK, 8 - 10 July 2013.

- Electrochem 2013 (Pster presentation), Amperometric dtermination of high density lipoprotein and total cholesterol in serum using a printed biosensor, University of Southampton, UK, 1-3 September 2013.

- CRIB Annual Meeting 2014 (winner of the best Poster presentation prize), Development of an electrochemical sensor for high density lipoprotein cholesterol and total cholesterol using modified silver screen printed electrodes, UWE, Bristol, UK, 10 Jan 2014.

- Sensors in medicine 2014 (Poster presentation), A biosensor for the amperometric determination of high density lipoprotein cholesterol based on a printed lyotropic surfactant layer, Royal Geographic Society, London, 25 - 26 Mar 2014.

- Biosensor 2014 (Poster presentation), Amperometric determination of serum high density lipoprotein cholesterol and total cholesterol using a printed surfactant lyotropic layer , Melbourne, Australia, May 2014.

- ESEAC 2014 (winner of the best Poster presentation prize), A biosensor for the amperometric determination of high density lipoprotein cholesterol based on a printed lyotropic surfactant layer, Switzerland, Jun 2014.

- HLS Postgraduate Research Conference (Oral presentation), A biosensor for the amperometric determination of HDL-cholesterol based on a printed lyotropic surfactant layer, UWE, Bristol, UK, 27 Jun 2014.