Sensing Devices for Illicit Drug Detection

A THESIS

Submitted by

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In partial fulfillment for the award of the Degree of

5-year Bachelor of Science-Master of Science

Under the guidance of

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Confidentiality and Disclaimer

The project "Sensing device for illicit drug detection" is an initiative of Future Industries Institute (FII), UniSA, Adelaide in collaboration with the industry partner- Alcolizer. The information contained in the thesis is strictly confidential and should not be made available to any other person or entity without consent.

Certificate

This is to certify that this dissertation **"Sensing devices for illicit drug detection"** is entitled towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune. This represents the work carried out by **A R Anandapadmanabhan** at **Future Industries Institute, University of South Australia** under the supervision of **Dr. Beatriz Prieto Simon and Prof. Nico Voelcker** during the academic year of 2016-2017.

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Signed: 

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Acknowledgments

It is my privilege to express my sincere and deep sense of gratitude to my supervising guide **Dr. Beatriz Prieto Simón and Prof. Nico Voelcker**, for their excellent guidance and constant encouragement throughout the project. They steered me in a right direction whenever they thought I needed it. I am thankful to them for providing me with such an opportunity to work in their group, which will help me in future to a great extent. I would also like to thank **Dr. Xavi Cetó Alsedà** and **Dr. Sandra Pérez Rafael,** who were very supportive and encouraging. Without their passionate participation and input, the project would not have been materialized.

I would also like to acknowledge **Prof. Sulabha Kulkarni** and **Dr. Pramod Pillai** of Indian Institute of Science Education and Research (IISER), Pune, India as the thesis advisory committee (TAC) member and I am gratefully indebted to their valuable comments on this thesis.

Thanks, are due to my colleagues and friends for their support, encouragement and for keeping me motivated.

Finally, I express my heartfelt gratitude to my parents for their continuous encouragement, care, understanding and unfailing support throughout my life. This accomplishment would not have been possible without them.

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Abstract

This project is an initiative of the Future Industries Institute (FII) in collaboration with Alcolizer to design and build a novel biosensor for the detection of illicit drug, specifically tetrahydrocannabinol (THC). This report covers the requirements of such device, literature review, methodology and further work consideration.

With increasing cases of drug abuse, the demand of various sensing devices continues to increase. Chemists and engineers are contributing immensely to develop devices that can detect drugs with high selectivity and sensitivity. Biosensors have grown from a niche academic discipline with a couple of minor products in 1970's to a major commercial area with thousands of researchers active in the field. However, only a fraction of their real potential has been exploited in clinical diagnostics, pharmaceutical developments and applications, food and process controls, environmental monitoring, defense and security. In last few years, scientists have been significantly working in the development of electrochemical biosensors for the detection of illicit drugs.

Herein we report on the development of a disposable amperometric magneto immunosensor for the detection of tetrahydrocannabinol (THC) in saliva. The biosensor involves the use of selective capture antibodies immobilized on the surface of Protein G modified magnetic particles for specific detection of THCs. Competition takes place between THC, free in solution, and using horseradish peroxidase (HRP)-conjugated THC, added at a fixed concentration, for their binding to the anti-THC modified magnetic particles. The HRP used as an enzymatic label, enables the amperometric detection by measuring the current intensity at -0.2V against Ag pseudo reference electrode upon addition of the enzymatic substrate using hydroquinone (HQ) as electron mediator. We have successfully shown that using this methodology THC can be detected at very low concentration and shorter analysis time.

1. Introduction

1.1 Project Background

This project focuses on the development of a novel sensing platforms for the detection of THC in biological fluids like saliva. Here we will focus on exploring new sensing approaches based on magnetic particle-based sensing platforms for the detection of THC.

Cannabis, or more commonly known as marijuana, has been in use for more than thousands of years both as psychoactive drug and for medical purposes, being one of the most consumed drugs in the world [1]. The plant Cannabis sativa from which cannabis is derived, contains more than 500 known compounds which include approximately 60 cannabinoids. Of these cannabinoids, the most prominent ones are THC and cannabidiolic acid (CBDA) [2]. THC is the main psychoactive component of *Cannabis sativa*, causing cognitive decline and impaired motor skills. Because cannabis is the most common illicit drug (other than alcohol) consumed when driving, its detection is of high interest.

