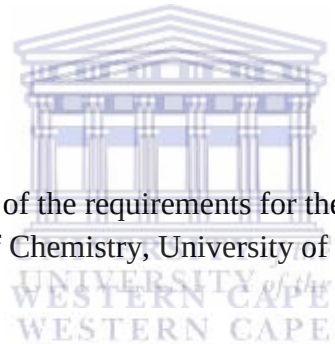
ELECTROCHEMICAL IMMUNOSENSOR BASED ON CYCLODEXTRIN SUPRAMOLECULAR INTERACTIONS FOR THE DETECTION OF HUMAN CHORIONIC GONADOTROPIN

BY

LINDSAY WILSON



A thesis submitted in fulfilment of the requirements for the degree of Magister Scientiae in the Department of Chemistry, University of the Western Cape.

WESTERN CAPE

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KEYWORDS

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β-Cyclodextrin epichlorohydrin polymer

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Co-operative effect

Cyclic Voltammetry

Square Wave Voltammetry

Enzyme Linked Immunosorbent Assay

Amperometric

Human Chorionic Gonadotropin

ABSTRACT

Glucose oxidase (GOx) and horseradish peroxidase (HRP) are important enzymes for the development of amperometric enzyme linked immunosensors. The selectivity of each enzyme towards its analyte deepens its importance in determining the sensitivity of the resultant immunosensor. In designing immunosensors that have customized transducer surfaces, the incorporation with FAD and iron based enzymes ensures that electron kinetics remains optimal for electrochemical measurement. Various different immobilization strategies are used to produce response signals directly proportional to the concentration of analyte with minimal interferences. The combination of self-assembled monolayers and supramolecular chemistry affords stability and simplicity in immunosensor design. In this work, two electrochemical strategies for the detection of human chorionic gonadotropin (hCG) is presented. This involves the modification of a gold surface with a thiolated β-cyclodextrin epichlorohydrin polymer (βCDPSH) to form a supramolecular inclusion complex with ferrocene (Fc)-functionalised carboxymethyl cellulose polymer (CMC). Cyclic voltammetry indicated that ferrocene is in close proximity to the electrode surface due to the supramolecular complex formed with βCDPSH. Furthermore, strategy (a) for the detection of hCG used α-antihCG labelled (HRP) as reporter conjugate. Strategy (b) maintained the CMC bifunctionalised with Fc and recognition antibody for hCG hormone. However, the system was functionalised with a HRP enzyme and detection is done by using GOx reporter conjugates for *in situ* production of hydrogen peroxide. The reduction of H_2O_2 was used for the amperometric detection of hCG by applying a potential of 200 mV. The sensitivity and limit of detection of both strategies were calculated from calibration plots. For strategy (a) the LOD was found to be 3.7283 ng/mL corresponding to 33.56 mIU/mL and a sensitivity of 0.0914 nA ng^{-1} mL^{-1}. The corresponding values for strategy (b) are 700 pg/mL (6.3 mIU/mL) and 0.94 nA ng^{-1} mL^{-1}.

DECLARATION

I declare that the *Electrochemical Immunosensor based on Cyclodextrin Supramolecular Interactions for the Detection of Human Chorionic Gonadotropin* is my own work that has not been submitted for any degree or examination in any other university, that all sources I have used or quoted have been indicated and acknowledged by means of complete references

Lindsay Robin Wilson December 2012

Signed …….

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‘My son, forget not my law: but let thine heart keep my commandments’, a verse from Proverbs chapter 3. To **God Almighty**, whom has given me strength, wisdom and vision, I THANK YOU. For always having Your merciful Hand over me and guiding me through hardships and good times. Ask and you shall receive. Giveth and you shall receive 10 fold in return. Father, your Love and Guidance gave me the strength to complete this work.

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DEDICATION

This project is dedicated to

The

Almighty God

And

My Dad, My Mom and My brother

Mr Cyril Wilson, Mrs Joslyn Wilson and Joshua Wilson

Contents

LIST OF PUBLICATIONS

Wilson, L. Ortiz, M. O'Sullivan, K. Iwuoha, E. Supramolecular platform for the detection of Human Chorionic Gonadotropin. Biosensors and Bioelectronics. Submitted 2012

Wilson, L. Ortiz, M. O'Sullivan, K. Iwuoha, E. Development of stable cyclodextrin platforms for anchoring of biomolecules for electrochemical immunosensors. ISE Conference. Abstract Submitted 2012

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LIST OF ABREVIATIONS

CD	Cyclodextrin
Fc	Ferrocene
CMC	Carboxymethyl cellulose
CV	Cyclic Voltammetry
SWV	Square Wave Voltammetry
ELISA	Enzyme Linked Immunosorbent Assay
$I_{p,a}$	Anodic peak current
$I_{p,c}$	Cathodic peak current
$E_{p,c}$	Cathodic peak potential
$E_{p,a}$	Anodic peak potential
hCG	Human Chorionic Gonadotropin
PBS	Phosphate buffer
SAM	Self-Assembled Monolayer
NMR	Nuclear Magnetic Resonance
FTIR	Fourier-Transform Infrared Spectroscopy
SNFTIR	Subtractive Normalised Fourier Transform Infrared Spectroscopy

CHAPTER 1

1. Background

1.1. Introduction

Among the vast different types of analytical immunoassays, biosensors afford a more flexible method due to the variety of materials and recognition elements that are used. They achieve much greater reproducibility, selectivity and rapid immunoassays. Such immunosensors are driven by their remarkable selectivity in there molecular recognition occurring between antibodies and antigens. The strength in the selectivity derives from antibody-antigen complexes which interact through ligand binding associations [Luppa *et al*., 2001]. The most commonly used immunoassay in laboratory practice, Enzyme linked immunosorbent assay offers good selectivity with slow operation times. It is imperative to design new methods that offer rapid response, selective and sensitive detection, which are currently under study for point of care analysis. Currently, miniaturized systems for point of care analysis are being studied which incorporates either chemiluminescence, fluorescence, optical or electrochemical detection. Since its initiation, electrochemical sensors have been shown to meet the requirements for point of care analysis, capable of providing rapid information with a reduction in costs. Various electrochemical immunosensors are based on similar concepts to ELISA operation methods, colorimetric assays which force the drive towards implementing electrochemical immunosensors with dissimilar operation methods. The design in terms of tailoring immobilization strategies which meets the equilibrium between the different types of biomaterial immobilized and their relationship between the transducer and immobilized biomaterial is of significant importance. Tailored immobilization strategies are currently being evaluated which includes electropolymerization, sol-gel entrapment, physical

adsorption, electrostatic interactions and covalent binding [Sassolas *et al*., 2012]. Designing material for tailored immobilization for novel strategies includes use of self-assembled monolayers (SAM's). Self-assembled monolayers are characteristic for reducing random orientation of recognition elements, reducing non-specific interactions, increasing surface area of immunosensor as well as improving stability. These materials (SAM'S) show interchangeable characteristics, with few nm-thickness to well over several hundred nm-thickness.

SAM's occur through covalent linkage onto metal surfaces by using sulphides, amines, thiols or silanes. SAM's with thiol terminated ends on gold surfaces has been the most extensively studied due to the thiol-gold bond's high stability and affinity. Cyclodextrin modified with thiols immobilized onto gold surfaces has been extensively studied, since they provide an improved orientated recognition layer, improved sensitivity and significantly reduced non-specific binding. The incorporation of the SAM character of thiolated cyclodextrin and its supramolecular interactions, host-guest inclusion complexes, has gained much attention. Since these host guest complexes provide a stable, well orientated and reversible strategy for redox activity for immunosensors. The development of such strategies which can incorporate ferrocene or adamantane moieties has demonstrated characteristic improvements in electrode surface engineering [Ortiz *et al*., 2011].

1.2. Problem Statement

During the length of pregnancy, multiple different forms of hCG exist in urine and serum. Namely, non-nicked hCG (whole hormone), nicked hCG, free and nicked α- and β- subunits, hyperglycosylated and hypoglycosylated [Coleet *et al*., 1997]. Various immunoassays measure non-nicked hCG and one of the seven different forms. After nicking hCG (whole hormone), increase in nicked and free β-subunit occurs which may be used in the detection of pregnancy but the stability of resulting forms decreases dramatically. The concentration increase of these two forms and decrease in there stability causes fluctuations in true concentration of the two forms. These fluctuations cause inconsistent results when doing immunoassays of hCG when using both nicked and free β-subunit as references. It is imperative to design immunoassays for hCG solely by reducing such fluctuations. Most commonly employed immunoassay in clinical diagnostics, enzyme-linked immunosorbent assay [ELISA], utilizes antibodies specific for β-subunit on hCG, label enzymes and a colorimetric substrate for producing amplified signal for antigen. The assay is time-consuming with long incubation steps.

Therefore tailoring platform architectures for immunosensor design for improved rapid response, stability, selectivity and reproducibility depends on the alteration of materials at the molecular scale [Ortiz *et al*., 2011] taking into account immunoassay detection design. Such design requires simple, pre-organization and well-orientated strategies for immobilization of bio-recognition elements improving toleration of analytes.

1.3. Aim of Research

This work utilizes a supramolecular based platform in immunosensor design and detection strategy for hCG.

Two design strategies are implemented, both bearing thiolated β-cyclodextrin polymer as a support layer on a gold surface. Supramolecular association between ferrocene and β-cyclodextrin is completed by using ferrocene moiety covalently attached to carboxymethyl cellulose (CMC) linear polymer to achieve multiple complex host-guest interactions.

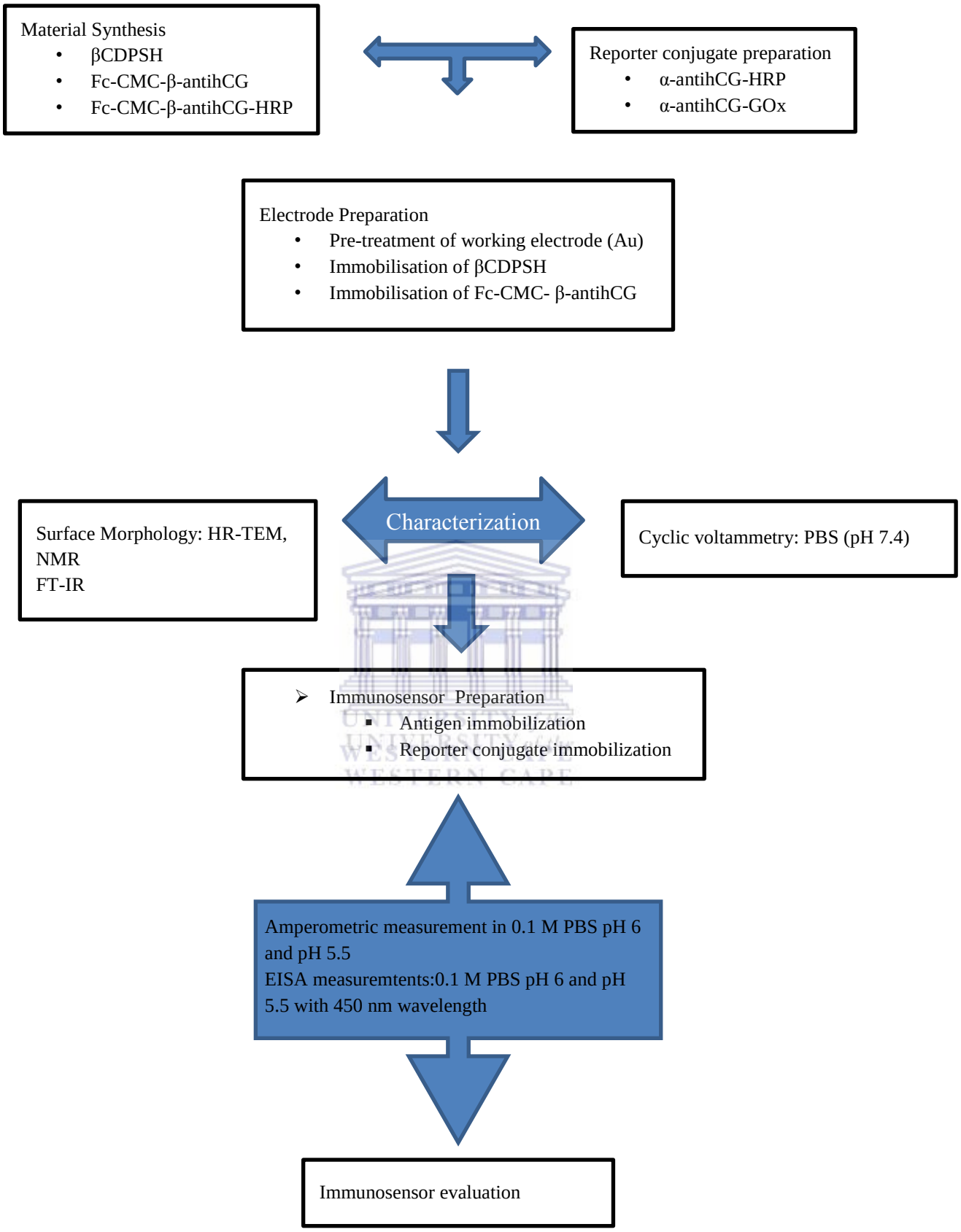
- Synthesis of thiolated β-cyclodextrin polymer.
- Synthesis of ferrocene functionalised carboxymethyl cellulose bearing capture antibodies (β-antihCG, strategy a) and further bi-functionalised with horseradish peroxidase (HRP) (strategy b).
- Electrochemical characterization of inclusion complex between β-cyclodextrin and ferrocene.
- Synthesis of reporter conjugates bearing HRP-α-antihCG antibody (strategy a) and glucose oxidase- α-antihCG antibody (strategy a).
- Enzyme activity, reporter probe concentration optimization and capability of bi-enzymatic system (strategy b).
- Development of amperometric immunosensor for the detection of human chorionic gonadotropin.

1.4. Rationale and Motivation

Various platforms have been utilized for the qualitative and quantitative detection of hCG. Polymeric based covalent attachment of hCG antibody [Truong *et al*., 2011], nanocomposites of carbon nanotubes [Yang *et al*., 2011], nanoporous gold and graphene have been evaluated for fast and label-free amperometric detection of hCG. An important concept is utilizing simplicity in immunosensor design to achieve these goals for accurate and selective immunosensor systems. Thiolated self-assembled monolayers on gold surfaces for electrode modification with functional molecules have shown to induce control of the microenvironment at the interface of biomolecule. The multivalent interaction between β-cyclodextrin and ferrocene increases the strength in the inclusion process.

1.5. Research Framework

Research methodology leading to final results followed sequential steps. A summarised flow diagram below shows study objectives in line with experimental procedure.



Scheme 1: Research Framework

1.6. Outline of thesis

This thesis will be presented as follows:

Introduction outlines pregnancy and relation between human chorionic gonadotropin and its different forms. The importance of the detection of hCG hormone accurately during the first weeks of pregnancy and how pregnancy tests accuracy is significantly questionable in Chapter 1. The use of biosensor and there development for improved diagnostics as well as the use and advantages of using self-assembled monolayers of modified β-cyclodextrin. The objective of this study, framework and limitations are also discussed in this chapter.

A literature review which relates the importance of protein detection and immunosensor design is described. Properties and characteristics of β-cyclodextrin, its polymer derivative and their application in biosensors are described in Chapter 2. The different parameters in biosensors, biomolecules and the immobilization are presented in this chapter. This chapter also covers a brief introduction into supramolecular associations and how they play a role in development of biosensors as well as the characteristics of supramolecular host guest inclusion complexes. Furthermore, the use of ferrocene in biosensors and its host-guest complex formation is presented. Characterization methods, such as cyclic voltammetry, square wave voltammetry and transmission electron microscopy are briefly discussed in this chapter. Chapter 3 contains information regarding chemicals used, instrumentation and research design with an overview of all the successive phases taken to meet the objectives.

Chapter 4 and 5 include results from experimental followed in Chapter 3. Chapter 4 contains a focussed discussion on the characterization of the development of polymers and supramolecular inclusion complexes and their behaviour on an electrode surfaces, as well structural analysis for synthesis of polymers. Chapter 5 discusses the immunosensor response

for the detection of hCG with chronoamperometry. A more detailed description of immunosensor and its analytical characteristics such as limits of detection, linear range and more important the co-operative effect is presented in this chapter.

Chapter 6 concludes the results from both the structural and electrochemical characterization of β-cyclodextrin and ferrocene-carboxymethyl cellulose with gold and silver electrodes. Electrochemical characterization of supramolecular complex formed on gold surface, ELISA measurements and detections and finally the electrochemical detection of human chorionic gonadotrophin is observed in this chapter.

CHAPTER 2

2. Literature Review

2.1. Surpamolecular Associations

Supramolecular chemistry was inspired by biological molecular architectures, mainly proteins and lipids and their interactions. It is the foundation for almost all essential biochemical process in life, the building blocks of complex biological molecules. It goes beyond the covalent bond in molecular chemistry, the chemistry of the intermolecular bond [Lehn *et al*., 1995]. It is the study of non-covalent interactions between molecules and how they form multimolecular complexes and ultimately the ability to have control over the intermolecular bond. It encompasses a range of different systems, from host-guest systems to machines, molecular recognition and more advanced systems called self-processes [Steed et al., 2009], self-assembly and self-organisation.

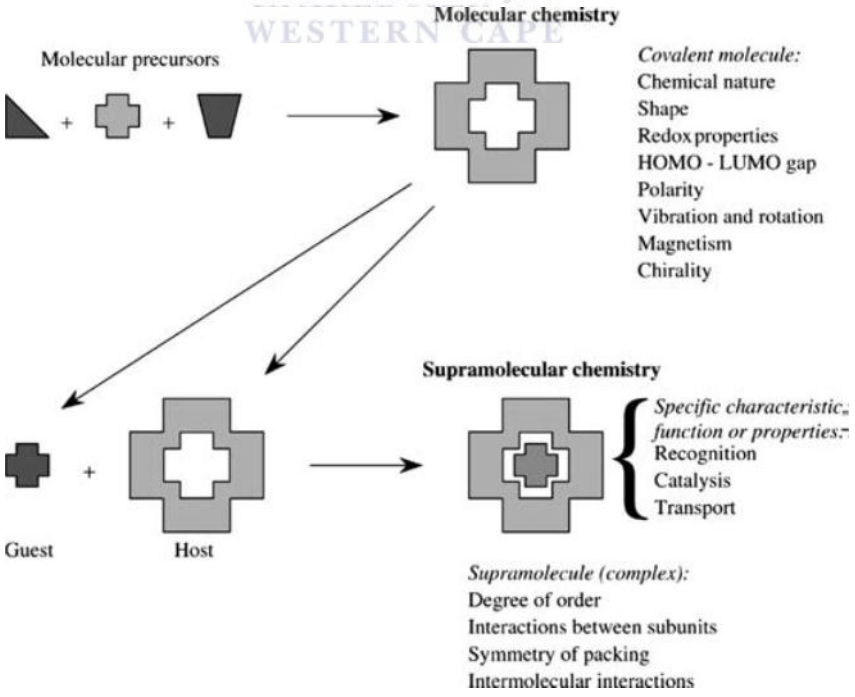


Figure 1. Schematic diagram showing comparison between molecular and supramolecular chemistry

Control over the intermolecular bond initiates by mimicking nature and its complex biological assemblies by creating functional small building blocks as new synthetic materials, creating assemblies in the micro and nanoscale range [Xu *et al*., 2009]. The capability to design and synthesize multimolecular supramolecular systems maintaining comparable functions and characteristics to lipids or oligonucleotides [Uhlenheuer *et a*l., 2010] remains a complex nature. These supramolecular systems result from two or more chemical molecules linked together through intermolecular forces such as hydrogen bonding, dipole-dipole, ion-dipole, cation-pi interactions and the hydrophobic effect. The hydrophobic effect derives from the association of organic molecules in aqueous media. It is been seen as the single most important factor in biological molecular recognition and the major factor in protein folding.

One important aspect in biotechnology and bioengineering is molecular complexation. This tool is important for achieving good selectivity, separation and more importantly solubilizing many biomolecules. In nature, most of the complexing agents that are readily available are proteins, antibodies and zeolites, inorganic agents. Synthetic molecular agents are costly to synthesize and have a very limited capability such as fullerenes and crown ethers. Cyclodextrins are good replacements as molecular agents since they attain a cage-like structure, used for supramolecular interactions such as cryptands, calixarenes and cyclophanes. These classes of cage-like compounds are characteristics for chemical reactions of intermolecular interactions where covalent bonding is not formed. This can be seen as the hydrophobic effect.

2.2. Cyclodextrin and its properties

Cyclodextrins were discovered over a hundred years ago by A Villiers. Villiers named the compound cellulosine and determined its composition. He then identified 6 and 7 membered cyclic oligomers α and β-cellulosine. Later, microbiologist F Schardinger isolated 2 compounds, 1 and 2, from bacterial digest of potato starch and his studies revealed that the second compound was identical to Villiers β-cyclodextrin. It wasn't until Freudenberg discovered ϒ-cyclodextrin and showed that they are in fact cyclic oligosaccharides. It was during the first half of the 20th century, that cyclodextrins was shown to form stable aqueous complexes. All though the three main cyclodextrins were discovered not much was known about the chemical structure and physiochemical properties. Freudenberg showed that cyclodextrin are α (1-4) linked with a central cavity. Towards the second half of the century, all three natural occurring cyclodextrins had been chemically and structurally characterised [Loftsson *et al*., 2007]. Following this work, was exploring the capabilities of encapsulating different molecules within the hydrophobic core of cyclodextrins. Fewer glucopyranose units than 6 forms unstable cyclodextrins, since there is a high degree of steric hindrances. Cyclodextrins with higher glucopyranose units, 9 or 10 units are unstable and contain impurities when synthesized. Associated problem with higher units of glucopyranose in cyclodextrins is that the cavity is prone to collapsing and may only contain smaller molecules. Cyclodextrin with 6, 7 and 8 glucopyranose units has diameters of approximately 5, 6.3 and 7.9 Å. The size and depth of the cavity determines the size of the guest molecule for encapsulation and binding strength.

Table 1: Properties of cyclodextrins

Cyclodextrin Properties			
Property	α-cyclodextrin	β-cyclodextrin	γ-cyclodextrin
Number of glucopyranose units	6	7	8
Molecular weight (g/mol)	972	1135	1267
Outer diameter (Å)	14.6	15.4	17.5
Cavity Diameter (Å)	4.7-5.3	6.0-6.5	7.5-8.3
Height of torus (Å)	7.9	7.9	7.9
Cavity volume ($Å^3$)	174	262	427
Solubility in water (g L^{-1}, 25^oC)	14.5	18.5	23.2

In aqueous solutions and in the presence of hydrophobic molecules, cyclodextrin forms inclusion complexes where the hydrophobic molecule situates itself in the cavity of the cyclodextrin. The hydroxyl groups on outer surface of cyclodextrin can form hydrogen bonds with surrounding water molecules or other molecules. In saturated aqueous solutions, both inclusion and non-inclusion complexes exist in solution. Modifications for cyclodextrins are done at the hydroxyl groups at position 6, 2 and 3. Depending on the nature of the hydroxyl group, functionalization could be done either by nucleophilic or electrophilic attack. The three types of hydroxyl groups, position 6 are the most basic and more nucleophilic, the OH's at position-2 are most acidic and those at postion-3 are the most inaccessible out of the three.