Conventional techniques used for THC and 11-nor-9-carboxy-delta-9-tetrahydrocannabinol (THC-COOH) detection involve chromatographic techniques and immunoassays [3]. However, these techniques require the use of sophisticated and bulky laboratory instruments and are time consuming involving lengthy protocol, which makes them non-suitable for on-road testing. Even more, the stringent specifications required to test illicit drugs such as low limit of detection, short time analysis and low sample volume support the need of new approaches. Very recently Jung-Rok Lee *et al.* developed a portable biosensor to detect THC in saliva based on giant magnetoresisitance (GMR) [4]. In this direction, electrochemical biosensors offer several advantages over current drug testing technology.

THC being electroactive, their direct electrochemical detection has been achieved by absorptive stripping voltammetry (ASV) [5] and by square wave voltammetry (SWV) [6]. However, due to the high potentials required to achieve its oxidation developed sensors lack the specificity to be used in complex matrices even after the use of electron mediators to reduce those potentials. This leads to the use of a specific receptor for the targeted drug such as molecular imprinted polymers (MIP), antibodies or DNA aptamers. In this project, antibodies (anti-THC) were preferably used as bioreceptors for the targeted drug. Additionally, to improve the sensitivity and minimize any possible matrix effect, the use of magnetic particles to capture and preconcentrate the drug was considered. Moreover, the feasibility of magnetic particles to be quickly dispersed and collected onto a magnetized electrode, can also have an important effect on reducing the analysis time.

1.2 Project Aim

The main goal of our project is to achieve the development of an electrochemical biosensor towards THC at the low ng/mL level, so it can be later applied to its determination on saliva samples. To this aim, the immuno-sensing strategy involving anti-THC antibodies-functionalized magnetic particles for competitive binding to free THC in solution and a tracer (THC labeled with horseradish peroxidase (HRP)) was performed. Indirect detection of THC was done through the amperometric quantification of the enzyme HRP used as label.

2. Literature Review

2.1 Introduction

There has been significant growth in biosensor related research over the last two decades. Scientists have developed several biosensing concepts and techniques. Until the mid-20th century there was not much research in biosensing devices. It all started in 1962 when Leland C. Clark Jr. and co-workers introduced the first glucose oxidase biosensors [7]. His biosensor was based on an oxygen electrode where glucose oxidase was entrapped with the help of semi-permeable dialysis membrane and any change in the oxygen concentration was proportional to concentration of glucose. [8]. Since then, the biosensor field has grown enormously, ranging from clinical research to national defense applications.

When a specific biochemical mechanism and detection are combined, biosensors can be developed for label-free detection, which presents shorter analysis time and simplicity over labelled strategies. Biosensors provide high sensitivity, selectivity and robustness to detect target analytes at low cost. Another important feature is their ability to be miniaturized and therefore potential for portable in-situ analysis. All these characteristics taken together with the unique properties of nanostructured/magnetic materials provide an attractive means for the development of novel platforms with improved sensitivity [9].

Technically biosensors can be defined as an analytical device that combines a biological sensing element with a transducer to generate a signal proportional to the analyte concentration [10]. So basically, it boils down to two major components: bioreceptor and transducer. The bioreceptor is the biological recognition element of the sensor that selectively recognizes the target analyte. This biorecognition element can be enzymes, membrane receptors, antibodies, nucleic acids, organelles, cells, tissue or even a microorganism.