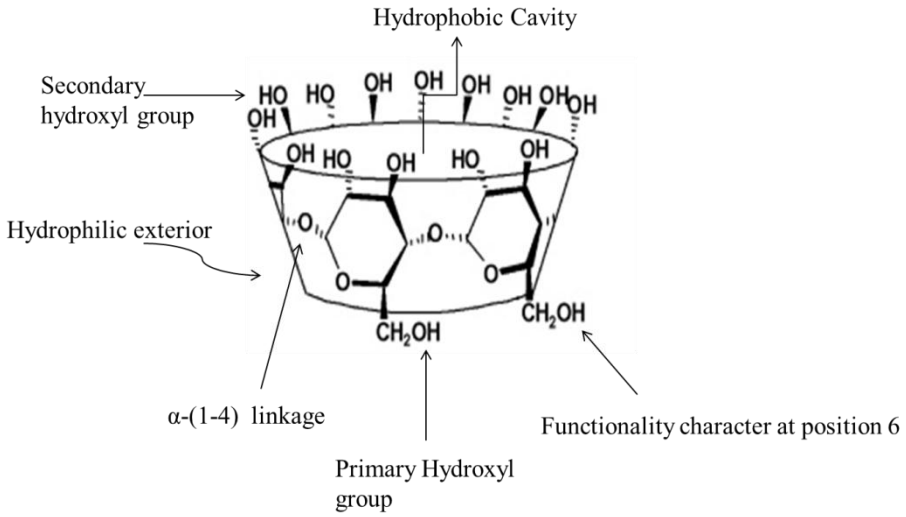


Figure 2. Structure of β-cyclodextrin

2.2.1. Cyclodextrin Polymers

Drawback of β-cyclodextrin (β-CD) is its reasonably poor aqueous solubility. This is due to its intrinsic structure, the molecular dimensions can induce formation of a ring of intramolecular hydrogen bonds that counteract hydration of βCD [Kurkov *et al*., 2012], reducing the solubility. Overcoming this problem arose the chemical substitution of multiple β-cyclodextrin hydroxyls by covalent attachment of organic species to produce different derivatives of β-cyclodextrin, namely 2-hydroxypropyl (HPβCD and HPγCD). Chemical modification greatly improved aqueous solubility. Another direction to improve the aqueous solubility is by using an epichlorohydrin cross linker which forms a β-cyclodextrin epichlorohydrin polymer (β-CDEPI). The reaction is done in alkaline media. Cross-linked βCD polymers improve the stability constants for inclusion complexes it forms with lipophilic guests [Renard *et al*., 1997]. Various methods have been developed for chemical polymerization of βCD such as radical polymerization of acryloyl cyclodextrin monomers, cross linkage with maleic anhydride in anhydrous N,N-dimethyl formamide (DMF) [Girek *et al*., 2000], cross linkage with succinic anhydride with anhydrous DMF [Girek *et al*., 2005]

and cross linkage with epichlorohydrin which has been extensively studied. After polymerization, βCD can be functionalised with different functional groups. The ease of functionalization of βCD lays its structure, availability of hydroxyl groups at position 2 and 6.

2.2.2. Cyclodextrin Host-Guest Complexes

Host-guest inclusion complexes of CD are molecular complexes characterised by the entrapment of lipophilic molecules within the hydrophobic CD cavity with molecules such as hydrocarbons to polar molecules, acids, amines and parts of larger compounds. In the pharmaceutics industry the major use of CD is for improving solubility and bioavailability of drugs [Davies *et al*., 2004]. CD inclusion complex formation is considered to be a reversible process. Analysis of inclusion complexes are done by use of nuclear magnetic resonance, monitoring the change in proton environment due to increase in hydrogen bonding from guest to host. Another useful technique, mass spectroscopy is also used for structural analysis of inclusion complexes. Generally, inclusion complexes are formed by addition of a guest to saturated solution of cyclodextrin. All lipophilic guests are captured within the hydrophobic cavity of CD with the ring assuming a more symmetrical conformation [Semlyen et al,. 1996].

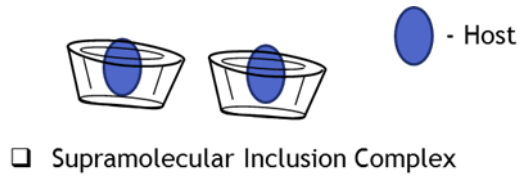


Figure 3. Supramolecular inclusion complex with cyclodextrin with 1:1 stoichiometry

During a 1:1 inclusion complex formation between CD and lipophilic guest molecule (G), the following equation is obtained:

$xG + yCD \leftrightarrows G_xCD_y$

Where x guest molecules of G forms an inclusion complex with y CD molecules with a stoichiometry of x: y. The stability constant for this inclusion complex for a stoichiometry of 1:1 can be represented as:

$$K_{1:1} = \frac{[G/CD]}{[G] * [CD]} \quad (1)$$

In equation 1, K represents the binding constants, and [ ] represents corresponding concentrations of CD and guest molecule. An interesting study done by Liu and colleagues showed that the release of high energy water molecules from the CD cavity is not the driving force for complex formation, due to water molecules attaining higher conformational freedom [Lui *et al*., 2002].

2.3. Ferrocene and its inclusion complexes

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Since early developments in organometallic materials, there applications grew in vast directions, from non-linear optical devices, light emitting diodes to electrochemical sensors [Hudson., 2001]. Ferrocene is a metallocene which consists of a ring system pair, an organometallic compound bearing two cyclopentadienyl rings bound on opposite's sides of a central iron atom. Iron centre is assigned a +2 charge. The two corresponding C_5H_5 rings provide 10 molecular orbitals which overlap with iron's *p* and *d* orbitals and yields the 18 electron configuration stable metallocene. It is one of the most extensively studied metallocenes. It's characteristic for maintaining structural stability when oxidised to form its corresponding ferrocenium ion. Ferrocene is stable in organic solvent under electrochemical study, with rapid response to electroactive substance [Zhang *et al*., 2011]. The low solubility of ferrocene in aqueous media limits its electron transport capabilities. To overcome this, chemical modifications to ferrocene has been done to achieve much better solubility in

aqueous media such that it can be utilized in biochemical studies [Kolivoska *et al*., 2011]. Chemical modifications of ferrocene is a complicated processes, and requires harsh conditions. Ferrocene undergoes Friedal-crafts substitution to produce acetylferrocene derivative, from which ferrocenecarboxaldehyde can be synthesized. Such derivatives allow the incorporation of ferrocene into various applications.

Ferrocence is one of the most studied electroactive species in supramolecular electrochemistry. It forms supramolecular complexes with vast hosts, cyclodextrin, cucurbit[n]urils, cavitands, cyclophanes, through non-covalent interactions, mainly hydrogen bonding and hydrophobic interactions. These interactions are used to produce ferrocene that is soluble in aqueous media by forming host-guest complexes. Such complexes uses the hydrophobic inner core of the host to form the inclusion complex with ferrocene, where the outer regions of the host remains hydrophilic, rendering ferrocene soluble in aqueous media and maintaining its electron transfer capability in such media. Li 2012 showed the inclusion complex formed between ferrocene and its derivatives with cucurbiturils are stable, required mild conditions, with binding constants from 10^{9} M^{-1} to 10^{12} M^{-1}. A decrease in binding affinity is observed from deprotonation and Fc one electron oxidation to 10^{7} M^{-1}. Podkosienly showed that inclusion complexes formed between ferrocene derivatives and cavitand decreased the electrochemical kinetics in aqueous solutions as result from stable inclusion complex as compared to free guest [Podkosielny *et al*., 2008].

Ferrocene adsorbs poorly onto electrode surfaces, due to hydrophobic/hydrophilic barrier between the surface of an electrode and ferrocene. Inclusion complexes attached onto the surface of an electrode through covalent or non-covalent bonds allows outer hydrophilic

region of hosts to overcome the barrier [Huang *et al*., 2012]. The host-guest complex with ferrocene can be used to attach the mediator onto electrode surface.

2.4. Human Chorionic Gonadotropin (hCG)

2.4.1. hCG Hormone and its Properties

Human chorionic gonadotropin (hCG) is a hormone produced during pregnancy and to certain extent non-pregnant women. The hormone is a glycoprotein that has two dissimilar units, α- and β-subunit which are bound to each other through non-covalent charge interactions [Montagnana *et al*., 2011]. These two subunits are important for biological and immunological activity of hCG hormone. The α-subunit is similar in structure and hence activity to thyroid stimulating hormone and luteinizing hormone, where the β-subunit is more specific for the biologic and immunologic activity of the hormone. It is produced at high concentrations through the placental trophoblasts. Clinically, hormone concentration above 5 mIU/mL is an indication of pregnancy with various home pregnancy tests having limits of detection between 6.3 - 20 mIU/mL. Abnormally high concentrations of hCG hormone in non-pregnant women could be an indication of trophoblastic tumour growth [Stenman *et al*., 2004]. Using hCG as a biomarker, detection of preeclampsia can be done which is a vasoconstrictive disorder that occurs after the 20^{th} week of pregnancy. Bearing in mind that this disorder may result from a host of factors, one being abnormal high concentrations of hCG of 69.70 kIU/L, with normal concentrations being around 24.65 kIU/L [Bouvier *et al*., 2005]. Human chorionic gonadotrophin is a 237 aminoacid glycoprotein hormone, which is produced during pregnancy. It is secreted from the syncytiotrophoblast cell layer after conception. Due to the hormones structural heterogeneity it exists in fluids in different forms or isoforms namely, nicked hCG, free β-subunit, free α-subunits, intact active hormone and

β-core fragment. The average molecular weight of the hormone as estimated by mass spectrometry gives 37.5 kDa, 23.5 kDa for β-subunit and 14 kDa for α-subunit. Average of 30% of the hormone weight is due to carbohydrate content. The α-subunit contains 92 amino acids, 2 N-linked carbohydrate chains and 5 disulphide bridges whereas the β-subunit contains 145 amino acids, 2 N-linked and 4 O-linked carbohydrate chains, and 6 disulphide bridges. The physiology of hCG extends functional life to the corpus luteum, maintains a high progesterone concentration during early pregnancy and at high concentrations becomes thyrotropic.

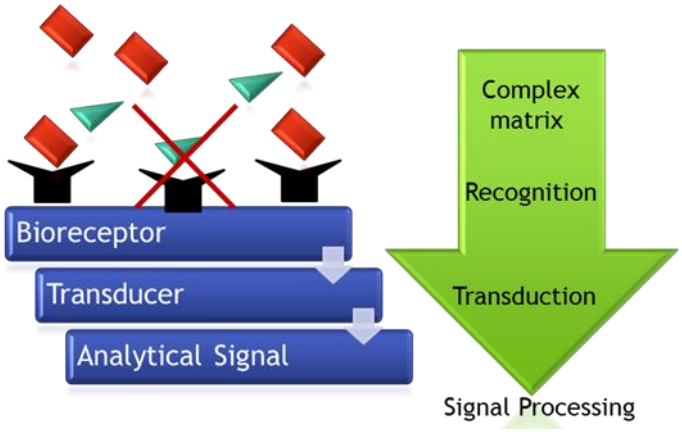
2.4.2. Diagnostics Methods

The measurement of hCG is used for diagnosing pregnancy, pregnancy-related disorders, prenatal screening and in gynaecological cancers [Montgnana *et al*., 2011] with pregnancy diagnosis being the leading application. The works of Zondek *et al* and Friedman et al gave birth to the first pregnancy diagnostic tests in early 1931 [Zondek., *et al* 1930, Friedman., *et al*., 1931]. These tests used urine samples and injected into rabbits to induce ovulation. Since the rabbit test, more highly sensitive and specific immunoassays have been developed using rabbit monoclonal antibodies [Cole., 2010]. The detection mechanism is based on sandwich type assays, either using one or two antibodies which binds to hCG and tracer antibody, enzyme labelled antibody are used for either spectrometric or luminescence detection [Montagnana *et al*., 2011]. The basis of all immunoassays for pregnancy diagnosis follows sandwich type assays, with later developed dual antibody immunometric systems for point-of-care pregnancy diagnosis that are available over-the-counter. Regardless of recent developments in point-of-care systems for pregnancy diagnostics, there remains a high degree of inaccuracy of such tests. False positives and false negatives are frequently encountered with significant consequences. This occurs as a result of interference from proteins found in

urine not fileted through the kidney. Most over-the-counter pregnancy test-kits are not calibrated with a set standard of hCG for accurate measurements, with such test-kits claiming low limits of detection and 99% accuracy. [Cole *et al*., 2009]

2.5. Biosensors

Science and technology maintains to have a great influence in life and communication. With regards to fast and efficient means of improving life through science combined with technology. There has been increase need to develop technological miniaturized devices. This lead to an increase in expectation and development of analytical devices that could serve as reliable monitoring, detection and quantification of chemical substances. These small and easy to use analytical devices are commonly known as sensors in electrochemical research. Application of these sensors is seen in the clinical, industrial, environmental and agricultural sectors (analysis). Sensors are electrochemical devices that convert a physical quantity into a signal in the form of current. The distortion in current which gives the signal can be read of a variety of instruments [Paddle *et al*., 1996]. Chemical sensors consist of a recognition layer and a transducer material which is combined in a direct spatial contact with each other. The former acts as the basis of a specific supramolecular interaction occurring with the chemical species of analytical interest. Chemical sensors range from potentiometric, amperometric and impedimetric electrodes and other various optical detectors that utilize colour indicators [Jianrong et al., 2004].



Scheme 2. Representation of a Biosensor

Biosensors are an important aspect of bio-analytical chemistry. The IUPAC definition of biosensors fall under bioelectronics which states that the application of biomolecular principles to microelectronics occurs in biosensors and biochips. They utilize immobilized biomacromolecules such as enzymes, antibodies and receptors and to some extent higher integrated systems to recognize and detect analyte molecules. Different biological elements may be combined with different sorts of transducers provided that the reaction of biological element with the substrate can be monitored [Lui *et al*., 2009]. On the basis of the transducing element, biosensors are broadly classified as electrochemical, optical, piezoelectric, and thermal. They are also classified as immunosensors, enzyme electrodes, microbial electrodes, and genosensors depending on whether an antibody, enzyme, microbe (or tissue slice), or DNA is used as the principal biological section of the recognition layer [Paddle *et al*., 1996]. Hence achieving an electrical response between an electrode and an enzyme is an pivotal step in the development of amperometric biosensors.

2.5.1. Electrochemical Biosensors

A chemical reaction which produces or consumes ions or electrons generates alterations in electrical properties of the reaction solution. These alterations represent a measurable quantity, and can be sensed by this class of biosensors. The change in electrical property is proportional to the concentration of analyte present. Dependent on the operating principle, electrochemical biosensors can be categorised as potentiometric, impedimetric and amperometric.

2.5.1.1. Potentiometric Biosensors

Potentiometric biosensors operate by measuring the difference in potential between two electrodes, where potential is dependent on concentration of analyte. Ion selective electrodes (ISE) and ion sensitive field effect transistors (ISFET) are the bases of potentiometric biosensors, with their surfaces composing of membranes with selective composition for applying potentials primarily associated with the ion of interest. Membranes consist of glass or inorganic crystal or a plasticized polymer. The outputting signal derives from ions accumulated at the ISE-membrane interface [Phanka *et al*., 2008]. Redox reactions of an analyte with specific receptors cause alterations in potentials due to the change in flow of current. Direct potential difference or voltage where reaction occurs indicates reaction of ions or analytes of interest.

2.5.1.2. Impedimetric Biosensors

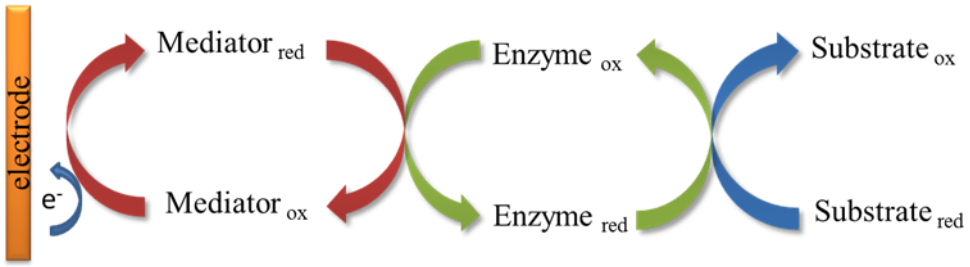
The measurement of electrical resistance or conductance of a system affords powerful analysis. Basic operation occurs by application of voltage perturbation with a small amplitude sinusoidal voltage signal and recording the current response from surface phenomena and

alterations in bulk properties [Lisdat *et al*., 2008]. Electrochemical reactions produce and/or consumes ions and electrons which result in an overall change in either resistance or conductance. These direct changes are measured, analysed and provide essential information about electrode kinetics, conducting polymers, semiconductors, enzyme kinetics and antibody-antigen interactions [Guan *et al*., 2004]. In many studies, immunosensors consist of antibodies immobilised on conductive platforms to enhance electrochemical signal or to probe changes in electrical properties of antibody-antigen complex formations. Impedance properties can be used to study antigen-antibody molecular recognition occurrences. Capacitive immunosensors monitor alterations within the dielectric double layer due to antigen-antibody complex formation.

2.5.1.3. Amperometric Biosensors

Amperometric biosensors is a system built for detection and measurement of redox reaction involving specific recognition of analyte of interest and reaction events by producing a signal in the form of current through electrochemical transducer from an applied voltage. In this case, enzymes are used to amplify the signal. Amperometric measurement relies on the continuous turnover of substrate which produces a significant signal that can be measured. Oxidoreductases are enzymes that are mostly used and immobilized on electrode surfaces. A three electrode system is used to maintain control over potential at electrode surface. A constant potential is applied to the working electrode producing electron flow from oxidized substrate to electrode surface, regenerating the active form of a cofactor of an enzyme and the resulting current is measured [Mehrvar *et al*., 2004]. Enzyme activity are best at set optimal conditions, with fixed temperature and pH, such that the current signal generated from the redox reaction at electrode interface is directly proportional to concentration of substrate

species present in solution. Therefore the current produced is proportional to the rate of substrate conversion in the enzymatic reaction.



Scheme 3. Redox reaction in Biosensors

Interferences are a common factor that affects biosensor performance. To reduce such interferences, chemical electron acceptors known as mediators are incorporated into electrode-enzyme interface. The development of amperometric biosensors has seen great improvements since the glucose biosensor developed by Clark et al by detection of hydrogen peroxide. Saurina et al 1999 developed a biosensor for determination of lysine by immobilizing rigid-conducting composites on electrodes. Lysine oxidase membranes were attached onto graphite-methacrylate electrodes and enzymatic conversion of lysine produces hydrogen peroxide which is detected by application of 1 V. The addition of a second enzyme into composite, peroxidase, showed a reductive electrochemical detection and reduced interferences by oxidizable amino acids. Stantca et al 2004 utilized a tyrosinase based biosensor for the determination of phenols. Tyrosinase was entrapped in an amphiphilic polypyrrole matrix and produced an increase and stabilized background current, significant increase in sensitivity towards phenol and good stability. Liu et al 2008 developed a reusable amperometric immunosensor for the detection of prostate-specific antigen. Anti-prostate specific antigen was conjugated to HRP on a self-assembled monolayer consisting of

phenylborionic acid on gold surface. Detection is done by monitoring the change in the electrocatalytic response of HRP-anti-PSA in presence of H_2O_2 and thionine. Kurtinaitiene et al 2008 combined ferrocenecarboxlyic acid and *N',N',N',N'*-tetramethylbenzidine (TMB) as mediators for detection of antibodies (anti-*g*p51) against bovine leukaemia protein. The immunosensor performance showed high sensitivity towards anti-*g*p51 antibodies by using TMB as a mediator with sensitivity five times higher than corresponding competitive ELISA assays. Zhao et al 2007 used the inhibition character of HRP for enzyme immunosensor for detection of vibrio parahemolyticus (VP). An agarose nano-gold membrane on screen printed electrode was used as platform for biomolecule immobilization. HRP was conjugated with VP antibody. The presence of VP inhibited the active site of HRP for catalysing the oxidation reaction of thionine by H_2O_2. Chawla et al 2012 detected haemoglobin A1c in whole blood by using fructosyl amino acid oxidase on nanoparticle/polymer composites on a gold surface.

2.6. Analytical Methods

2.6.1. Fourier Transform Infra-Red Spectroscopy

Infrared spectroscopy provides information regarding the vibrational states of molecules, compounds. Spectral position and intensity of IR absorptions is used to identify structural elements such as functional groups on molecules. Among the most typical functional groups are hydroxyls or hydrogen bonding, and carbon-oxygen vibrations. The identification of functional groups allows determination of chemical and electrochemical process. Thus infrared spectroscopy has been an essential analytical tool for structural analysis. Firstly, when an IR spectrum is recorded, with dispersive IR spectrometer, the data points show transmitted light at different frequencies. Fourier transformation is algorithm technique which

converts the complete spectrum from time-domain into the frequency-domain, such that the spectrum of molecule is seen [Skoog *et al*., 2004].

2.6.2. Nuclear Magnetic Resonance

Atomic nuclei can be depicted as a positively charged sphere that rotates on its own axis. This spin is characteristic of nuclei, such that the charge's mobility causes a small magnetic field which is parallel with spin axis. The physical phenomenon in NMR derives from the resonance frequency which is proportional to the external magnetic field strength [Jacobsen *et al*,. 2007]. The frequency of radiation for absorption of energy follows a criteria, namely the nucleus, chemical environment of the nucleus, and lastly the spatial location in the magnetic field. Nuclei spins arise from magnetic moment from spin of positive charges. When placed in an external magnetic field, this nuclei spin is disrupted and is no longer random. The nuclei spin state will have one orientation probability. Therefore, a two-state description is used which describes nuclei spin quantum number I = 1/2. For proton NMR, nuclei spins of a nucleus with I = l/2 should be in one of the two states.

2.6.3. High-Resolution Transmission Electron Microscopy (HR-TEM)

Transmission electron microscopy (TEM) is a widely used microscopic technique which uses a beam of electrons with high energy that the electrons are transmitted through a ultra-thin sample. Samples are physisorbed onto copper grids. The beam of electrons interacts with sample as it passes through it. Images are formed when electrons bombard and pass through the specimen, known as backscattered electrons. Magnification of the image is increased, focused and an imaging device, namely backscattered electron detector processes the image through a CCD camera. These transmission electron microscopes achieve resolution far

below what the conventional light microscopes can achieve. High resolution transmission electron microscopy allows the analysis of fine details as fine as single column of atoms. HR-TEM allows imaging of crystallographic structures of a sample at an atomic scale, providing essential information regarding nanoscale properties.

2.6.4. Enzyme Linked Immunosorbent Assay

Enzyme linked immunosorbent assay is based on labelling of antibodies with enzymes, under the sandwich principles. It is a powerful technique for qualitative and quantitative analysis of proteins or any biomolecule found in a complex matrix. The analysis is done by incubating proteins in microplate wells using specific antibodies. Direct assays operate by immobilizing antigens directly into microplate wells followed by labelled antibodies. Capture ELISA sandwich assays operate by immobilizing antibodies first into microplate wells, following washing and blocking steps, immobilization of antigen followed by immobilization of enzyme labelled antibodies [Engvall *et al*., 1971]. Indirect assays, operate similarly to direct assay but uses a secondary labelled antibody for signal generation. There are four key points in doing ELISA, coating and capture of antigens, plate blocking by proteins that has no interferences between binding between antibody and antigen. As well as detection which relies on incubation times, affinity of antibodies for antigens and finally the detection of the signal generated.

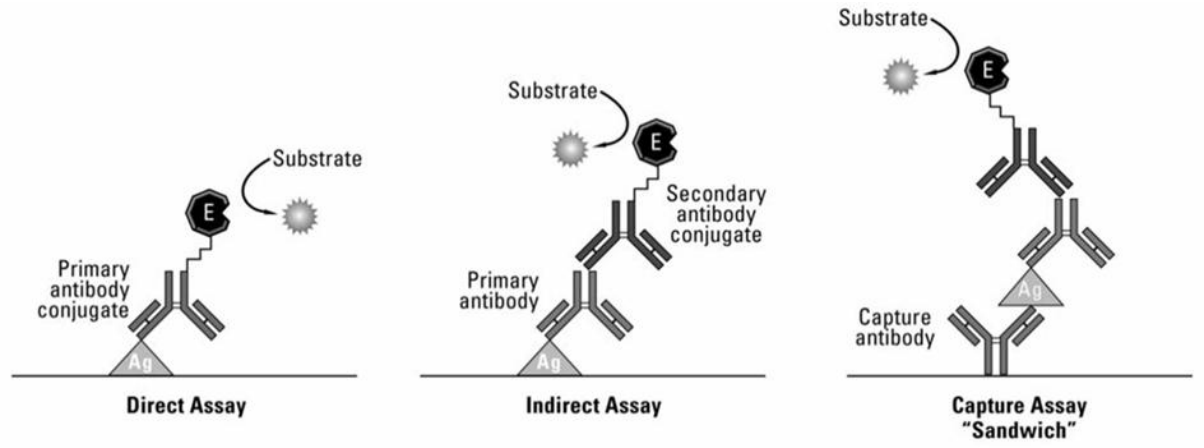


Figure 4. Schematic representation of different assay strategies used in ELISA

2.6.5. Cyclic Voltammetry

Cyclic Voltammetry is the most widely utilized electrochemical technique for studying and analysis of electrochemical reactions. The electrochemical technique works by scanning a potential from an initial potential to a final potential and then essentially back to initial potential. Throughout electrochemical measurement, solution and electrodes are kept constant. During the measurement, the potentiostat measures the resulting current generated from applied potential. Hence the cyclic voltammogram plot is current vs. potential. Figure 5 represents current vs. potential plot. Typically, the forward scan represents the oxidation of reductant material to their corresponding oxidized form and on the backward scan is the reduction. The forward scan results in a loss of electrons from reductant material which is observed in cyclic voltammogram as peak, known as the anodic peak. The backward scan or reverse scan is a transfer of electrons to the oxidised species to its reduced form and this is referred to as the cathodic peak in a cyclic voltammogram. The loss and gain of electrons generates peak currents that are referred to as the anodic ($E_{p,a}$) and cathodic ($E_{p,c}$) peak potentials and the anodic ($I_{p,a}$) and cathodic ($I_{p,c}$) as the peak currents.

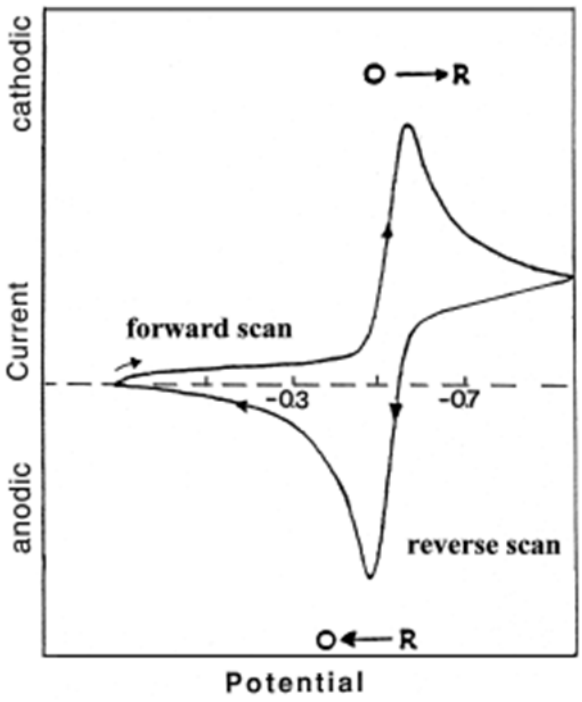


Figure 5. Typical cyclic voltammogram for a reversible process

Cyclic voltammograms can either present a reversible, quasi-reversible or irreversible process, dependent on the electrochemical reaction [Wang., 2000]. Reversible process occur when a electroactive species gets oxidized in the forward scan and reduced in the reverse scan with E_p's and I_p's which represent reversible process. Regardless of the direction of scan, this process is in equilibrium throughout the potential scan. Irreversible process occurs when electrochemical reaction only goes in one direction. This is typically observed as either a single anodic peak or single cathodic peak with a weak or no peak on the reverse scan. If there is a peak on the reverse scan, the peak potential separation is typically very large. The process which results in irreversible reaction results from slow electron transfer kinetics at the working electrode. Quasi-reversible process lies in between reversible and irreversible reactions [Brett., 1999].

Table 2. Summary of different parameters for reversible , quasi-reversible and irreversible processes

Parameter	Reversible	Quasi-reversible	Irreversible
E_p	Independent of scan rate	Shifts with scan rate	Shift cathodically or anondically by 0.03/αn V
$E_{p,a}$ - $E_{p,c}$	- 0.059/mV	Can approach 60/n at slow scan rates.	No reverse peak observed
$I_{p,a}/I_{p,c}$	= 1, independent of scan rate	= 1, only if α = 0.5	Typically no peak current on reverse scan.

2.6.6. Square Wave Voltammetry

Square Wave voltammetry has gained much interest due its suitability to examine redox reactions by overlapping waves. Here, a waveform which consists of a symmetrical square wave which is superimposed on a staircase potential is applied to the working electrode. The forward pulse corresponds with the staircase. The difference in current or net current is the difference between the forward and reverse currents from forward and reverse pulses. The E_p is known as the formal potential for analyte species. As in CV, the peak height of the wave voltammogram is proportional to the concentration of analyte species. SWV reduces the charging of background current, which allows for very low limits of detection, 1 x 10^{-8}. The resulted low LOD shows SWV has higher sensitivity and improved rejection of background current when compared to cyclic voltammetry. The scan rate is higher, which in adjacent to signal averaging increases the signal to noise ratio and importantly experiments can be run successively.

2.6.7. Chronoamperometry

Chronoamperometry involves the stepping of potential where redox reactions occur to a potential where concentration of electroactive species is zero. This occurs at the surface of the electrode. Stationary working electrode with a still electrolyte solution is used. As potential is applied, the resulting current is measured which is proportional to concentration of analyte species in solution. The process occurs solely by diffusion, where the current-time curves show the change in concentration gradients.

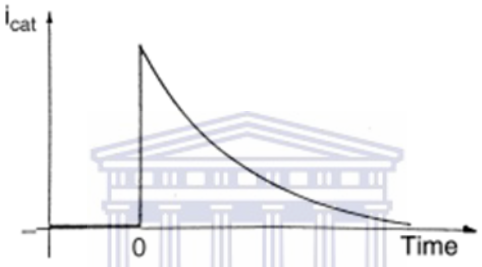


Figure 6. Representation of current-time response with constant potential.

2.7. Electrochemical Immunosensors

Analytical tools that incorporate both high specificity between biological molecular recognition events, receptors and the electrochemical output techniques are known as electrochemical immunosenors. These combined tools permit direct electrochemical detection of a vast range of analytes with high sensitivity and specificity. Immunosensors consists of having either antibody or antigen immobilized on the surface of an electrode. The three main types of immunosensor detection are electrochemical (amperometric, potentiometric and impedimetric), optical and microgravimetric and thermometric. These systems can than further be subdivided into direct or labelled immunosensors. Direct immunosensors detect physical alterations during immune complex formation, whereas

labelled immunosensors use a more sensitive strategy, by employing signal enhancing labels within the complex [Luppa *et al*., 2001]. Most proteins are not able to act as redox partners in electrochemical reactions, therefore they are labelled with electrochemically active labels. These labels produce a current flow generated from electrochemical reaction at constant voltage. The most commonly used labels which are enzymes such as the peroxidase family, glucose oxidase, lactate oxidase, alkaline phosphatase, and luciferase or catalyse. Multiple accounts of labelled immunosensor derives from there excellent stability and high turnover rates.

2.7.1. Antibodies

Antibodies are highly specific and are famous bio-components used in analytical assays. They are abundantly used in immunoassays, construction of immunosensors as well as immunoaffinity columns for protein purification. The highly selective and specific complexes formed between antibodies and antigens used in immunochemical techniques. This character of complexes renders such techniques highly sensitive, selective as well as simple and inexpensive. This high affinity of antibodies for antigens provides excellent sensitivity for immunoassays. To improve such immunoassays, tailoring biomolecules has become an excellent option since the development of antibody fragmentation or recombinant antibodies for complex matrix conditions.

2.7.2. Antibody Production

2.7.2.1. Immune System

The production of antibodies forms part of an immune response of mammals for alien species such as viruses, parasites and bacteria. [Harlow *et al.*, 1988]. The immune system identifies and then eradicates alien species. Innate immune system known as non-specific immune system is the first line of defence. This occurs by preventing the host from developing infections from alien species in a non-specific custom. The adaptive immune system is more specific immune system, by generating cells and processes that eradicate and prevent pathogenic growth and spread. This is one of the more important defence systems for analytical sciences. Cells such as lymphocytes have selective cell surface receptors, which produces proteins known as antibodies to bind to alien species (antigen) for eradication. There are three main divisions of lymphocytes, all having specific receptors on their surface for antigens. A process known as tolerance occurs in the manner in which lymphocytes generate antibodies. The most important lymphocyte is the B cells (lymphocyte B), which produces antibodies of significant importance in analytical chemistry and drug delivery development systems. Failure of tolerance system leads to autoimmune disease, such as AIDS. The first immune response to alien invades is slow. Followed by a second exposure, the immune response is faster and results in a strong immune reaction, since lymphocytes produced during first exposure recognises the antigen species early and very selectively. The immune response for production of antibodies depends on the infection.

2.7.2.2. Antibody Structure

Antibodies are considered to be a large family of glycoproteins, produced from vertebrates' immune system as a defence against infections. They are classified into 5 main classes, known as IgG, IgA, IgM, IgD and IgE, where IgG is the most abundant immunoglobin found in serum. Antibody structure consists of two identical light chains and two identical heavy chains, which yields two identical binding sites for antigens. The antigen-bind region of the antibody is known as the variable region or V region. The region which constitutes the effector functions of the immune system on the antibody is known as the constant region or C region. The overall structure of antibody is known to be roughly that of Y-shaped structure. The leg of Y originates from the paring of carboxyl-terminal halves of the two heavy chains. They are linked together through polypeptide chains which are known as the hinge region. The average molecular weight of an antibody is approximately 150 kDa, 50 kDa per heavy chain, and 25 kDa per light chain. The two identical chains (H-chains, or L-chains) are linked through strong and stable disulphide bridges. The disulphide bridges between identical chains and non-identical chains are responsible for the overall stability of antibodies. These chains can be further subdivided, with the heavy chains into four subclasses and light chain into two subclasses. The variable region V and constant region C are used for classification of subclasses. Heavy chain has three C-regions, C_H1, C_H2, C_H3 and one V-region, V_H. Light chain has one C-region, C_L and one V-region, V_L [Cohen., 1975]. The base of Y known as the crystallised fragment or Fc region forms from two C_H2 and C_H3 domains, where each of the arms known as Fab (fragment antigen binding site) forms from the binding from C_H1 with C_L and V_H with V_L. C_H1 and C_H2 are responsible for rotational and lateral movement of Fab fragments for binding to antigen sites. Amino groups are found at the end of light chains, or Fab fragments, with the terminal carboxyl groups situated at the Fc fragment. Binding sites are situated between V_H and V_L.

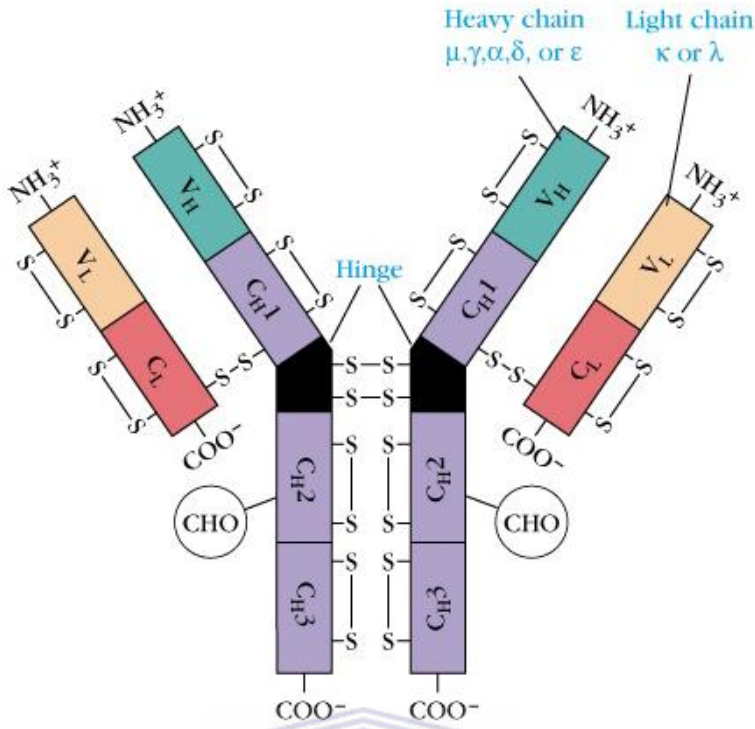


Figure 7. Chemical structure and attributes of an antibody

Other domains carry importance for interaction with immune system. Such as C_H2 contains carbohydrate binding sites and C_H3 is responsible for interaction with the immune system. Importantly, heavy chains differ from each immunoglobulin. With IgE a ε- chain, IgD a δ-chain, IgG a γ-chain and IgM a μ-chain. The different chains yields IgG1, IgG2, IgG3 and IgG4 which all differ in Fc fragment and functions in immune systems

2.7.2.3. Affinity antibody-antigen interactions

Chemical structures of antibodies elaborate on their functions and activity towards the binding of antigens. Binding versatility, biological activity and binding specificity are all criteria for activity of antibodies. Binding between antibodies and antigens is driven by non-covalent interactions, which is a reversible interaction. The typical reaction mechanism observed for antibody-antigen interactions are seen as:

[antibody] + [antigen] ⇆ [antibody-antigen] complex (2)

In equation 2, [ ] represents the concentrations of antibody, antigen and antibody-antigen complexes. The type of bond interaction found within these complexes occurs from intermolecular forces, mainly hydrophobic interactions, weaker van der Waals forces, hydrogen bonds and ionic bonds [Brownlee., 2007]. Bond formation occurs through amine groups of antigens and antibody biomolecules, such that conformational change is dependent on the strength and structure of both antigen and antibody. Antibodies bind very strongly and with extreme high affinity, such that binding in complexes can be explained through equilibrium constants. These association constants, K_A, range from 10^8 to 10^{11}. Equilibrium is explained by following equation 3 using association constant K_A, a measure of the affinity of antibody for antigen.

$$K_A = \frac{[\text{Antibody-Antigen}]}{[\text{Antibody}]*[\text{Antigen}]} \quad (3)$$

The dissociation depends on the association constants and dissociation constants. Antibodies with a high association constant binds tightly to antigens, such the complex remains stable for long periods of time. On the contrast, a high dissociation constant shows low affinity antibodies and that binds weakly to antigens and only for short periods of time. Association constants for monoclonal antibodies can be determined but it's more difficult to determine association constants for polyclonal antibodies due to their heterogeneous character with respect to specificity and affinity. The difference between polyclonal and monoclonal is that monoclonal antibodies maintain single and identical immunoglobin molecules, whereas polyclonal antibodies are a mixture of antibodies specific for different epitope sites.

Therefore it is essentially important to characterize immunosensors by the affinity of antibodies for antigens and the reverse, such that to high affinities which results in irreversible biosensors are avoided.

2.7.3. Enzymes

Enzymes are a class of biomaterial responsible for biological catalysis within living organisms. They are responsible for increasing or to initiate reactions that occur slowly, at moderate temperatures. They are characteristic for inducing reactions steps by creating reaction intermediates, slow down reactions and maintain thermal control. They particularly useful for controlling reaction kinetics for specified and reproducible reaction processes. Enzymes are generally more used biocatalysts in biosensors due to their specific properties in chemical and biological reactions. Enzymes are globular proteins, made up of a polypeptide chain folded into a 3D tertiary or quaternary structure held together by a range of bonds between sides chains of the polypeptide, with a separate active site rooted within the protein. Activity of such proteins is dependent on the tertiary or quaternary structure. The active site is only open to certain molecules with the correct shape and size for conversion through the protein shell and the binding sites. Enzymatic reaction mechanism works by a substrate (S) binding to the enzyme (E) which forms a reactive intermediate, the enzyme-substrate complex (ES). This complex (ES) than reproduces the enzyme with the converted product (P) from the active site. Enzymes are temperature and pH sensitive as well as prone to inhibition. Elevated temperatures and extreme pH values denature enzymes, by disrupting the intermolecular bonds such as the intrinsic hydrogen bonding and hydrophobic interactions.

Enzymatic reactions consume substrates and produces products, which are dependent on temperature, pH, enzyme concentration and substrate concentration. Active centres of

enzymes differs from enzyme to enzyme, many of which have metal ions embedded in there centre and others required additional active centres known as co-factors, NADH. Enzymes are used as catalytic biosensors or as markers in affinity biosensors. Catalytic biosensors use constant enzyme concentration with substrate in smaller concentrations. On the contrary, genosensors or immunosensors has an antibody or DNA labelled with an enzyme where substrate concentration is in excess.

2.8. Immobilization strategies for biomolecules

Immobilization of biological material onto transducer surface is an essential component for development and appropriate performance with good operation and storage stability, high sensitivity, high selectivity, and rapid response [Sassolas *et al*., 2012]. Theoretically, the optimal immobilization process should yield biomolecule immobilized on the surface of the transducer fulfilling the criteria as mentioned previously. Once immobilised, the biomolecule should be fully accessible for analytes, substrates, antigens or DNA. A key factor is that the transducer should at all times not be affected through the immobilization process of biomolecules, retaining its conversion characteristics to electrical signals. Various immobilization strategies have been developed and still under evaluation. Dependent on the objective of the study, each immobilization strategy carries disadvantages. The most common used immobilization strategies in biosensor developed can be categorised into physical and chemical methods. Chemical methods include covalent linkage of biomolecule to transducer surface or through cross linking which improves stability. Whereas physical methods include physical adsorption, entrapment and encapsulation

2.8.1. Physical Adsorption

Physical adsorption of biomolecules onto transducer surfaces has been extensively studied and is a simple technique [Barker., 1987]. Biomolecules adsorb onto solid surfaces through low energy bonds (weak bonds) such as hydrogen bonds, van der Waal forces, hydrophobic interactions and charge-charge interactions. The surface of transducer is incubated with biomolecule for a selected period of time and then washed for removal of excess of unbound biomolecules. The disadvantage is that because of the low energy bonds, the stability of the biomolecule on transducer surface is relatively poor and may be affected by solution ionic strength, temperature and pH. To overcome this, techniques that utilize protein A or protein G on the transducer surface provides improved orientation for immobilization of biomolecule receptors such as antibodies. The basis behind using protein A or protein G is that they are specific for the Fc region of antibodies and provides free interaction of Fab fragments with antigen epitopes

2.8.2. Entrapment

Biomolecules can be entrapped in a three dimensional matrix such as electropolymerised films, amphiphilic networks, silica gel or polysaccharides. This is done by providing a polymer gel around the biomolecule increasing stability and activity. These networks are formed by polymerising a three dimensional structure or for increased biomolecule stability, crosslinking two-dimensional polymer strands. Electrochemical polymerization for controlled immobilization of biomolecules onto electrodes surface is more simple approach. It is a one-step approach by applying potential, current to the transducer in aqueous solution which contains both monomer and biomolecule. Cross linking biomolecules with inert proteins such

as bovine serum albumin forming a gel matrix, where active biomolecule is entrapped in the gel and are often only accessible by small molecules. A loss in binding affinity or enzyme activity is also observed. Since there is a certain degree of heterogeneous character in gel structure, leakage of biomolecules can be observed from the less densely polymerised regions.

2.8.3. Covalent Binding

Covalent attachment of biomolecules onto transducer surfaces is a popular chemical immobilization technique for development of stable, selective and rapid response with a low to zero percentage of non-specific adsorption. Biomolecules are bound to the surface of transducers through functional groups. Such as amino acids' amine groups and carboxyl groups that are not important for activity can be attached on supports such as glass, natural cellulose or synthetic polymers such as nylon. Membranes which are pre-activated can also be used. The amino groups used for covalent bonding originate from lysine or arginine and the carboxyl groups from aspartic and glutamic acid. Sulfhydryl groups originate from cysteine.

2.8.4. Encapsulation

Encapsulation is one of the simple methods used for immobilization of biomolecules onto transducer surfaces. Biomolecules are retained in a semi-permeable membrane, such as dialysis membranes. Advantage of encapsulation relies on the ease of substrates and product's to cross the barrier and biomolecules retaining its activity.

2.9. Supramolecular cyclodextrin and ferrocene inclusion complexes

2.9.1. Cyclodextrin platform in immunosensors

The development of immunosensors requires the recognition element (antibodies/antigens) to be properly immobilized on electrode surfaces, with an orientation that is optimal binding to analytes. Immunosensors suffer from non-specific adsorption, so blocking agents needs to be immobilized. Blocking agents such as Tween 20 or Bovine serum albumin are the most commonly used. Self-assembled monolayers on electrode surfaces significantly reduce non-specific adsorption of unwanted materials. SAM's are usually functionalised onto noble metal surfaces such as gold or platinum. These SAM's increase the stability of biomolecule receptors. Thiol-terminated SAM's has been highly studied because of the high affinity of thiol groups for gold surfaces [Aria *et al*., 2009]. Alternative methods to immunosensor development from classical methods, is the fabrication of highly ordered molecular systems in junction with supramolecular chemistry [Ortiz *et al*., 2011]. The use of βCD coated electrode surfaces affords a reversible route for immobilization biomolecules. Thiol modified βCDP affords SAM's of organized nature. SAM's of thiol βCDP has intrinsic character for blocking non-specific adsorptions by using supramolecular host-guest interactions with mainly adamantane or ferrocene. These supramolecular complexes dock recognition elements to the electrode surface. Evan though the binding constant for inclusion complex formation is moderate, the inherent stability results from the multivalency of host-guest interactions.

2.9.2. Redox activity of cyclodextrin and ferrocene inclusion complexes

Zheng et al 2008 prepared derivatives of βCD which incorporated ferrocene. There results indicated the bioelectrocatalytic efficiency of the complex with the mediator effect for the

bioelectrocatalysis of glucose oxidase. A study done by Zhang et al 2011 showed a water soluble inclusion complex of ferrocene and βCDP. After host-guest complex formation in a solution for 1:1 inclusion complex, ferrocene maintained its characteristic electrochemical behaviour. They showed a reversible electrochemical interaction of Fc inside the cavity of βCDP with transducer. Ortiz et al 2011 development a genosensor on the basis of the inclusion complex formed between βCDP and ferrocene functionalised CMC. They observed well pronounced reversible peaks for the modification of a gold electrode with βCDPSH-Fc-CMC-DNA. The presence of ferrocene increased sensitivity of genosensor. They also found that peak to peak separation for cyclic voltammogram of genosensor was higher than 0 mV for surface confined species which is due to the polydispersion of Fc-CMC-DNA.