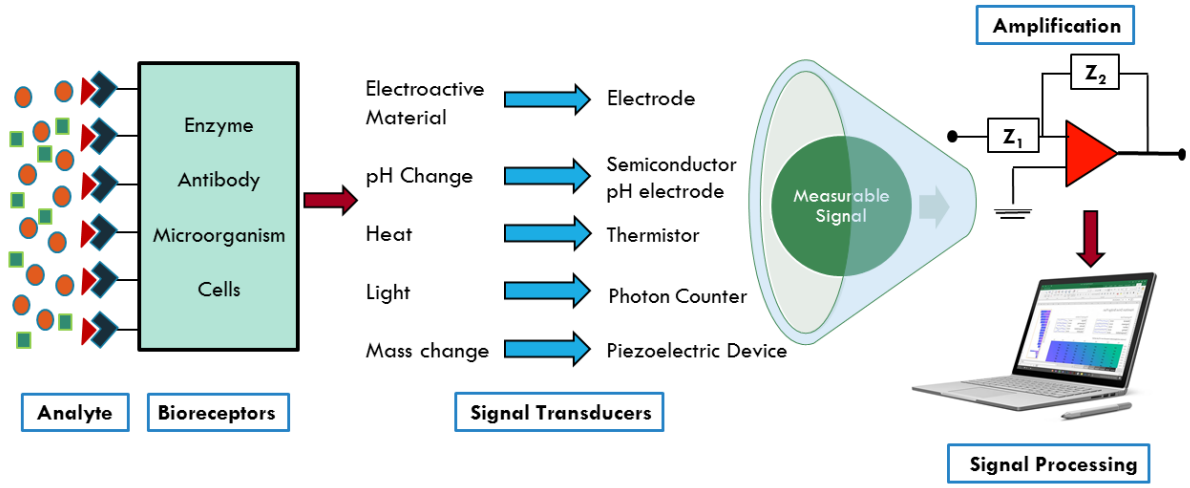


Figure 1. Elements of a biosensor

Once the bioreceptors recognizes the analyte, the corresponding biological response is converted into electrical signals with the help of transducers. The electrical signals are then later amplified using the detector circuit which is then converted to meaningful data using computer software. (as shown in Fig.1.)

2.2 Classification of biosensors

Depending on the type of bioreceptors and the nature of transducers used, biosensors can be of various forms [11]. Fig.2 shows different types of biosensors.

2.2.1 Bioreceptor based

A bioreceptor is a molecular element responsible for the recognition of the target analyte. Based on the nature of the biorecognition process, biosensors can be classified into two main groups i.e. affinity biosensors and biocatalytic biosensors [12,13].

2.2.1.1 Affinity biosensors

Affinity biosensors are those biosensors in which the shape and size of the binding site to the target analyte is the basis of molecular recognition [12]. This includes biorecognition element such as antibodies, oligonucleotides and membrane