2.9.3. Bienzymatic system for biosensors

The construction of biosensors with redox enzymes requires electron transfer capability with an electrode surface. To produce electron transfer, researchers utilize mediators to access the redox centre of the enzyme [Senel *et al*., 2011]. Ferrocene has been extensively studied as a mediator since it is well known for its usable properties for construction of sensitive, selective and rapid response biosensors. The use of bienzymatic system for immunosensors is mainly for increasing sensitivity and decreasing limit of detection. The development of a bienzymatic system for the detection of anti-gliadin antibodies was done by Ortiz and colleagues 2011. Lactate oxidase and horseradish peroxidase were used as enzyme labels. The addition of sodium lactate produced H_2O_2 *in situ*, which gave a higher sensitivity than just single enzyme labels. Perez et al 2012 developed bienzymatic biosensor for the detection of $_L$-Lactate in wine and beer samples by using lactate oxidase and horseradish peroxidase enzymes which were encapsulated into carbon nanotube/polyslfone membrane. Detection

mechanism is similar to that of Ortiz et al 2011. In this case, potential of -100 mV was used for the amperometric detection of $_{L}$-Lactate

CHAPTER 3

3. Experimental Methods

3.1. Materials

Mixed bed resin TMD-8, sodium meta-periodate, cystamine dihydrochloride, 1,4-diaminobutane, carboxymethyl cellulose sodium salt, (+/-)-epichlorohydrin, horseradish peroxidase, glucose oxidase, D ± glucose, phosphate buffer saline, carbonate buffer, phosphate buffer saline with 0.05% Tween 20 (dry powder), 2-(N-morpholino)ethanesulfonic acid (MES) buffer, hydrogen peroxide 30%, potassium ferrocyanide (III) and potassium ferricyanide (II), 3,3';5,5'-tetramethylbenzidine dihydrochloride hydrate, 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate system for ELISA were all supplied from Sigma Aldrich. Sulphuric acid, nitric acid and hydrochloric acid were supplied from Scharlau. Aqueous solutions were prepared with Milli-Q water (Milipore) and all regents were used as received. hCG proteins were obtained from Scipac with stock concentration of 2 mg/ml (which contains 900 IU/mg).

3.2. Buffers and Solutions

- All solutions for electrochemical characterisation, morphology, structural characterisation consisted of phosphate buffer.
- All ELISA experiments utilised phosphate buffer saline, and phosphate buffer containing 0.05 % Tween20.

- 0.5 M H_2SO_4 was prepared in 500 mL volumetric flask and diluted to the mark using Millipore water.
- All conjugates and antigen solutions were made up with phosphate buffer saline.
- A solution of Potassium ferricyanide, $K_3[Fe(CN)_6]$ was prepared in 250 ml flask.

3.3. Instrumentation

Electrochemical characterisation of materials was carried out by using a BAS100W Bioanalytial System. Electrochemical detection was carried out by using an Autolab PGSTAT 10 System. All electrochemical studies were carried out in a straight three electrode cell. A 1.6 mm diameter gold electrode and 1.6 mm platinum electrode were used as working electrodes, silver/silver chloride electrode as reference electrode and platinum gauze as counter electrode. FTIR-Spectrometer Perkin Elmer (Spectrum 100), SNFTIR (Type MISE-PS/09 Vertex 80v). NMR spectra were recorded on a GEMINI 200MHz NMR spectrometer. Spectra were recording using 5 mm tubes using D_2O solvent. A multiplate reader Wallac Victor 2 1420 Multilabel counter from Perkin-Elmer was used to perform absorbance measurements.

3.4. Electrode Surface Modification

3.4.1. Preparation of thiolated cyclodextrin-epichlorohydrin polymers

The first step involved the cross-linking of β-cyclodextrin with epichlorohydrin in alkaline medium. For this, β-cyclodextrin (5 g, 4.4 mmol) was dissolved in 50 mL of 10% (w/v) NaOH and 10 mL (90 mmol) of epichlorohydrin was added. The system was vigorously stirred for 8 hours, then another 5 mL of epichlorohydrin was added with stirring and the

mixture was kept overnight at room temperature. The solution was then concentrated to about 15 mL and precipitated by addition of cold ethanol (500 mL). The gummy precipitate was crushed several times with ethanol in a mortar until a fine precipitate was obtained. The precipitate was then washed again with ethanol and acetone and dried under high vacuum overnight. A mixture of βCDP (0.8 g, 0.7 mmol of βCD units) and $NaIO_4$ (200 mg, 0.9 mmol) in 20 mL water was stirred overnight at room temperature in the dark. The solution was then deionised by passing through a column (1 x 10 cm) containing a mixed bed ionic exchange resin (TMD-8, H+ and OH form, 16–40 mesh, Aldrich). The appropriate fractions were collected and cystamine (0.5 g, 3.3 mmol) was added and stirred for 30 minutes at room temperature. Afterwards, sodium borohydride (100 mg dissolved in 1–2 mL water, 2.6 mmol) was added and the solution left stirring overnight. The product was precipitated by addition of ethanol, filtered, washed with ethanol and vacuum dried [Fragoso *et al.*, 2009].

3.4.2. Preparation of ferrocene functionalised carboxymethyl cellulose

WESTERN CAPE

Carboxymethyl cellulose was oxidized by dissolving 100 mg of the polymer in 5 mL of H_2O and treated with 400 mg of sodium metaperiodate under continuous stirring at 4^o C in the dark for 2 hours. The oxidation reaction was stopped by adding 100 µL of ethylene glycol and stirred for 1 hour, and further dialysed against distilled H_2O. 1,4-Diaminobutane (10 mmol) was further added to the activated polymer solution, and then treated with 20 mg of $NaBH_4$ for 4 hours under continuous stirring. The modified polymer solution was dialysed against distilled H_2O and finally lyophilized [Villalonga *et al.*, 2006]. 0.5 g of aminated CMC2 was dissolved in 25 mL of Milli-Q water. A solution of 0.32 g (1.5 mmol) of ferrocenecarboxaldehyde in 2 mL of DMSO was then added dropwise with continuous magnetic stirring. After 3 hours an excess (60 mg, 15 mmol) of sodium borohydride was

added and the solution stirred overnight at room temperature. The mixture was concentrated to about half the initial volume by rota-evaporation and dialysed for 24 hours to remove impurities and then dried in vacuum to give Fc-CMC [Ortiz *et al*., 2011].

3.4.3. Preparation of Horseradish peroxidase secondary labelled antibodies

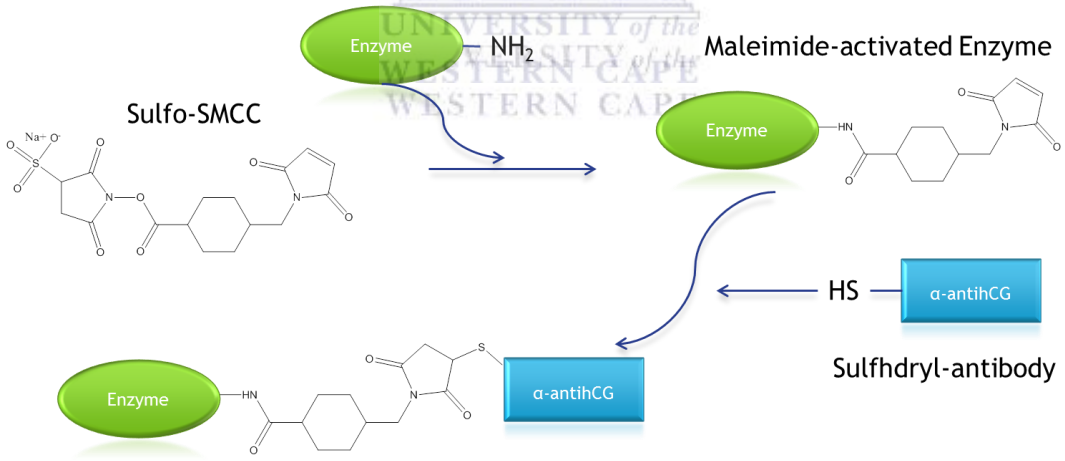
Preparation of conjugates with HRP commenced by the addition of 1 mg of α-antihCG antibody in 0.1 M MES buffer (containing 0.15 M NaCl, pH 7.2). SATA protocol was followed by the addition of SATA in a molar ratio of 9:1 of SATA to antibody. The reaction was left for 45 minutes in the dark with slow stirring. A further 100 µL of 0.5 M hydroxylamine, 5 mM EDTA in phosphate buffer was added per 1 mL of reaction solution to produce sulfhydryl groups. Unreacted material was removed by passing the reaction solution through a Sephadex G-25 desalting column and simultaneously performing a buffer exchange into a solution which contained 10 mM EDTA, which reduces disulphide bond formation. SATA-activated α-antihCG was immediately reacted with maleimide activated HRP in a 1:4 molar ratio in the dark for 90 minutes. HRP conjugates were purified by passing reaction mixture through desalting column and then concentrated with Amicon 100k centrifugal filter.

3.4.4. Preparation of Glucose oxidase secondary labelled antibodies

Preparation of conjugates with GOx commenced by dissolving 2 mg of α-antihCG antibody in 0.1 M MES buffer (containing 0.15 M NaCl, pH 7.2). SATA protocol was followed by the addition of SATA in a molar ratio of 9:1 of SATA to antibody. The reaction was left for 45 minutes in the dark with slow stirring. A further 100 µL of 0.5 M hydroxylamine, 5 mM EDTA in phosphate buffer was added per 1 mL of reaction solution to produce sulfhydryl

groups for 2 hours. Unreacted material was removed by passing the reaction solution through a Sephadex G-25 desalting column and simultaneously performing a buffer exchange into a solution which contained 10 mM EDTA, which reduces disulphide bond formation.

Glucose oxidase was activated with sulfo-SMCC by dissolving 10 mg of GOx in 1 mL phosphate buffer pH 7.2. The reaction commenced by adding a 15 molar excess of cross linker and reaction left for 45 minutes in the dark. Unreacted material was removed by passing reaction mixture through a desalting column. Maleimide activated GOx was then immediately reacted with SATA-activated α-antihCG in a 5:1 molar ratio. The reaction was left for 90 minutes in the dark. GOx conjugates were purified by passing reaction mixture through desalting column and then concentrated with Amicon 100k centrifugal filter.



Scheme 4. General representation of SATA protocol for bioconjugate synthesis

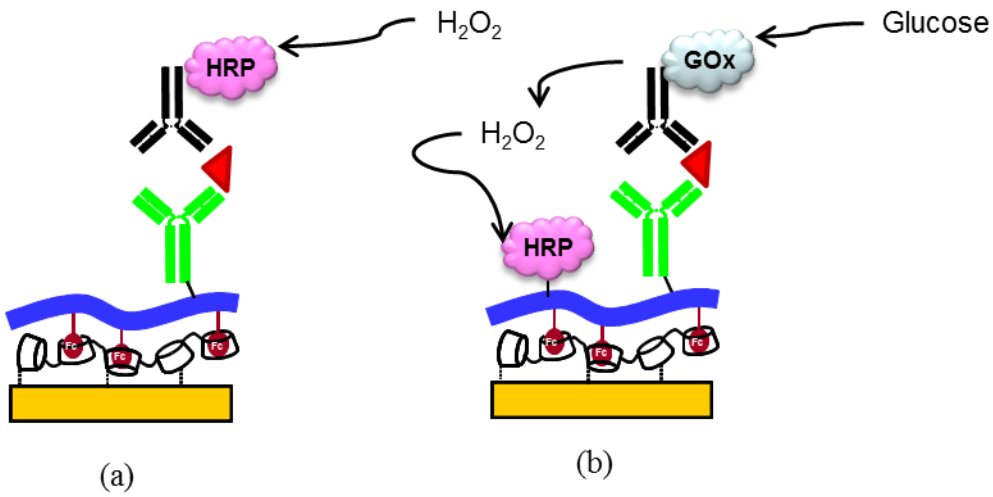
3.4.5. Enzyme Linked Immunosorbent Assays (ELISA)

All assays were done with Nunc plates as received. Direct sandwich assays were done to test conjugates that were prepared in section 3.4.4. All washing steps were done using a solution containing 0.1 M PBS, 0.15 NaCl and Tween 20 (0.05 %) at pH 7.4. To test conjugates prepared in section 3.4.4, hCG (0 – 20 µg/mL) was incubated for 1 hour into wells in binding buffer. Wells were washed with washing buffer followed by incubation of GOx-α-antihCG conjugate at different concentrations (0 – 100 µg/mL) for 1 hour. After a final washing step, absorbance was carried out by adding 10 mM 3,3';5,5'-tetramethylbenzidine dihydrochloride hydrate, 50 µl of 50 U/mL horseradish peroxidase type (II) and 2 mM (±)-D-glucose. The reaction was left for 10 minute for colour development and the absorbance recorded at intervals of 5 minutes with a wavelength of 650 nm.

The immunoassay of hCG for strategy (a) was done by incubating 20 µg/mL (absence of βCDPSH) of Fc-CMC-α-antihCG for 1 hour. After incubation the wells were washed with washing buffer and blocked with PBS-Tween 20 (0.05%) for 1 hour. Wells were washed after blocking with washing buffer and different concentrations of hCG (0 – 4 µg/ml) was incubated for 1 hour. Further was washing was done, followed by incubating HRP-α-antihCG at different concentrations (0 – 10 µg/mL) for 1 hour. A final washing step was done followed by addition of commercial TMB (ELISA substrate for HRP) and the absorbance recorded. The immunoassay of hCG for strategy (b) was similar to that of strategy (a), by incubating 20 µg/mL Fc-CMC-α-antihCG-HRP for 1 hour. Further incubation steps and absorbance measurements were done as mentioned for the testing of conjugate prepared.

3.4.6. Electrode cleaning, modification and hCG detection

Gold and platinum electrodes were cleaned by repeated mechanical polishing in 1.0 µM, 0.3 µM and 0.05 µM alumina slurry, followed by sonication in ethanol for 10 minutes, and in water for 10 minutes. Mechanical cleaning and sonication were carried out three times. Electrochemical cleaning of gold electrodes was performed by potential cycling in 0.5 M H_2SO_4 between -0.2 and +1.7 V vs. Ag/AgCl at a scan rate of 0.2 V s^{-1}. Electrode cleanliness was tested with cyclic voltammetry in 1 mM $K_2[Fe(CN)_6]$ in phosphate buffer pH 7.4. Self-assembled monolayers of 1 mg/mL βCDPSH in water were formed by drop-coating 30 µL on gold electrodes and left overnight at room temperature. After rinsing gold electrodes with PBS buffer, 30 µL (20 µg/mL) Fc-CMC-β-antihCG for strategy (a) and 30 µL (20 µg/mL) Fc-CMC-β-antihCG-HRP for strategy (b) was drop-coated on to the functionalized gold electrodes and incubated overnight to form a inclusion complex. After washing, various concentrations of hCG antigen were incubated for 30 minutes following washing with PBS pH 7.4. HRP-α-antihCG (10 µg/mL) for strategy (a) was incubated for 15 minutes. Electrochemical detection commenced by measuring a blank sample (PBS pH 6.0) following the addition of 10 mM of H_2O_2 in a v/v of 3:1 (PBS : H_2O_2) and the electrochemical response recorded at 0.2 V. GOx-α-antihCG (50 µg/mL) for strategy (b) was incubated for 1 hour. Electrochemical detection commenced by measuring blank sample (PBS pH 5.5, with no substrate), at 0.2 V. D-(±)-glucose (10 mM) was then added in 0.1 M PBS pH 5.5 and electrochemical response was measured at 0.2 V.



Scheme 5. Amperometric Immunosensor strategies for detection of hCG, (strategy a, strategy b)

CHAPTER 4

4. Results and Discussion 1

4.1. Structural Analysis

4.1.1. NMR of βCD and βCDP

Self-assembly of a thiolated βCD polymeric material on a gold surface was used as a strategy for the immobilization of Fc-CMC-β-antihCG and Fc-CMC-β-antihCG-HRP. The importance of βCD polymeric material enables the generation of a high amount of immobilised guest molecules and improved stability. For this, βCD was cross linked with epichlorohydrin in alkaline medium to form a polymer. Figure 8 shows the ^{1}H-NMR for βCD monomer.

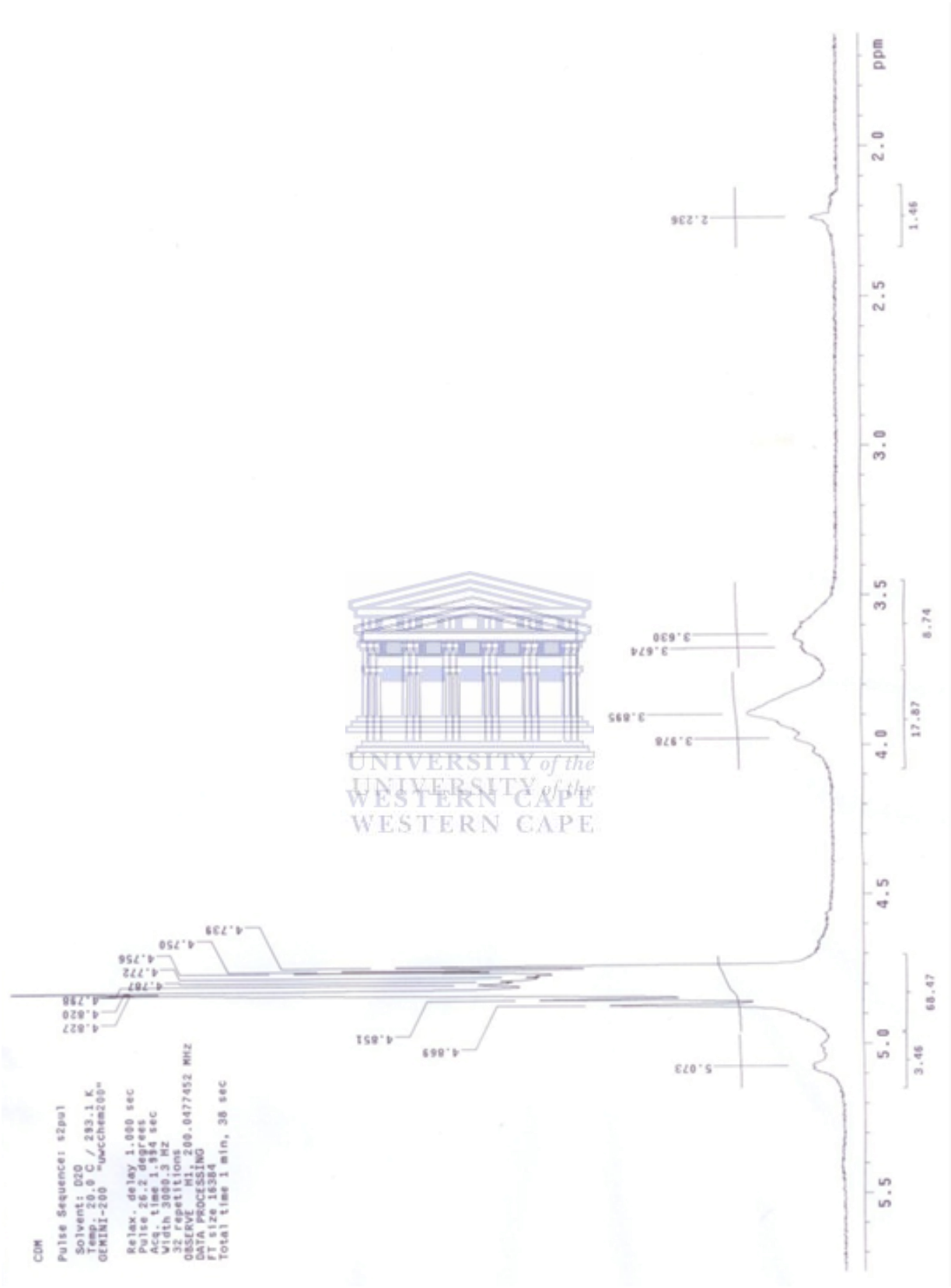


Figure 8. NMR spectrum of βCD monomer

Figure 8 shows the NMR spectra for βCD monomer. A singlet peak is observed near 5.0 ppm deriving from the anomeric proton attached to C-1 of the glucose unit. Two more broadened peaks are observed between 4.0 – 3.5 ppm which corresponds to the protons on the pyranose rings.

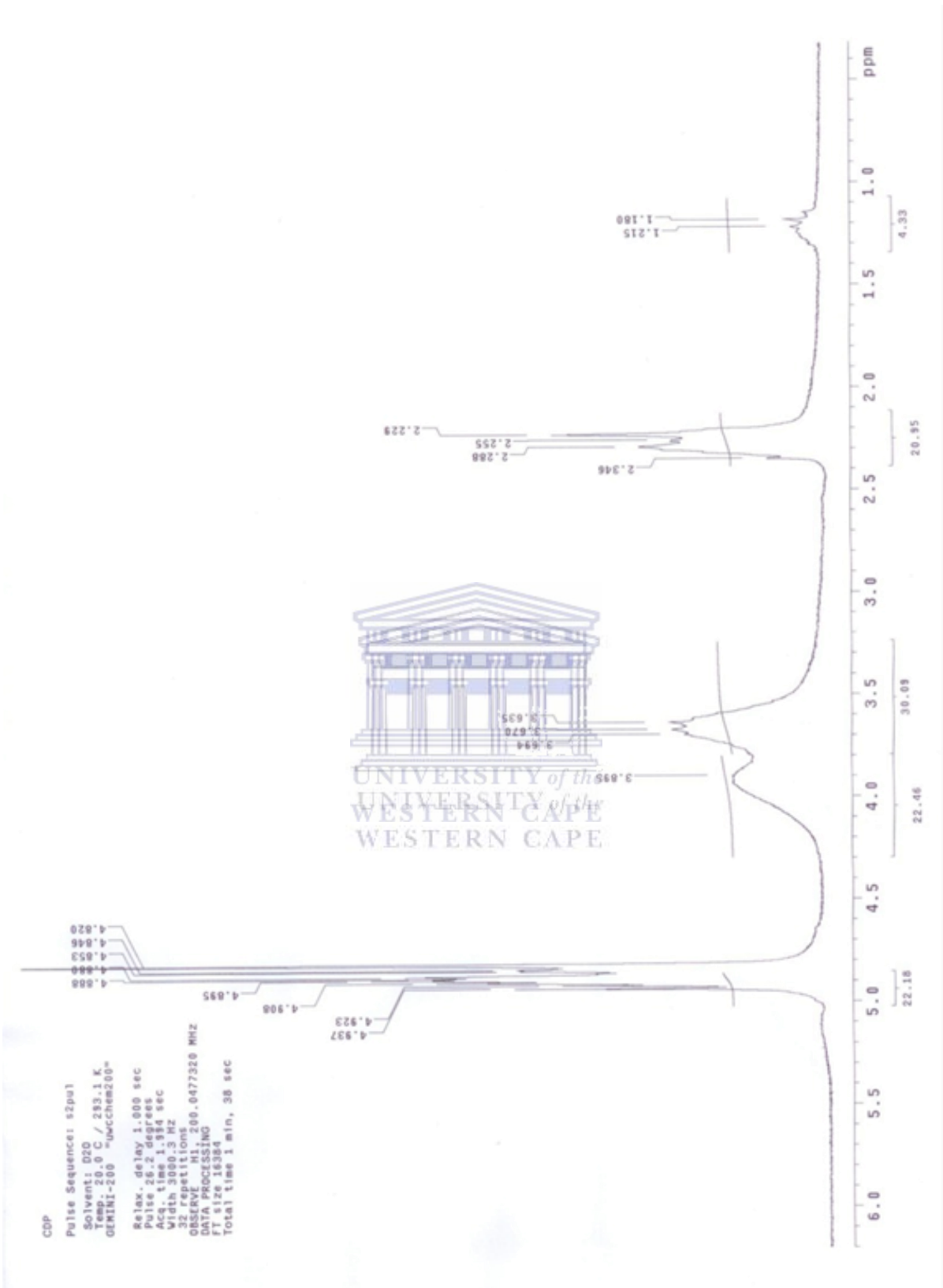


Figure 9. NMR spectrum of βCDP

The synthesis of βCDP with epichlorohydrin results in high molecular weight compound in alkaline media [Mura *et al*., 2002]. The use of high NaOH concentrations leads to substitution on one side of βCD resulting in high molecular weight compounds [Renard *et al*., 1997]. The hydroxyl groups on βCD reacts with one reactive group of the bifunctional agent, such that the side chain further reacts in one of two ways. The epoxide ring reacts with another hydroxyl group on a second βCD ring producing a glyceryl bridge connecting the two cage-like molecules or the epoxide ring is hydrolysed. In some cases, epichlorohydrin may react with itself to form homoploymers. In essence, the glycerol tails after stopping polymerisation and glyceryl bridges have different lengths which can be determined through Mass spectroscopy. Figure 9 shows the ^{1}H NMR spectra for βCDP. The observed peaks which correspond to the polymer protons appear elongated and broadened in comparison to signals seen for βCD monomer. This is due to the polydispersion of the polymer. Peaks seen from 3.5 – 4.6 ppm correspond to the skeletal carbohydrate protons from the $CH_2CH(OH)CH_2$ bridging units.

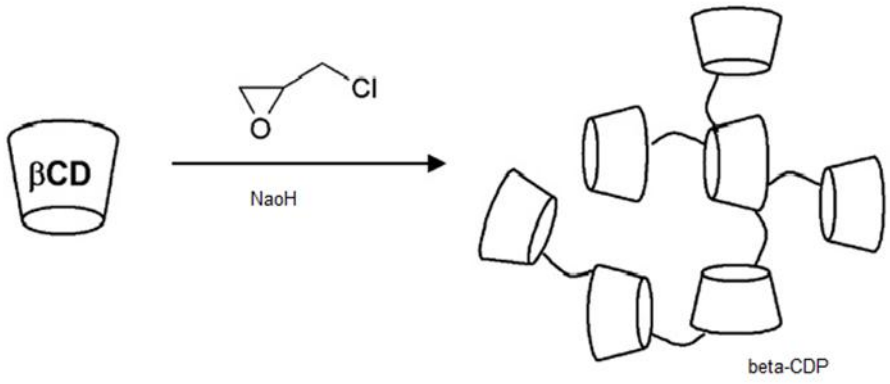


Figure 10. Synthesis of β-cyclodextrin polymer with $NAIO_4$ in alkaline media [Fragoso *et al*., 2009]

4.1.2. FTIR spectroscopy

4.1.2.1. FTIR spectra for βCD and βCDPSH

For interest of the study, FTIR spectroscopy was used to confirm the structure of βCDPSH and Fc-CMC. Stretching bands for ˉOH and –CH is observed for βCD monomer (Figure 11) from 3293 cm^{-1} and 2912 cm^{-1}. Characteristic peaks are observed for the C-O stretching at 575 cm^{-1} for the vibration of glucose rings and absorption peaks at 1035 cm^{-1}[Deveswara. *et al*., 2012]. Aromatic C-H bends are observed from 528 cm^{-1} to 856 cm^{-1}. The dialkyl stretch for C-O-C is seen at 1077 cm^{-1}, 1152 cm^{-1} and 1222 cm^{-1}.

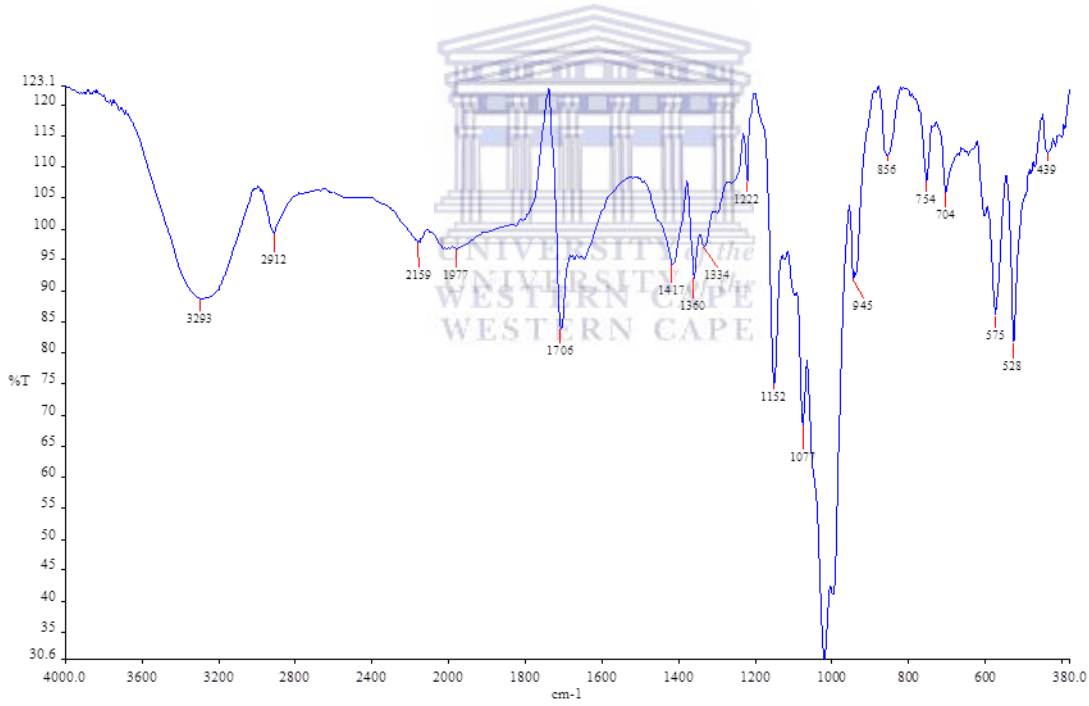


Figure 11. FTIR spectrum of βCD monomer.

The immobilization of Fc-CMC-β-antihCG-HRP recognition layer is based on the covalent deposition of a thiolated polymeric form of βCD. Procedure for producing βCDP has previously been mentioned. Importantly, thiol groups were introduced by oxidising vicinal ˉ

OH with sodium metaperiodate which produces a poly-aldehyde which is than further reacted with cystamine to produce thiolated polymer βCDP.

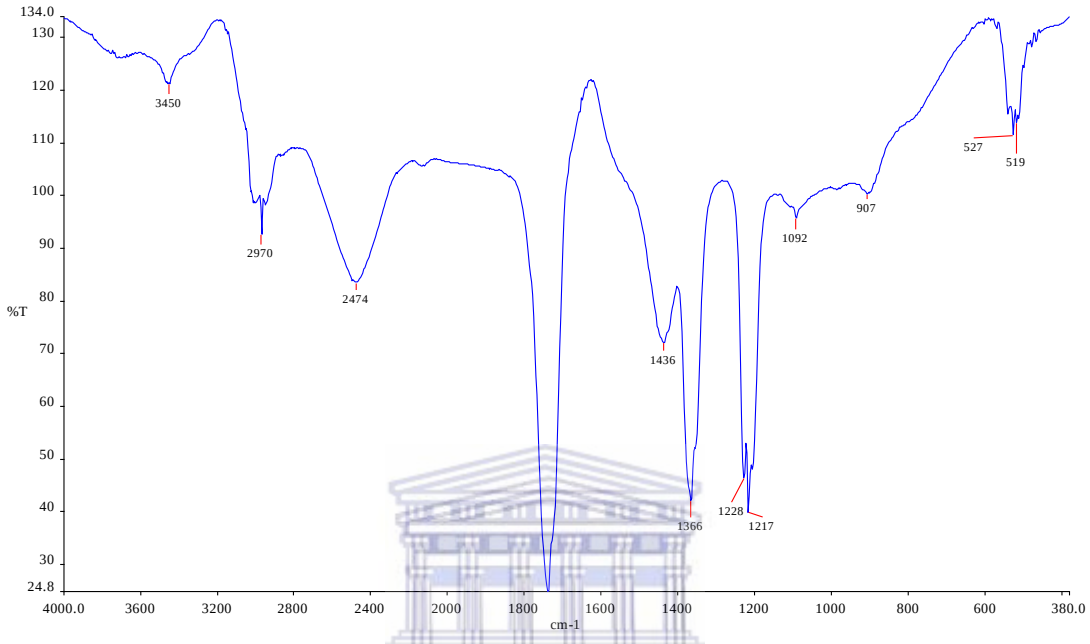


Figure 12. FTIR spectrum of thiolated βCD polymer

Adsorption bands characteristic for β-cyclodextrin are observed at 3450 cm^{-1} for ⁻OH, 2970 cm^{-1} for C-H stretch, 1366 cm^{-1} for C-H_2 is observed in figure 12. Aromatic C-H bends are observed at 519 cm^{-1} and 527 cm^{-1}. These bands indicate that the basic structural characteristics of βCD remain uninterrupted after polymerisation and thiolation. New bands appear at 2474 cm^{-1} owning to S-H stretch, an indication of thiol groups on βCDP as well as a band at 1436 cm^{-1} corresponding to N-H bend from $NHCH_2CH_2S$

4.1.2.2. FTIR spectra of CMC and Fc-CMC

Cellulose is a carbohydrate which is easily modified to sodium carboxymethyl cellulose by converting native CH_2OH group in the glucose units with carboxymethyl groups [He *et al.*, 2007]. The use of CMC lies in its structure which allows for modifications with different attachments of antibody probes and ferrocene moieties, mainly the carboxylic group at C-6 and vicinal diol group between C-2 and C-3 [Ortiz *et al* 2011].

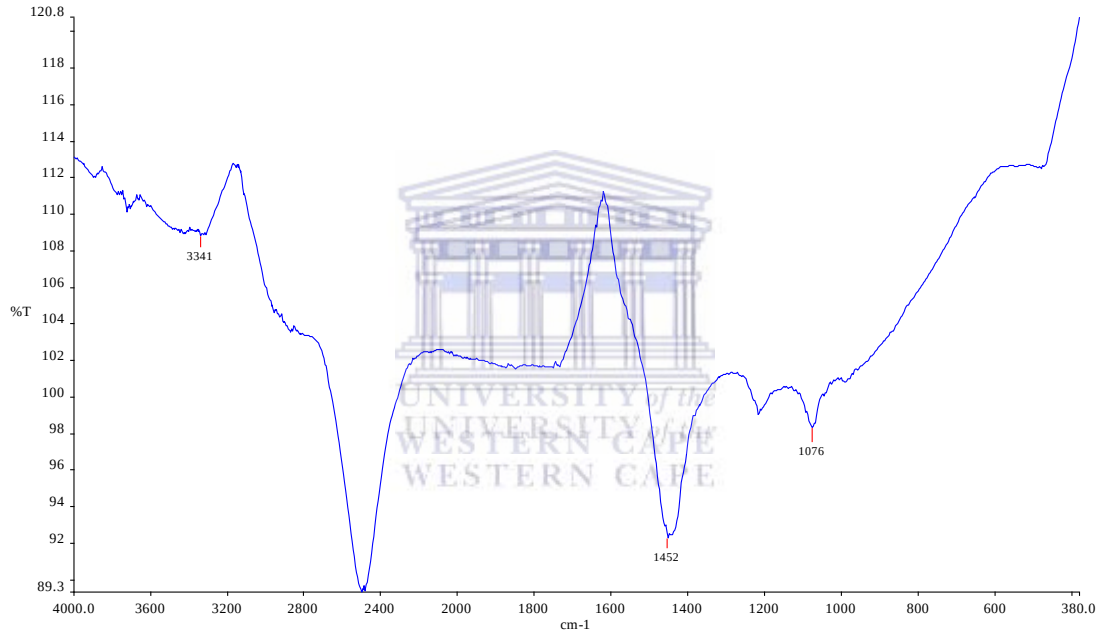


Figure 13. FTIR spectrum of carboxymethyl cellulose

Figure 13 shows the FTIR spectrum for CMC. An ^{-}OH stretching band is observed at 3341 cm^{-1}, with COO^{-} (symmetric) bending at 1452 cm^{-1}. Ether functionality shows a stretching band for (RCH_2OCH_2R) at 1200 cm^{-1} for C-O-C. A stretching band for C-O stretching is observed a 1076 cm^{-1}.

Covalent linkage of mediators onto polymers affords stable and readily fast response for biosensor development. The linkage between ferrocene and CMC provides a method for adopting duel synthesis of mediators and recognition layers and enzyme labels. Figure 14 represents the FTIR spectra of Fc-CMC. Bands seen at 1444 cm^{-1} results from COO^- stretching, bands at 1216 cm^{-1} indicate C-O-C stretching as we as the small shoulder observed at 1380 cm^{-1} which indicates the presence of Fc-aldehyde. It is essentially important to observe the characteristics band for Fc-aldehyde at 1680 cm^{-1} for C=O stretching is not observed which indicates that the aldehyde groups of Fc were completely reacted with amino groups generated on CMC.

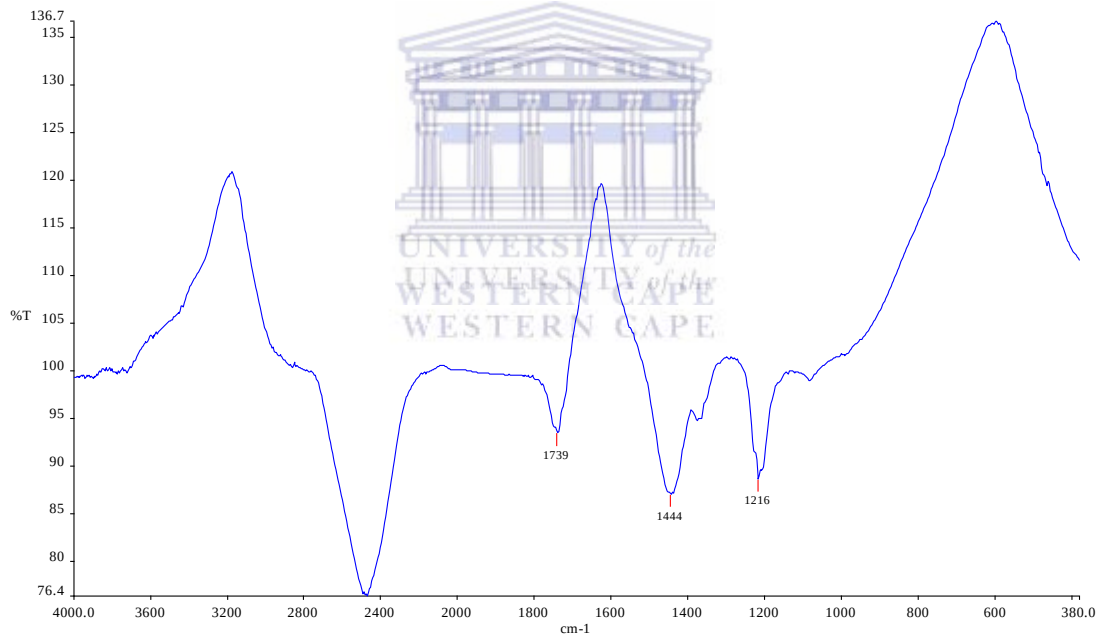
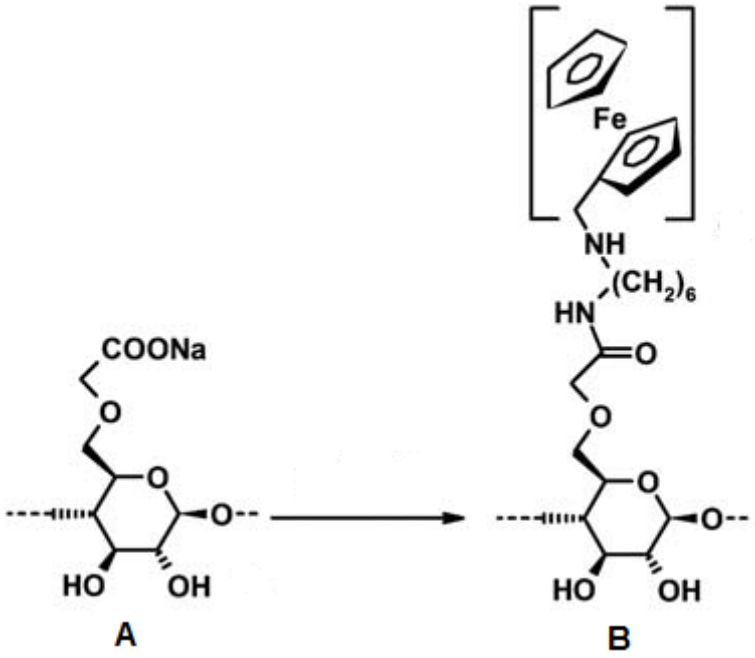


Figure 14. FTIR for Ferrocene-CMC



Scheme 6. Synthesis of Fc modified CMC [Ortiz *et al*., 2011]

4.2. Morphology studies: HR-TEM of βCDP and inclusion complex with Fc-CMC

Surface morphology of materials is particularly usefully in understanding how changes in morphology correspond to changes in electrochemical studies. As a means to understand the contribution of quantum phenomena towards development of bio/immunosensor, tailoring materials with control of surface morphology for applications can be used as analytical tools. The surface morphology of βCDP and the supramolecular inclusion complex formed with Fc-CMC studied with HR-TEM is shown in figure 15.

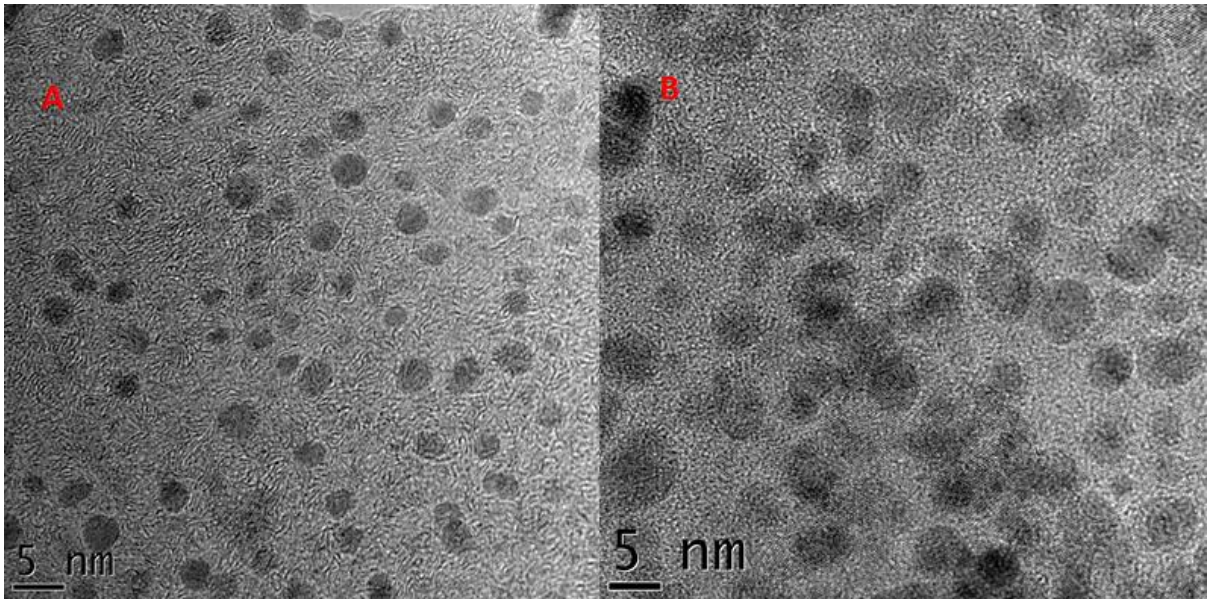


Figure 15. HR-TEM images for βCD polymer (A), and inclusion complex between βCDP and Fc-CMC (B).

Figure 15 (A) and (B) shows the high resolution transmission electron micrographs for βCDP and inclusion complex with Fc-CMC. These micrographs were recorded at 370 000 magnification resulting in scale of 5 nm. These micrographs were recorded on copper mesh grids. Figure 15 (A) resembles well dispersed βCDP particles after adsorption on copper grids. βCDP particles show average size of 3.5 to 5.1 nm. HR-TEM images for inclusion complex formed between βCDP and Fc-CMC in figure 15 B shows larger spherical particles. They seem to form an encapsulated morphology, as well particles being much more densely packed than for HR-TEM of βCDP. This encapsulation follows a crystal structure found in native cyclodextrins. The cage type complex formed has the cavity of one βCD molecule closed off on both sides by an adjacent βCD, such that the guest molecules do not contact each other. In the case of CMC functionalised with Fc, the polymer aids inclusion complex formation.

4.3. Electrochemical Characterisation of βCDP and Fc-CMC in PBS

4.3.1. Electrochemical characterisation on diffusion process

Mass transfer in electrolytes upon application of a potential causes diffusion to occur. Where by at least one of the components which is not uniformly distributed will direct mass transfer as to level concentrations gradients. Mass transfer of ions or neutral species towards electrodes alters in electrolyte solutions. When a current is flowing, the concentration of reactant and product in the near vicinity of the electrode surface changes compared to its bulk concentration. Hence diffusion develops as result of such concentration gradients.

4.3.1.1. Electrochemical response of βCDP and Fc-CMC in PBS solution with platinum electrodes

Bare platinum electrodes are immersed in 0.1 M PBS solution with pH 7.2, in the presence of 100 µg/mL of Fc-CMC. Cyclic voltammograms are used in conjunction with square wave voltammetry to study the effect of Fc-CMC and its diffusion process. This parameter is important for having a comparative study between adsorption onto gold electrodes for immunosensor and diffusion. Electrochemistry of CMC shows no observable peaks in CV, and there peaks in figure 16 are due to irreversible diffusion processes between Fc and Pt electrode.