receptors. Antibodies are the

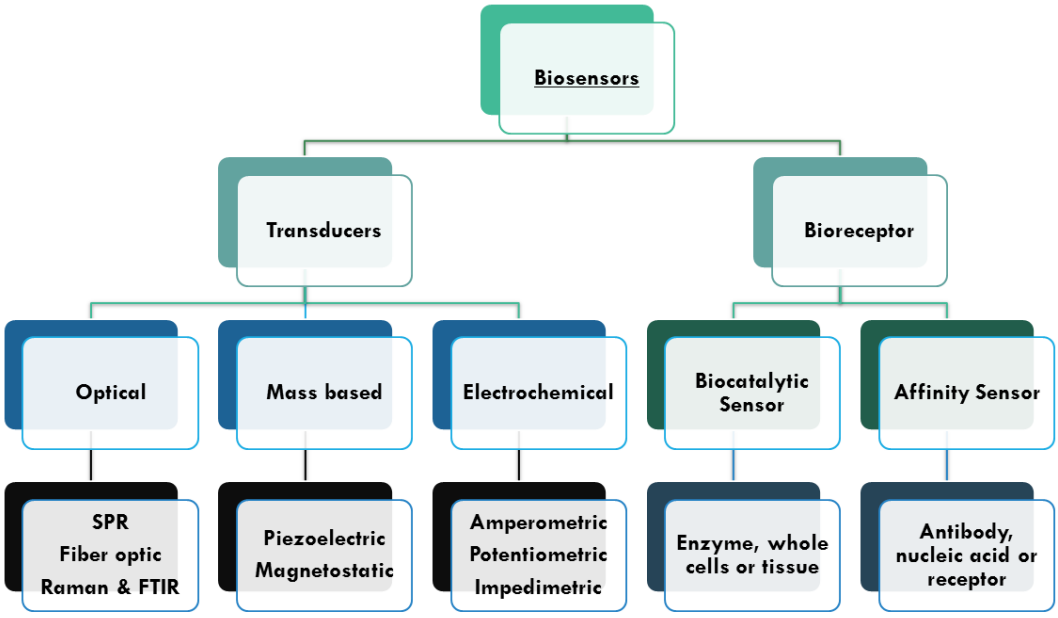


Figure 2. Classification of Biosensors

most commonly used bioreceptors, and the resulting biosensor are known as immunosensors. Antibodies are basically defensive proteins produced by a type of white blood cell know as B lymphocyte in response to the foreign substance (antigen) entering the body. Antibodies can be made to target specific proteins. In such cases, animals (often rabbit or mice) are injected with purified proteins which in course produce antibodies that can be collected in the blood. Antibodies can be produced from an identical immune cells or different immune cells, based on this they are divided into two: monoclonal and polyclonal antibodies. Monoclonal antibodies are produced from a single B lymphocyte and are specific to one epitope of the antigen whereas an activation of multiple B lymphocyte to produce immune response to an antigen results in polyclonal antibodies. Polyclonal antibodies show specificity towards multiple epitopes on any one antigen.

In an immunosensor the detection of the target analyte is based on its binding to the antigen binding site (Fab) of the antibody. The paratope of the antibody interacts with the epitope of the antigen by spatial complementarity which is analogous to the lock and key system [14]. This ensures that the 3-D structure of the antibody matches with that of the antigen attaining specificity and high selectivity in the process. This

makes immunosensors a powerful sensing tool for the detection of a wide range of target analytes, such as drugs, microorganisms [9].

The Fab-epitope interactions are non-covalent, reversible and non-specific due to the weak molecular forces (like electrostatic forces, hydrogen bonds, hydrophobic interactions, and van der Waals forces) involved. Since the interaction is reversible, it can be expressed as

$$[A_g] + [A_b] \Leftrightarrow [A_b A_g]$$

The equilibrium association constant is given by the following equation:

$$k_a = \frac{[A_b A_g]}{[A_g][A_b]}$$

Where,

K_a = affinity constant

$[A_b]$ = concentration of unoccupied binding sites of antibody

$[A_g]$ = concentration of unoccupied binding sites of antigen

$[A_g][A_b]$ = concentration of antigen-antibody complex

The affinity constant is the measure of the strength of binding which also describes the amount of antigen-antibody complex present at the point when an equilibrium is achieved. Normally in immunoassays, the antibodies or the analyte are covalently modified with a label to amplify the detection signal and thus forming a perfect molecular probe. The most commonly used labels are enzymes but fluorophores and radioactive isotopes have also been reported [9].

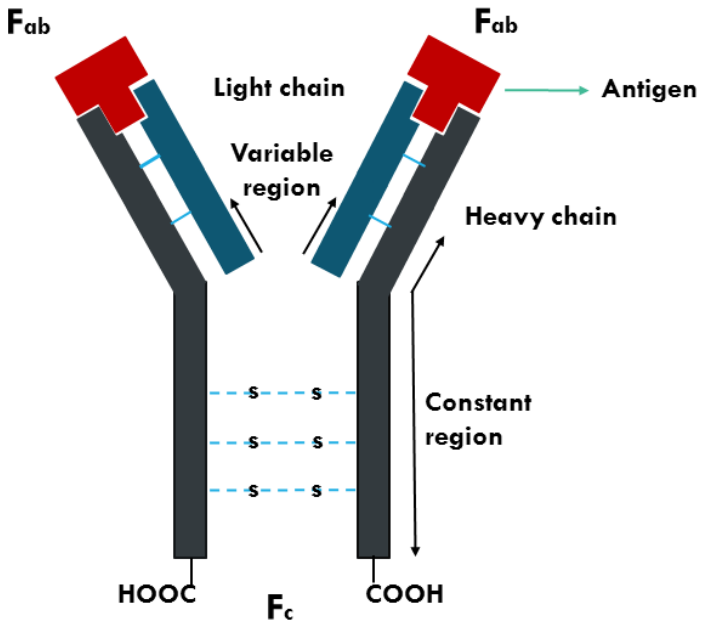


Figure 3. Antigen-antibody interaction

2.2.1.2 Biocatalytic biosensor

Biocatalytic biosensors are based on a reaction catalyzed by macromolecules such as enzymes, whole cells or tissues as the biorecognition element. Due to specificity and catalytic activity of enzymes, they are very often used as bioreceptors in biocatalytic biosensors [15]. The use of enzymes as bioreceptors was first introduced by Prof. Leland C. Clark in 1960's. He designed the first-generation glucose sensor in which the enzyme glucose oxidase was entrapped onto an oxygen electrode with the help of dialysis membrane [7,8]. Since then, different forms/generations of glucose oxidase biosensors have been developed and in fact they have led to the development of some of the most commercially successful biocatalytic sensors such as the blood glucose monitoring device. [16]

Enzymatic biosensors offer several advantages, the most important being its simple design, inexpensive instrumentation and exquisite specificity which helps in detecting

individual molecules in complex matrices. Enzymes offer high stability and sensitivity [9].