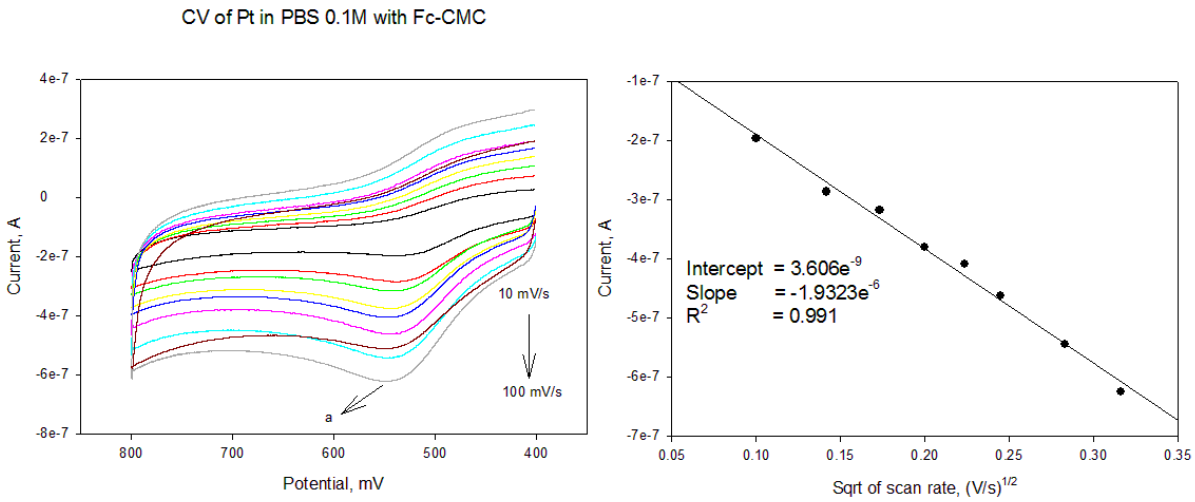


Figure 16. Cyclic voltammograms at different scan rates of Fc-CMC in PBS 0.1 M PBS with Pt electrode. Randel-Sevich Plot

Cyclic voltammograms at different scan rates is seen in Figure 16. Within a narrow potential range, Fc-CMC was mixed with electrolyte solution as to study diffusion process which occurs with platinum electrode. Oxidation peaks are observed at an E_{pa} of 548 mV, owing to the oxidation of Fc-CMC. Well known peaks for Fc is not observed in solution due to the insulation character of CMC. As scan rate increases, oxidation peaks increases with no change in E_p. The Randel-Sevick plot, current vs square root of scan rate shows a linear increase of I_{pa} with increase in scan rate. The process is irreversible, by the appearance of one oxidation peak. Very large activation energy is required to overcome the activation barrier which causes fracturing of original molecular species

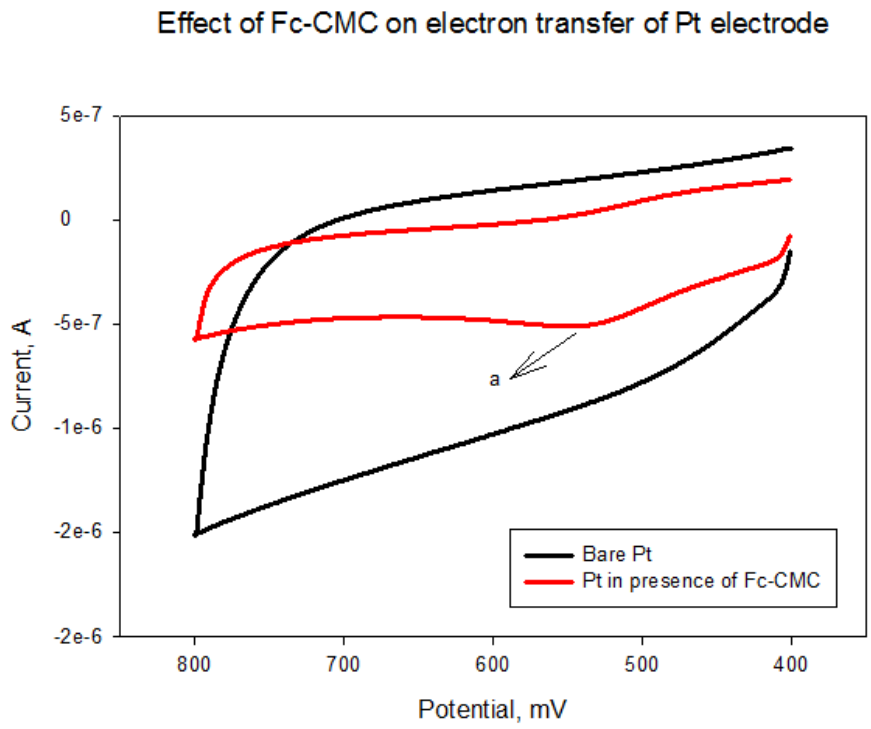


Figure 17. Overlay of bare Pt electrode and effect of Fc-CMC.

In figure 17, overlay of bare platinum with that of the electrode in PBS solution containing Fc-CMC shows a dramatic decrease in current wave seen in voltammogram. This insulation is due to CMC which significantly hinders electron transport. This blocking of electron flow is important for the development of immunosensor such that it forces electron flow from electrode to ferrocene and blocking of non-specific adsorption

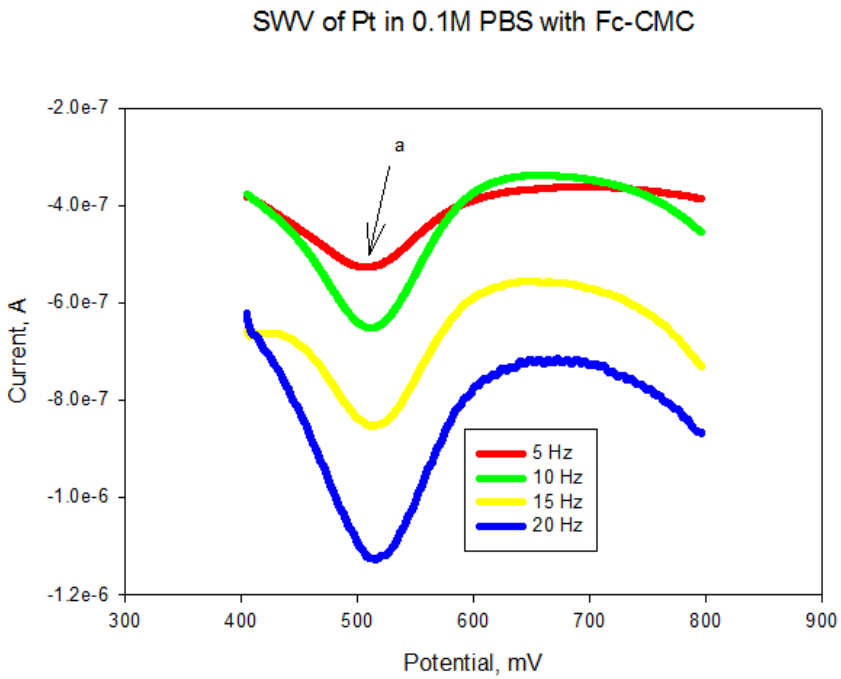


Figure 18. SWV of Pt electrode in 0.1 M PBS with Fc-CMC

Using SWV for characterisation allows the observation of more sensitive electron flow such that the peaks observed in CV are much more pronounced. An increase of frequency from 5 Hz to 20 Hz was done. In figure 18, anodic peak is observed at 495 mV, and increases proportionally with increase in frequency. The peak current for oxidation process is in well co-ordinance with that of the corresponding CV (Figure 16).

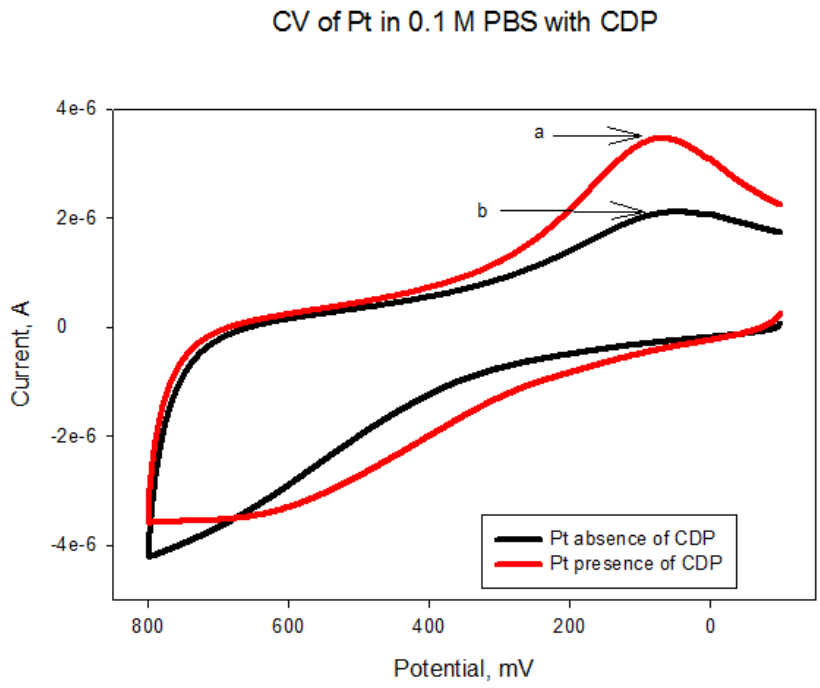


Figure 19. CV of Pt electrode in the presence and absence of βCDP in solution.

The cyclic voltammogram for βCDP in 0.1 M PBS is seen in figure 19. An I_{pc} for Pt electrode in the absence of βCDP is $-2.4e^{-6}$ A with an E_{pc} of 50 mV (peak a). The addition of βCDP causes a 75 % decrease in Pt electrode oxidation peak to $-1.76e^{-6}$ A (peak b).

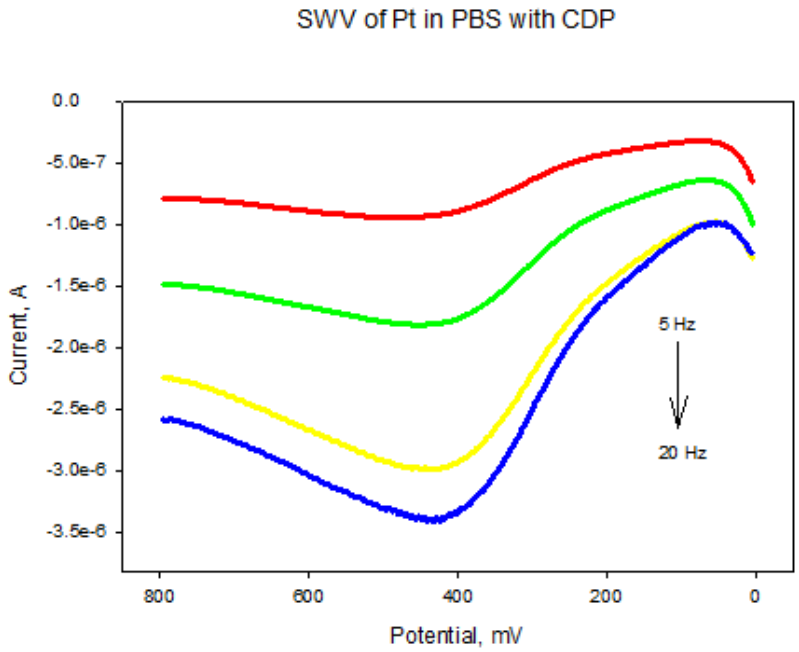


Figure 20. SWV of Pt electrode with βCDP in solution

In Figure 20, square wave voltammograms for oxidation process at different frequency's is observed. Formal potential is seen to 420 mV, with I_{pa} increasing from -9.8475e^{-7}A for 5 Hz to -3.405e^{-6}A for 20 Hz. The oxidation currents correspond to the oxidation of hydroxyl groups on βCDP.

4.3.1.2. Electrochemical response of βCDP and Fc-CMC in PBS solution with gold electrodes

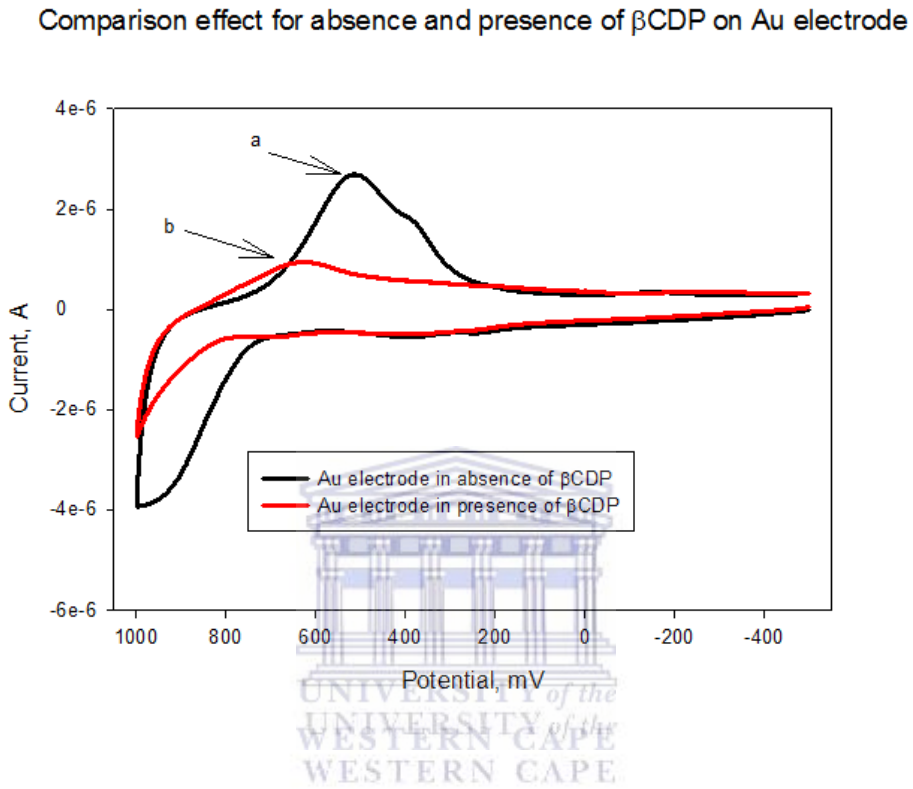


Figure 21. Comparison of bare Au electrode in the absence and presence of βCDP in PBS 0.1 M solution.

The electrochemical diffusion process for βCDP was studied using an Au electrode in a 0.1 M, pH 7.2 PBS solution. Addition of βCDP decreased the pH slightly but had an insignificant impact on the cyclic voltammograms. As seen in the behaviour of βCDP with Pt electrode, in figure 21 a shift in cathodic potential (E_{pc}) to more oxidative potentials occurs. The shift is due to electron transfer in conjunction with insulating polymer for Au electrode. Shift occurs from 522 mV (peak a) to 610 mV (peak b), which corresponds to an 88 mV shift. The reduction in I_{pc} is as observed for Pt electrode, a 75% decrease in Ipc is observed due to the βCDP.

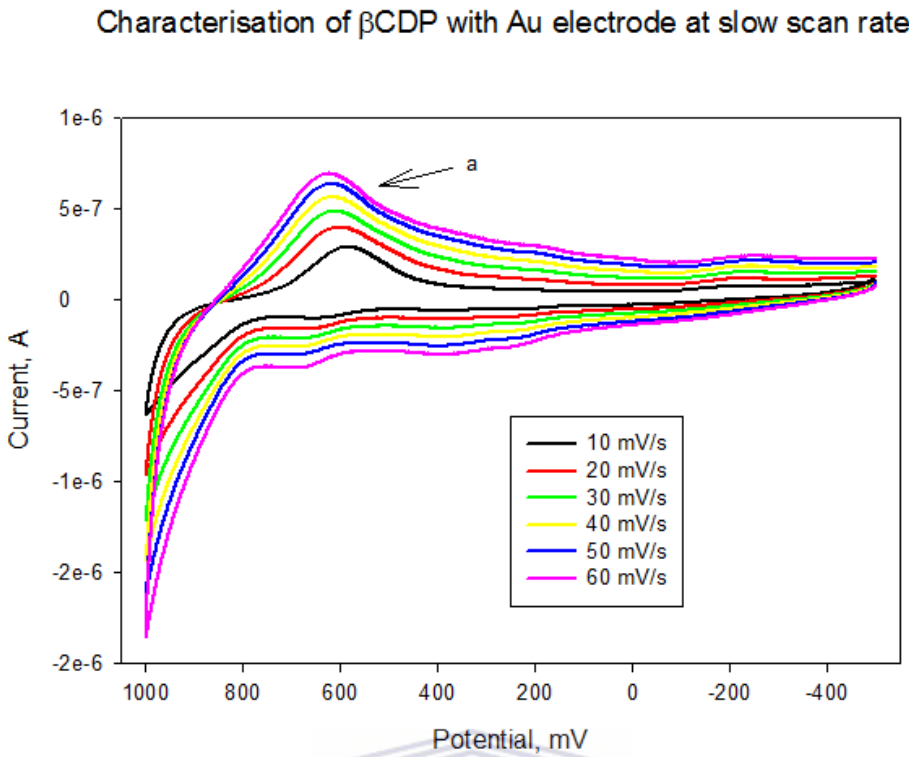


Figure 22. CV at slow scan rates of βCDP in PBS with Au electrode

Applying slow scan rates reduces the rate of electron transfer making it possible to observe small electronic changes or shifts as observed in figure 22. As different scan rates are applied, I_{pc} values increase proportionally. High molecular weight polymers have a significantly slow diffusion rate in comparison to their monomers. In the case of βCDP, bulkiness and structural effects causes the slow diffusion rate. At faster scan rates, electrochemistry of βCDP is much more challenging to observe. The fast electron transfer from transducer over shadows that of βCDP. As scan rates tend closer 200 mV/s, differences in peak heights decreases, this decrease is seen in the slopes, which is gives a lower diffusion rate.

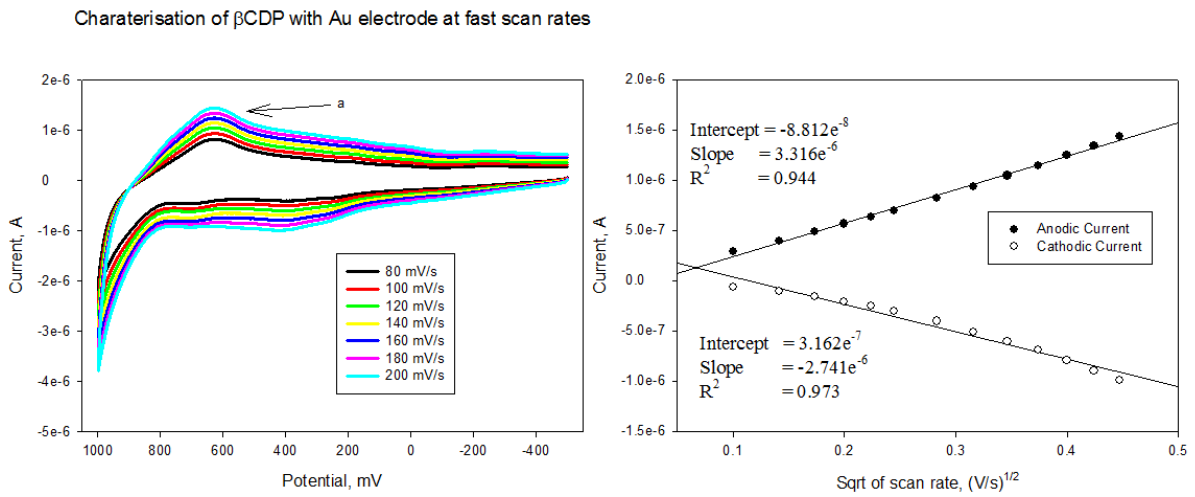


Figure 23. CV at fast scan rates of βCDP in PBS with Au electrode. Randel-Sevich plot

The Randel-Sevich plot seen in figure 23 shows a linear response for CV of βCDP with Au electrode in 0.1 M PBS at slow and fast scan rates. Peak currents increase linearly with increase of scan rate. For oxidation of βCDP, a slope of $-2.741e^{-6}$ A/(V/s)$^{1/2}$ and for reduction a slope of $3.316e^{-6}$ A/(V/s)$^{1/2}$ (peak a).

To further illustrate the electrochemical behaviour of βCDP in 0.1M PBS solution, SWV was done at different frequencies, namely 5 Hz, 10 Hz, 15 Hz and 20 Hz. Peak potentials remain unchanged at 615.8 mV as frequency increases. Peak currents increases with increase in frequency.

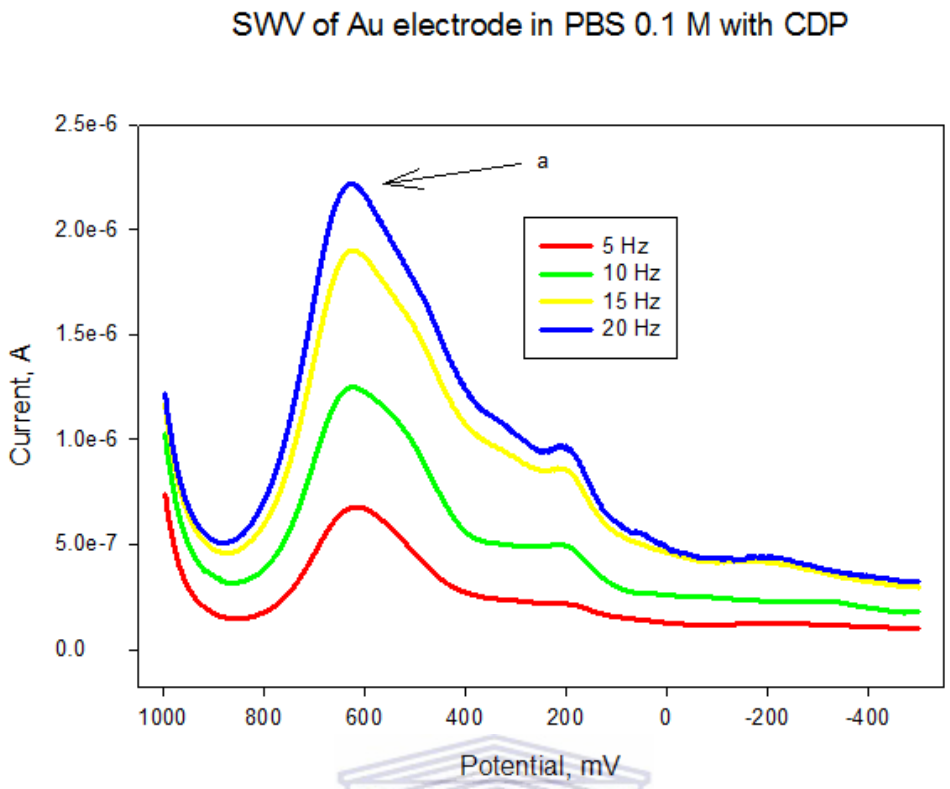


Figure 24. SWV for reduction of βCDP in 0.1 M PBS with Au electrode

Electrochemical characterisation of Fc-CMC with a platinum electrode in combination with Fc-CMC with gold electrode concludes the slow diffusion towards electrode surface and insulating character of CMC moiety. The well pronounced gold reduction peak at round 500 mV (peak a) disappears in the presence of Fc-CMC. Scan rate is 100 mV/s as observed in figure 25. One contributing factor is as mentioned for the electrochemistry of βCDP with gold electrode. The shear bulk of the polymer combined with steric effects reduces diffusion towards the electrode surfaces significantly.

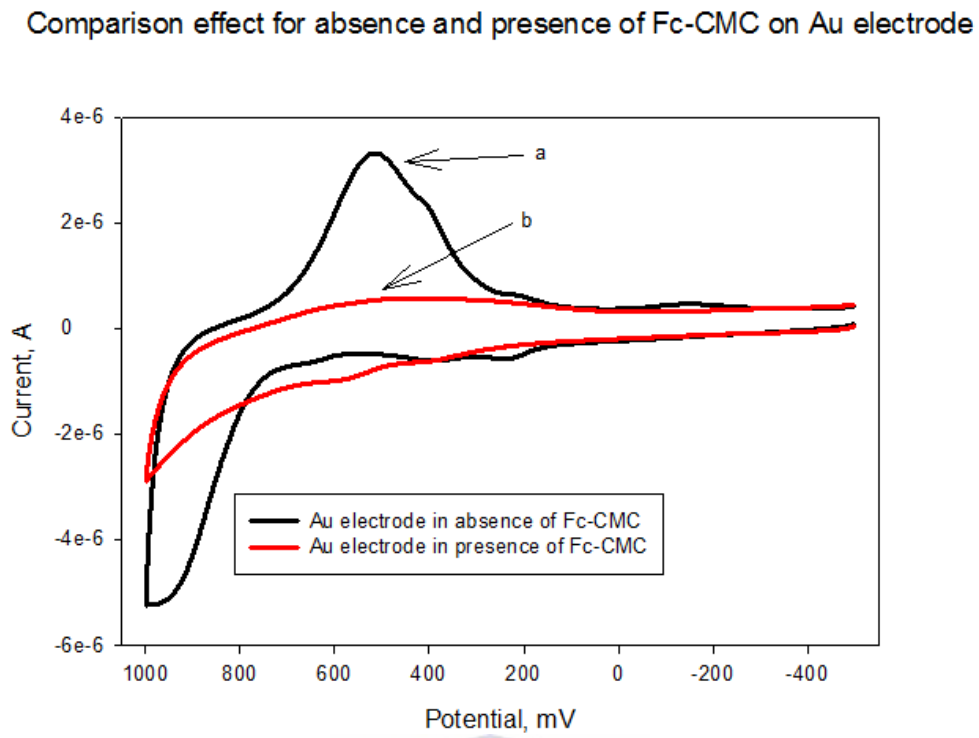


Figure 25. CV of Au electrode in 0.1 M PBS in the absence and presence of Fc-CMC

Characterisation of Fc-CMC in PBS at slow scan rates shows a pair of redox peaks at about 580 mV seen in figure 26. The redox peaks and current wave represents the presence of Fc in solution. The low peak current derives from the covalent linkage to CMC and thus reduction in diffusion towards the electrode surface. Slopes for oxidation and reduction for Fc-CMC at slow scan rates are $-4.456e^{-6}$ $A/(V/s)^{1/2}$ (peak b) and $2.426e^{-6}$ $A/(V/s)^{1/2}$ (peak a).

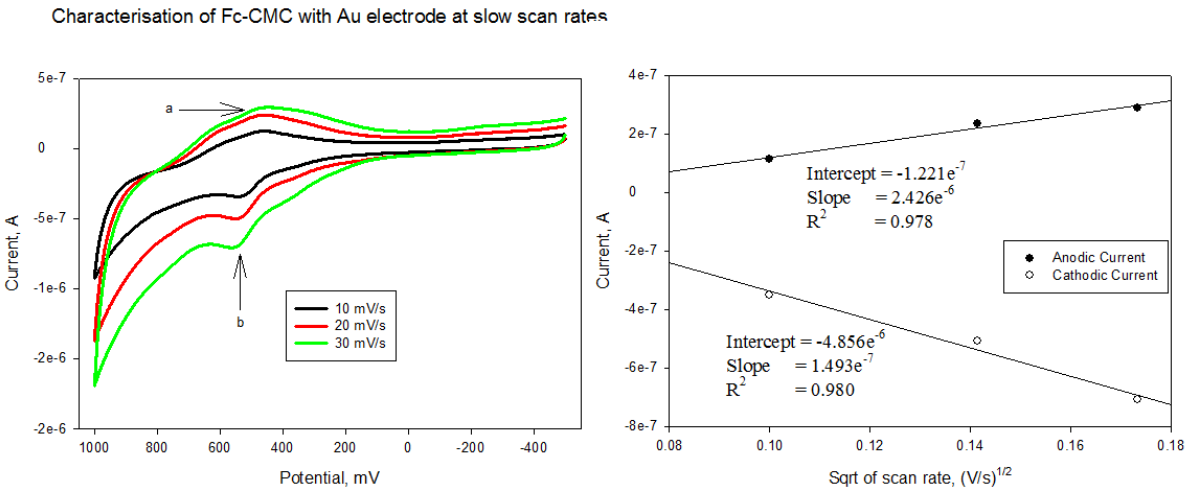


Figure 26. CV at slow scan rates of Fc-CMC in 0.1 PBS with gold electrode.

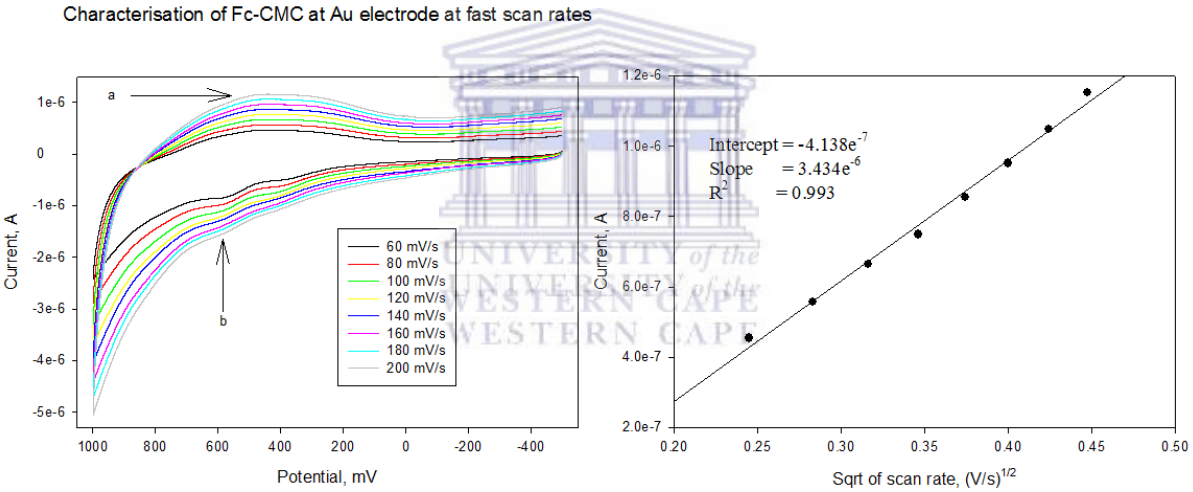


Figure 27. CV of Fc-CMC at fast scan rates in 0.1 M PBS with Au electrode. Randel-Sevich plot.

Figure 27 shows that at faster scan rates, the electrochemistry of Fc-CMC at bare gold electrodes is difficult to observe. Evan though I_{pc} increases linearly with scan rate.

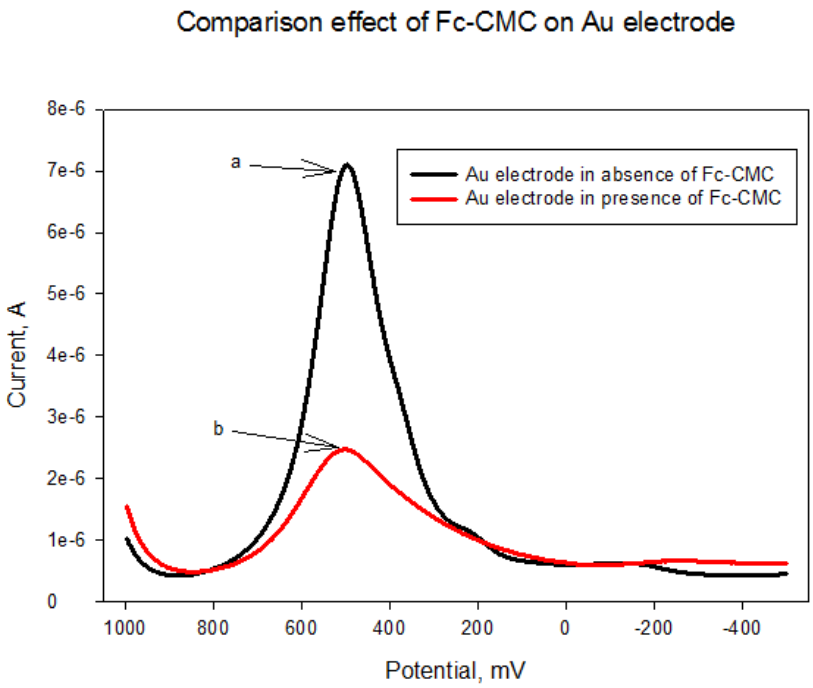


Figure 28. SWV for the reduction of Fc-CMC in 0.1 M PBS with Au electrode

The SWV of Fc-CMC was done with a gold electrode seen in figure 28. SWV of Fc-CMC in 0.1 M PBS solution shows a dramatic decrease in $I_{p,c}$ which occurs due to the presence of CMC. The $E_{p,c}$ at 560 mV remains unchanged. A large reduction in peak a is observed as compared to peak b. As previously mentioned, the insulating character of CMC blocks electron transport to and from the electrode surface.

4.3.2. Electrochemical Characterization of supramolecular complex on gold surface.

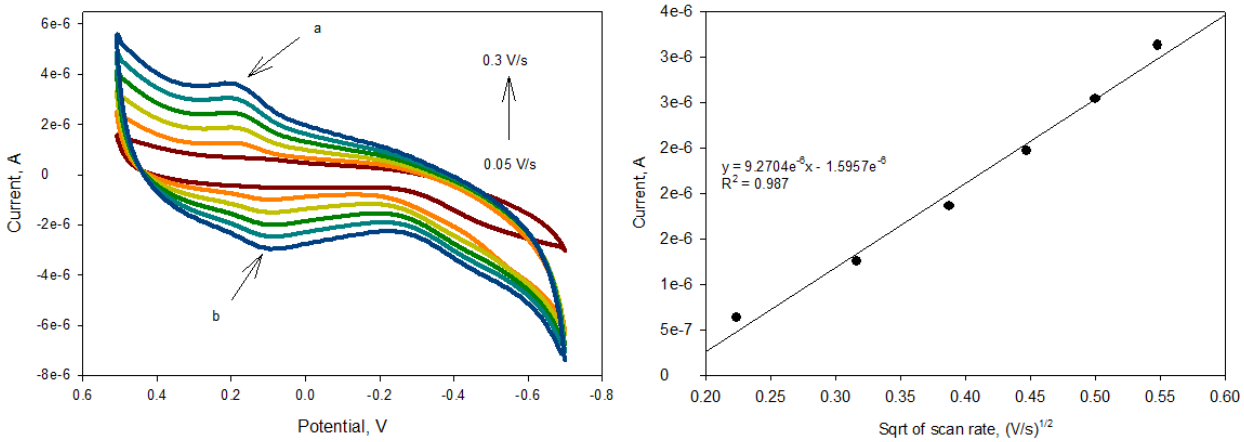


Figure 29. Cyclic voltammograms at different scan rates showing the presence of ferrocene immobilized on the cyclodextrin modified surface within immunosensor (strategy b).

Cyclic voltammetry was used to study the supramolecular interactions between Fc and βCDP on a gold electrode. The inclusion complex is favoured in terms of its entropy. Characterization of this supramolecular interaction was done by using different scan rates. The presence of Fc is observed by redox peaks at peak a and peak b, namely reductive peak at 0.1 V and oxidative peak at 0.2 V. These peaks are commonly observed for ferrocene on gold electrodes [Ortiz *et al.*, 2011]. Increasing scan rates, shows no change in E_{pc} and E_{pa}, an indication that Fc is surface bound and maintains its electro active character in the supramolecular inclusion. Randel-Sevick plot shows that current increases linearly with the square root of scan rate. This demonstrates that Fc is surface bound, parameter important for the electrochemical detection of hCG.

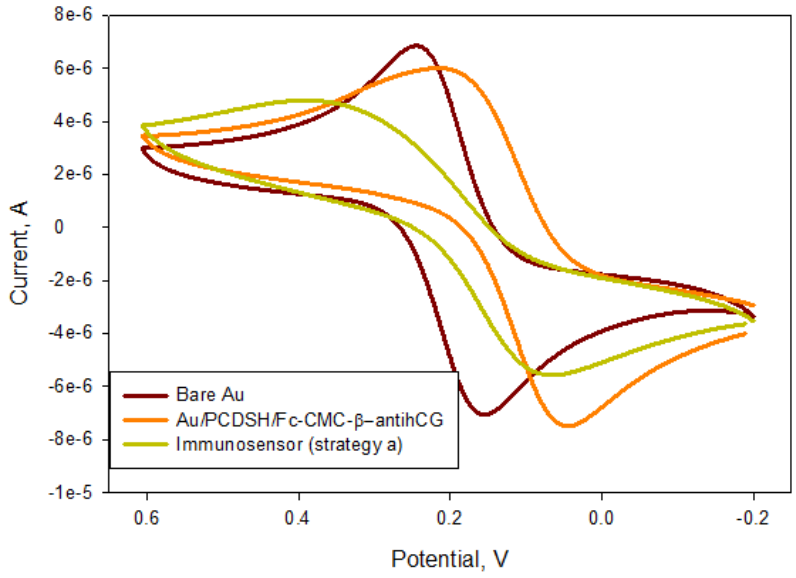


Figure 30. CV for different immobilization steps in immunosensor design. $[K_3Fe(CN)_6]$ in 0.1 M KCl

In Figure 30, a shift in $E_{1/2}$ of 40mV and 41mV of βCD modified gold electrode compared to bare gold electrode (b). A slight decrease in current is observed indicating the blocking of gold electrode area by βCD [Ortiz *et al.*, 2011]. This blocking of electrode surface is important for decreasing non-specific adsorption. Furthermore, immobilization of Fc-CMC-β-antihCG reduced redox peaks due to increase in electron transfer distance and increased the distance between peaks. This increase in distance between peak potentials shows an irreversible process occurring.

CHAPTER 5

5. Results and Discussion 2

5.1. ELISA measurements and electrochemical detection of hCG for strategy A and strategy B.

5.1.1. UV-vis absorbance of glucose oxidase conjugate

Synthesis of glucose oxidase reporter conjugates were done following well known SATA-protocol

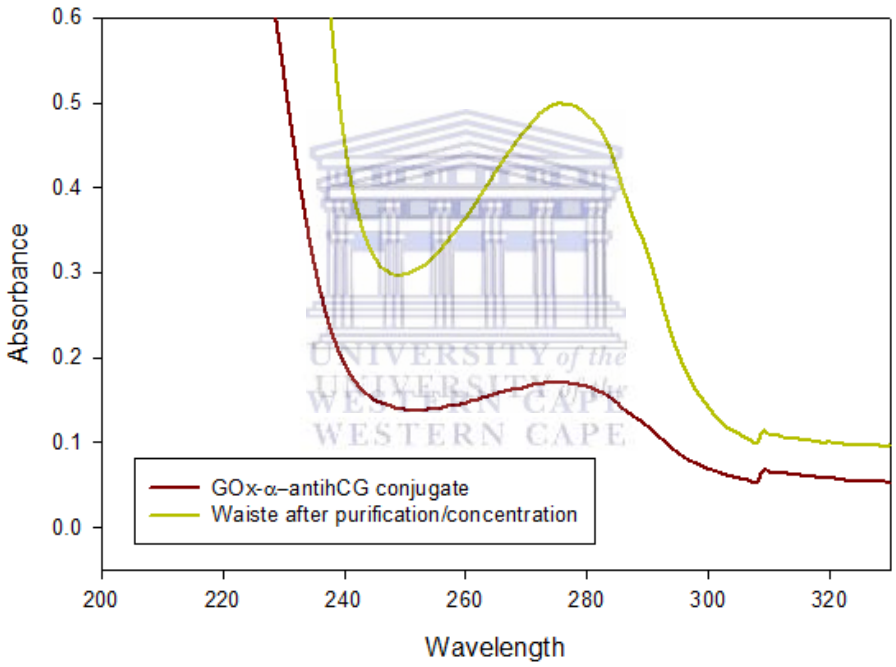


Figure 31. UV-vis absorbance of glucose oxidase labelled antibody

The determination of concentration of enzyme labelled antibodies is typically done by using UV-Vis spectroscopy. It is well known that amino acids of protein biomolecules absorb light at 280 nm wavelength. Molar absorptivity of proteins are found in literature, and from the absorbance measured, the Beer-Lamberts law is used to calculate concentrations of bioconjugates. These concentrations are essentially important for determination of conjugate

activity, enzyme activity and binding affinity of antibody after conjugation. Figure 31 shows the absorbance of glucose oxidase labelled antibody. As depicted, absorbance values at 280 nm indicated concentration of 6.3 mg/ml of GOx conjugate.

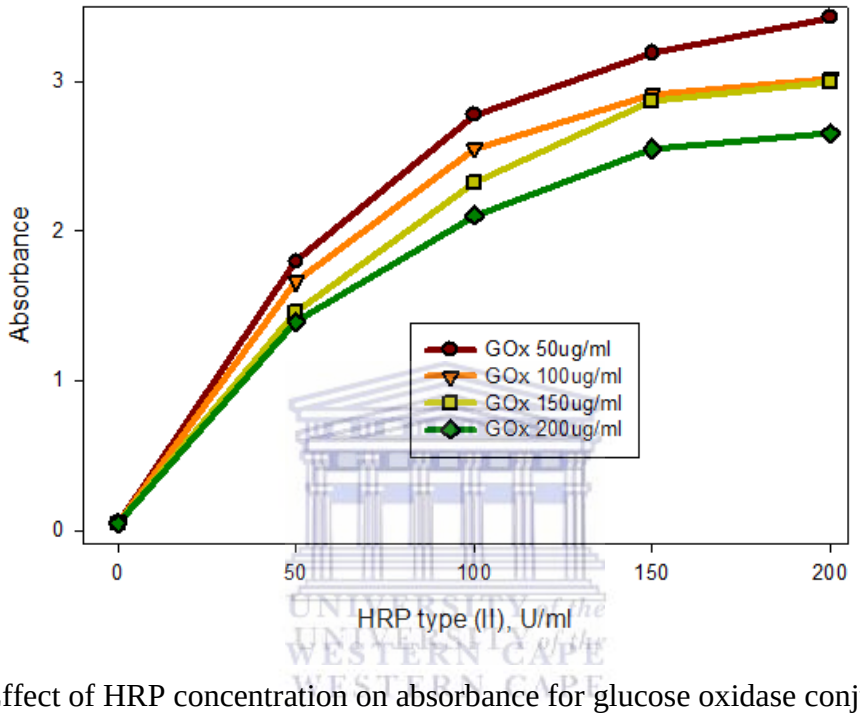


Figure 32. Effect of HRP concentration on absorbance for glucose oxidase conjugates

The co-operative effect between HRP and GOx is the bases for detection of hCG and the operating principle for the immunosensor. Since there is an *in-situ* production of hydrogen peroxide, the concentration of HRP should be optimal for reduction of H_2O_2. Figure 32 shows the effect of concentrations of HRP on absorbance. NUNC plates were used, with conjugated GOx free in solution, which contained 2 mM glucose, and 10 mM TMB powder. The lowest concentration of 50 µg/mL GOx gave the highest absorbance. The absorbance increases with increase of HPR enzyme concentration, with good absorbance results at different concentrations of GOx. Therefore the lowest concentration of HRP was used, where detection is done by 20 µg/mL of HRP for strategy b, and 10 µg/mL for strategy a.

5.1.2. Glucose oxidase labelled antibody enzyme activity and dependence on HRP concentration

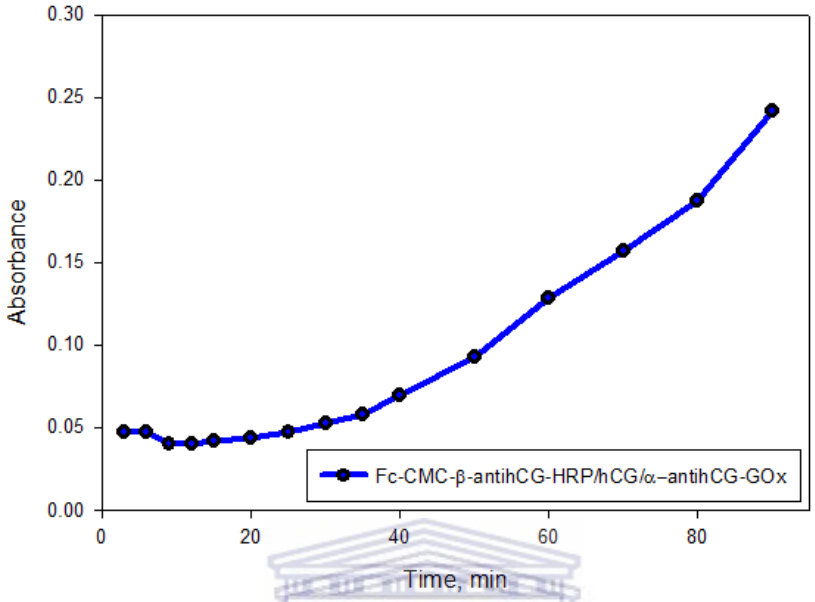


Figure 33. Enzymatic assay of glucose oxidase

It is crucial to determine the activity of glucose oxidase after conjugation and binding constant to glucose. Enzyme activity changes after conjugation, a loss in enzyme activity is expected but should be minimal. Figure 33 represents colorimetric assay of glucose oxidase with direct sandwich assay (figure 4) and monitoring absorbance as function of time. Glucose oxidase conjugate synthesised from SATA protocol were tested. Absorbance increases proportionally with time showing that the glucose oxidase conjugates remained active after conjugation and extended reaction time. Reaction time for oxidation of glucose is critical, since this reaction time should be optimal for the detection and assays of hCG. A reaction time of 40 minutes was chosen. Reaction kinetics within the assay is dependent on amount of HRP units present, concentration of TMB powder and concentration of glucose substrate. Reaction scheme used in enzymatic assay for glucose oxidase is as follows:

β-D-Glucose + O_2 + GOx → D-glucono-1,5-lactone + H_2O_2

H_2O_2 + TMB(reduced) + HRP → TMB (oxidized) + $2H_2O$

A 1 to 1 relationship between glucose and TMB is exists, so TMB and HRP is added in excess such that the reaction is dependent of the conversion rate of β-D-glucose to D-glucocono-1,5-lactone.

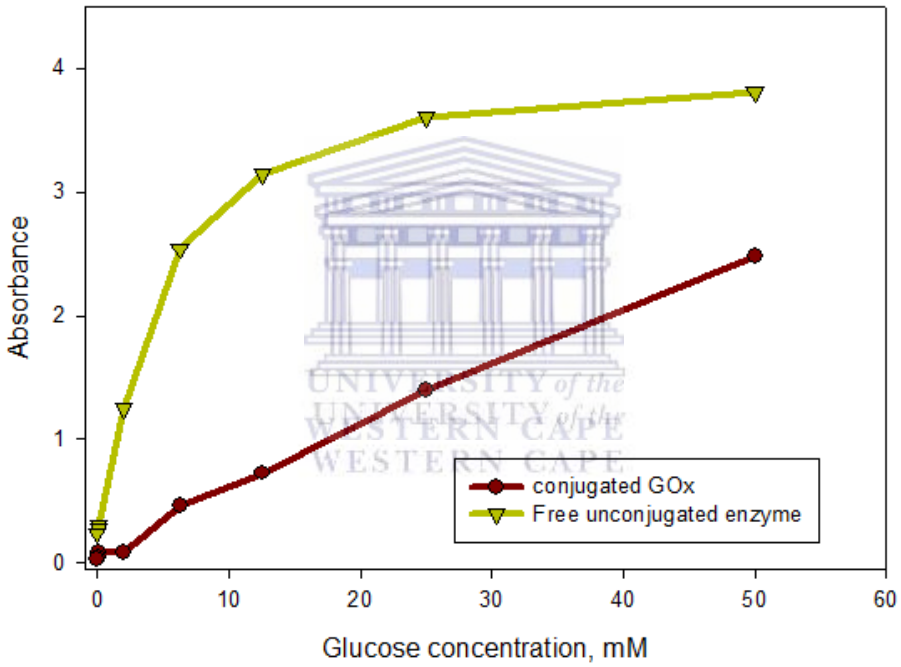


Figure 34. Enzymatic activity of free glucose oxidase and conjugated enzyme. (black line: conjugated GOx, red line: free GOx)

Enzyme activity after conjugation and antibody binding affinity for electrochemical detection is important since electrochemical signal is dependent antibody-antigen affinity and on the production of H_2O_2. Glucose oxidase catalyzes the oxidation of β-D-glucose to produce H_2O_2. The rate of the reaction or substrate oxidation is dependent on enzyme concentration, substrate concentration, enzyme activity and hosts of other factors. When maintaining

constant concentration of enzyme, the enzymatic catalysed reaction becomes dependent on glucose concentration. The reaction is monitored by increase in absorbance. The reaction is monitored as a function of time by keeping GOx and glucose concentrations constant. In figure 34, unconjugated enzyme is compared to conjugated enzyme to determine the percentage loss in enzyme activity after conjugation. Concentration of 50 µg/mL of non-conjugated and conjugated GOx was used. This corresponds to 0.951 units/mL of free enzyme and 0.326 units/mL of conjugated enzyme. A 12% loss in GOx activity is observed, particularly at the lower concentrations of glucose. Conjugated GOx was tested with direct sandwich assay using 10 µg/mL of hCG. It is important to note, that since direct sandwich assay with hCG was used, the concentration of GOx conjugate is dependent on the binding to hCG antigen.

Immunosorbent plates are suitable platforms for immobilizing recognition species for immunoassays. These assays are used for colorimetric assays and detection of bio-species. It is also a method commonly shown to determine binding between antibodies and antigens. The enzymatic assay was based on GOx conjugates for the detection of hCG [Ortiz *et al*., 2011]. Sensitivity is important when doing assays, therefore indirect sandwich assay was used for detection of hCG. Using Fc-CMC-β-antihCG as recognition layer and HRP conjugate as reporter probe, the detection of hCG was done by measuring the absorbance after the addition of TMB (ELISA substrate) at 650 nm. The β-antihCG is specific for the β-site of hCG such that random orientation is reduced when HRP-α-antihCG binds.

5.2. ELISA detection of hCG

5.2.1. Immunosensor design strategy A in the absence of βCDPSH

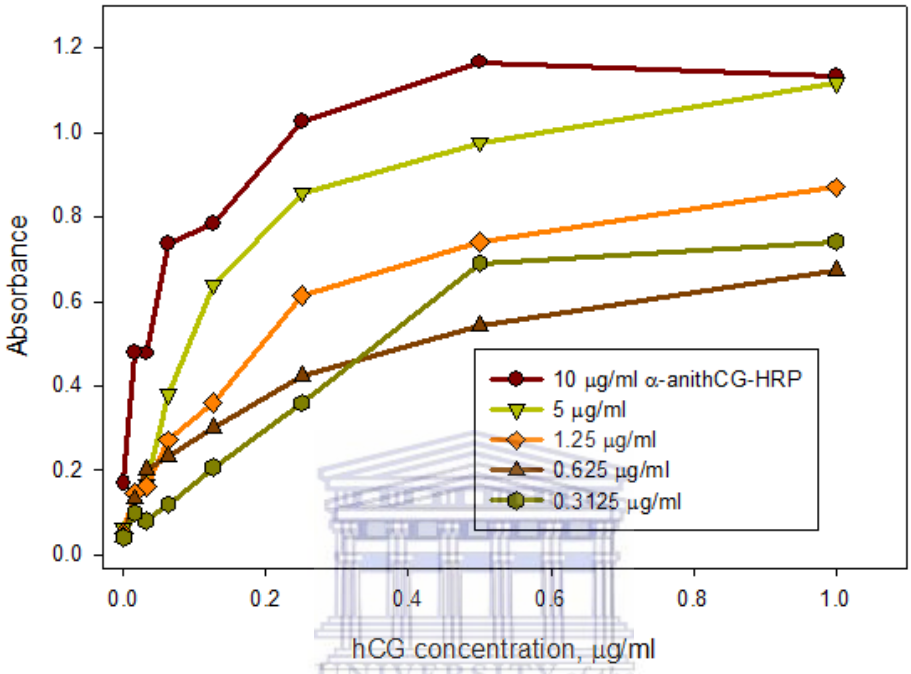


Figure 35. Detection of hCG using strategy (a) with ELISA

Figure 35 represents the immunoassay for the detection of hCG antigen at different concentrations for strategy a. Different concentrations of hCG and α-antihCG-HRP antibody were used, with a constant concentration of 10 µg/mL of Fc-CMC-β-antihCG used in the assay. Indirect sandwich assay is done to increase sensitivity when compared to direct assay and to maintain similar results to those of electrochemical detection. In the absence of hCG antigen the background signal is very low. This derives from the high specificity of the capture β-antihCG antibody for specific epitope on hCG (β-site) and good performance of assay. The use of Tween 20 reduces non-specific adsorption producing a good performance assay. The limit of detection was calculated from the calibration curve using 20 µg/mL concentration of α-antihCG-HRP antibody to be 239 pg/mL, which represents 2 mIU/mL.

5.2.2. Immunosensor design strategy B in the absence of βCDPSH

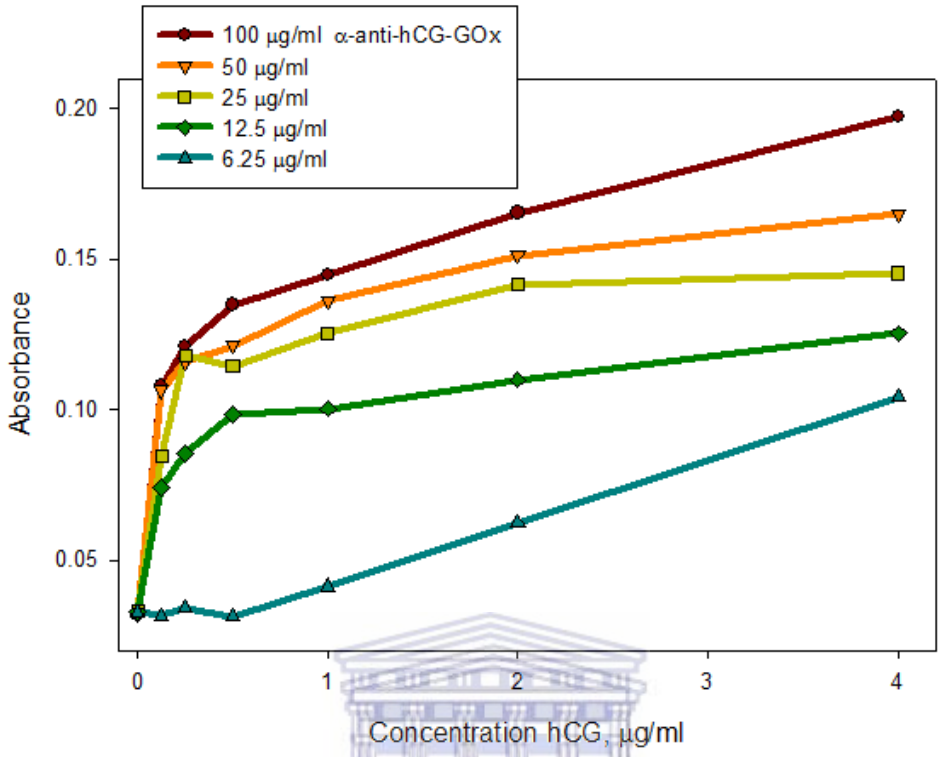


Figure 36. Detection of hCG with strategy (b) with ELISA

Figure 36 represents immunoassay for strategy b. Indirect sandwich assay is used as to maintain similar results when compared to the electrochemical detection. The incubation of Fc-CMC-β-antihCG-HRP was done for 1 hour followed by blocking. Various concentrations of hCG and α-antihCG-GOx conjugate are used. Absorbance signal increases proportionally to both reporter probe and hCG concentrations, which indicates good performance of the assay. The low absorbance as compared to figure 35 derives from the loss in enzymatic activity of GOx when conjugated as well as number of glucose oxidase units conjugated to α-antihCG. A unit ratio of 1:1 of GOx to α-antihCG is assumed. A detection limit of 2.35 ng/mL representing 21.15 mIU/mL of hCG was found. The lower absorbance is due to steric effects observed within the conjugate since both GOx and the antibody has molecular weights around 150 KDa. GOx also has its amino groups densely packed in its core, which further

reduces the bind between antibody and enzyme. HRP has a molecular weight of 45 KDa, and these steric effects are not observed since the enzyme is 3 times smaller than the antibody.

5.3. Electrochemical activity of HRP for strategy B on Screen printed carbon electrode.

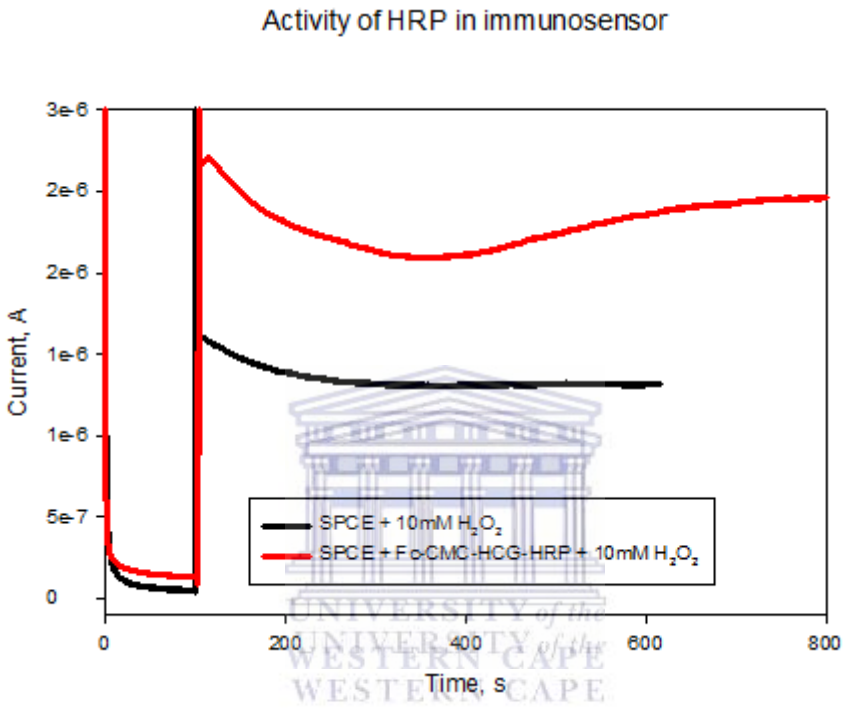


Figure 37. Electrochemical activity for the presence of HRP for strategy (b)

An important factor within immunsensor design for strategy b, is the electrochemical enzyme activity of HRP towards the reduction of hydrogen peroxide. After immobilization of thiolated βCDPSH and Fc-CMC-anti-βhCG-HRP, the amperometric response of recognition layer was done towards the addition of 10 mM H_2O_2. In figure 37, the presence and activity of HRP after conjugation is seen by increase in current compared to the absence of HRP. This shows that HRP attains its activity after conjugation which is important for the co-operative effect in the detection of hCG.

5.4. Optimization of incubation time for electrochemical detection

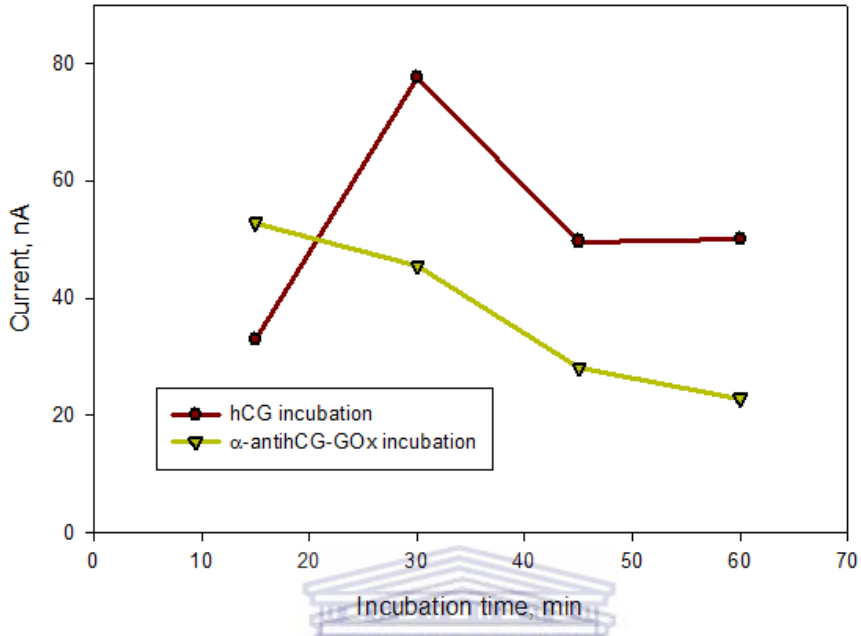


Figure 38. Electrochemical optimization of the incubation times for strategy (b).

To achieve the highest and most selective responses, the incubation times of hCG and α-antihCG were investigated. The effect of incubation times on immunosensors is a well-known factor for electrochemical detection [Ortiz *et al*., 2011]. Figure 38 shows the optimization of incubation times for the detection hCG for strategy. hCG was incubated at different times whilst keeping constant the incubation time of α-antihCG-GOx at one hour. The reporter probe, α-antihCG-GOx, was incubated at different times whilst keeping constant the incubation time of antigen at one hour. Optimal incubation times were 30 minutes for hCG antigen and 1 hour for reporter probe. These incubation times gave the highest response.

5.5. Electrochemical amperometric detection of hCG

The calibration curve obtained for the detection of hCG antigen using strategy (a) in scheme 5 under optimal experimental conditions is seen figure 39. An increase in hCG concentration shows an increase in the current response from the amperometric measurements. Therefore, using strategy (a) yielded a limit of detection of 3.7283 ng/mL (33.56 mIU/mL), a sensitivity of 0.09148 nA ng^{-1} mL^{-1} and regression coefficient of 0.963. This LOD is higher when compared to 238 pg/mL found for ELISA. Detection commences by the addition of 10 mM H_2O_2 in a 1:3 volume ratio to 0.1 M PBS. Current response derives from the enzymatic reduction of H_2O_2 to water and oxygen by HRP.

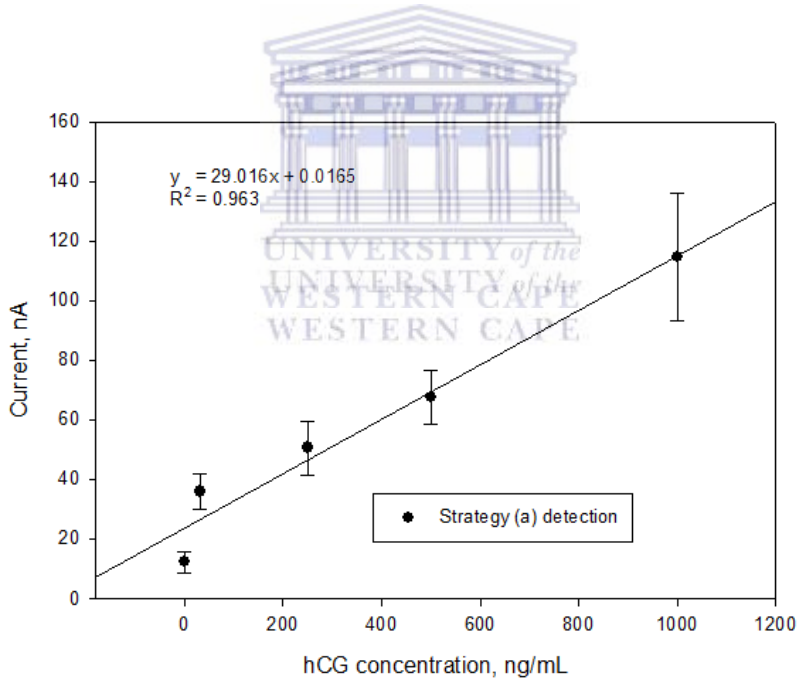


Figure 39. Calibration plot for the electrochemical detection of hCG with strategy (a)

The presence ferrocene on the electrode surface removes the use of further additional electron mediators such as TMB. The electrochemical detection system depends on the addition of H_2O_2 which is a limiting factor. The pH of electrolyte solution affects the amperometric

response of the immunosensor by affecting the enzymatic reaction occurring at the electrode surface. Since the detection is done by reduction of H_2O_2 by HRP, the optimal pH for the activity for HRP was chosen at pH 6 [Yang *et al.,* 2011]. Linear region lies between 62.5 ng/mL to 1 mg/mL of hCG antigen. Error bars shows reproducible responses, with a high error margin at concentration of 1 mg/mL. Reproducibility and stability in terms of response and performance of immunosensors are important factors in the development of immunosensors.

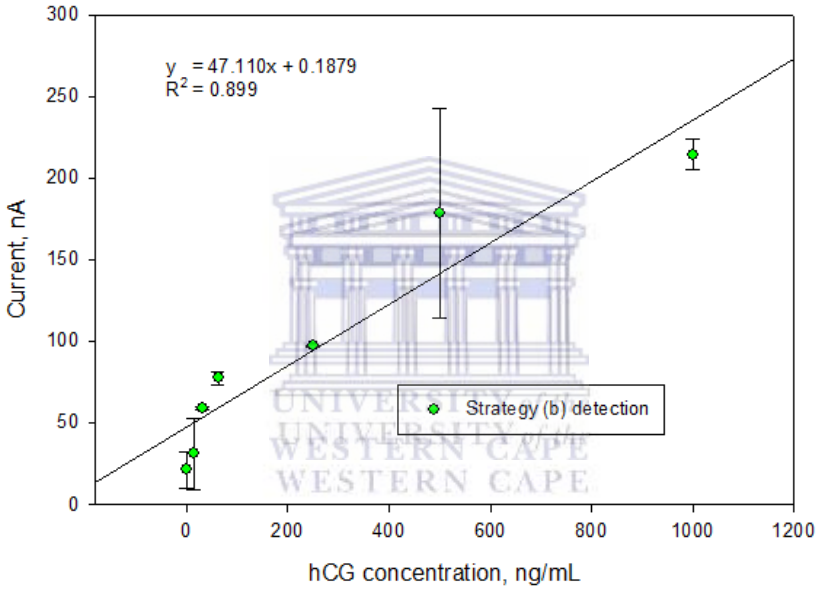


Figure 40. Calibration plot for the electrochemical detection of hCG with strategy (b).

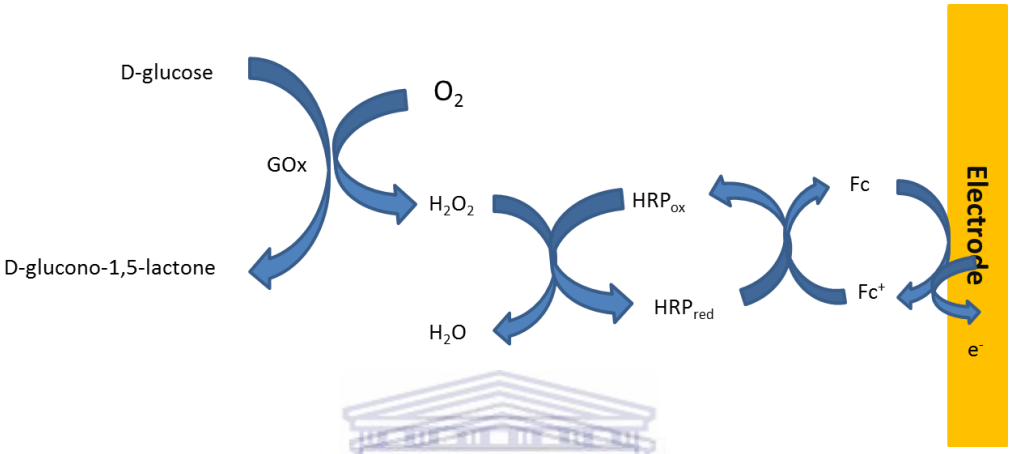
The calibration curve for the detection of hCG antigen using strategy (b) is shown in figure 40. An increase in hCG concentrations yields an increase in amperometric response. Enzymatic oxidation of glucose by glucose oxidase produces H_2O_2 *in situ,* which is further reduced by HRP enabling electrochemical detection. Detection is done by addition of 10 mM of D-(±)-glucose in 1:3 volume ratio to 0.1 M PBS with applied potential of 0.2 V. A linear behaviour is observed between 0 ng/mL to 62.25 ng/mL with a limit of detection of 700

pg/mL corresponding to 6.3 mIU/mL and sensitivity of 0.94 nA ng^{-1} mL^{-1}, which is one order higher than amperometric detection of strategy (a).

The absence of hCG gave low amperometric response, since no binding occurs between Fc-CMC-β-antihCG-HRP and GOx-α-antihCG antibody. The absence of GOx results in no electron transfer between HRP and electrode surface since there is no production of H_2O_2 [Chen *et al*., 2005]. The electrochemical signal increased two-fold as compared to strategy (a). Multiple supramolecular interactions between βCDP and Fc, enables multiple Fc units to be involved in the electron transfer process. This multiple host-guest interactions increase the stability of the recognition layer and the amperometric detection. These surface confined inclusion complexes combined with bienzymatic system enables increased signal. There is a continuous production of H_2O_2 from the enzymatic oxidation of glucose oxidase. This removes the amount of H_2O_2 limiting factor in the detection of hCG.

Non-specific signals due to non-specific adsorption are reduced by the blocking effect of β-cyclodextrin SAM and CMC on gold electrodes. It is well know that H_2O_2 diffuses readily to the electrode surface. The βCD and electron insulator behaviour of CMC allows the diffusion of H_2O_2 to the electrode surface. From literature, glucose oxidase has optimal enzyme activity at a pH 5.5. At this pH, there is maximum conversation of glucose to H_2O_2. Therefore this was the chosen pH for the electrochemical detection. Using glucose oxidase in close proximity to other biomolecules within a detection strategy, particularly in bienzymatic systems, can result in insignificant current signals due to the high steric hindrances [Ortiz *et al*., 2012].

Steric hindrance is not a factor in this strategy since glucose oxidase is conjugated onto reporter antibody through amine coupling providing sufficient molecular space for enzymatic reactions to occur. Strategy b shows improved performance compared to strategy a, this is due to the co-operative effect between the peroxidase and oxidase enzyme.



Scheme 7. Reaction mechanism for electrochemical detection of hCG.

Mechanism depicted in scheme shows the electron transfer from the oxidation of D-glucose to oxidation of ferrocene. In presence of oxygen, oxidation of D-glucose provides *in situ* production of H_2O_2 which is then further reduced by HRP. This reduction is an enzymatic reduction. The application of oxidative potential of 200 mV results in the current dependent on the concentration of hCG present. The oxidative potential withdraws an electron from HRP further through Fc mediator.

CHAPTER 6

CONCLUSION

FTIR and NMR structural analysis revealed compounds βCDPSH and Fc-CMC. Furthermore, the use epichlorohydrin in the polymerisation of βCD ensured the structural integrity of βCD remained unchanged. Morphology studies showed particle sizes up to 3 nm for βCDPSH and 4.5 nm for inclusion complex formed with Fc-CMC. Electrochemical characterization of βCDP and Fc-CMC in a solution of 0.1 M PBS under diffusion process showed significant decreases in overall current waves as compared to the inclusion complex immobilized on the surface of gold electrode. SAM of βCDPSH on gold electrode followed by host-guest complex formation showed that Fc maintained its electrochemical redox behaviour since it was in close proximity to the electrode surface. The bienzymatic strategy for the detection of hCG antigen has been shown. An increase in sensitivity from βCDP and ferrocene supramolecular inclusion complexes was observed. The co-operative effect of enzymes in strategy (b) increased the sensitivity and decreased the limit of detection in comparison to strategy (a). Electrochemical detection of hCG using strategy A showed a limit of detection of 2.35 ng/ml with α-antihCG-HRP antibody, whereas a lower limit of detection of 700 pg/ml for hCG is shown using strategy B.

The stability of this inclusion complex and aided directing orientation of recognition layer makes it possible for protein detection. This approach for detection of proteins offers more realistic, affordable and simple when compared to current methods.

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