Enzymes, being a biological catalyst increases the rate of the reaction by lowering the energy of activation for a reaction (as shown in Fig.4.a). The substrate binds to the enzyme at its binding sites and the enzyme converts these into products. The enzyme catalyzes the whole reaction but is not used up in the reaction.

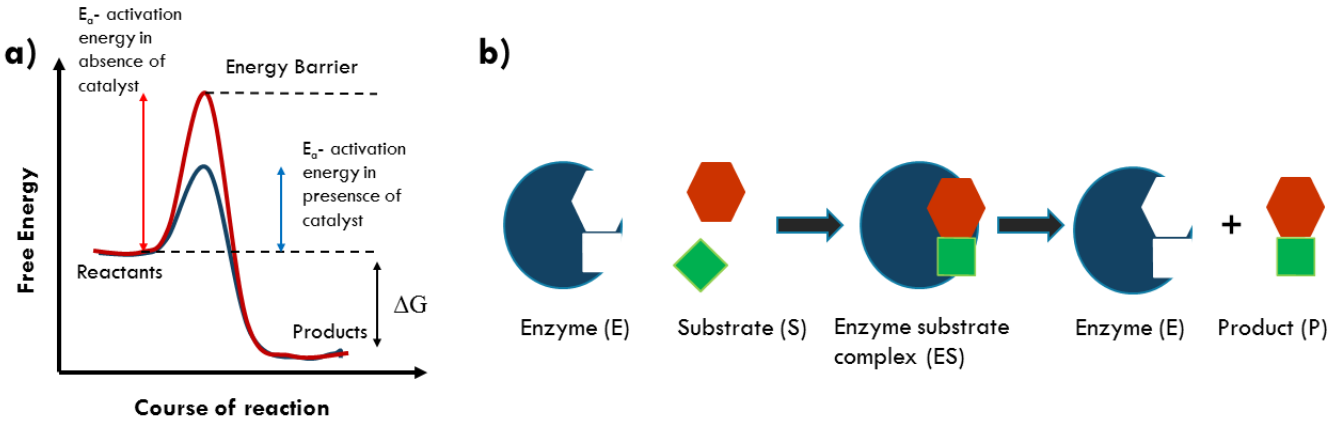


Figure 4.a) Graph of energy of activation with and without enzyme b) diagram depicting enzyme substrate complex

Many papers have been published where these enzymes are extensively used in developing biosensors. [17]

2.2.2 Transducer based

Technically, transducers are devices that convert a signal in one form of energy to signal in another form. In a biosensor, the transducer converts the biological response produced when the bioreceptor interacts with an analyte into electrical signals. Biosensors can be categorized into various forms depending on the transduction method employed [11]. They can be classified into optical, piezoelectric and electrochemical sensors, among others and each of them is further divided into various subclasses.

2.2.2.1 Optical and Mass-sensitive biosensors

Optical biosensors employ several techniques for the detection of analytes viz. fluoresce spectrometry to study different Spectro-chemical properties of analytes, surface plasma resonance, Fourier transform infrared (FT-IR) spectroscopy and other optical techniques. When light is reflected at sensing surface a change in the

physical parameters such as adsorption, fluorescence or refractive index takes places. This form the basis for optical sensor techniques. Another type of sensors are those based on measuring small changes in mass using piezoelectric crystals as transduction elements, where the transduction is based on small changes in mass. Such type of biosensors are called mass-sensitive biosensors.

2.2.2.2 Electrochemical biosensors

As the name suggests electrochemical biosensors are biosensors in which the transduction is purely by electrochemical means. Based on the type of the electrochemical detection techniques involved, electrochemical biosensors can be grouped into impedimetric, amperometric, potentiometric and conductometric sensors.

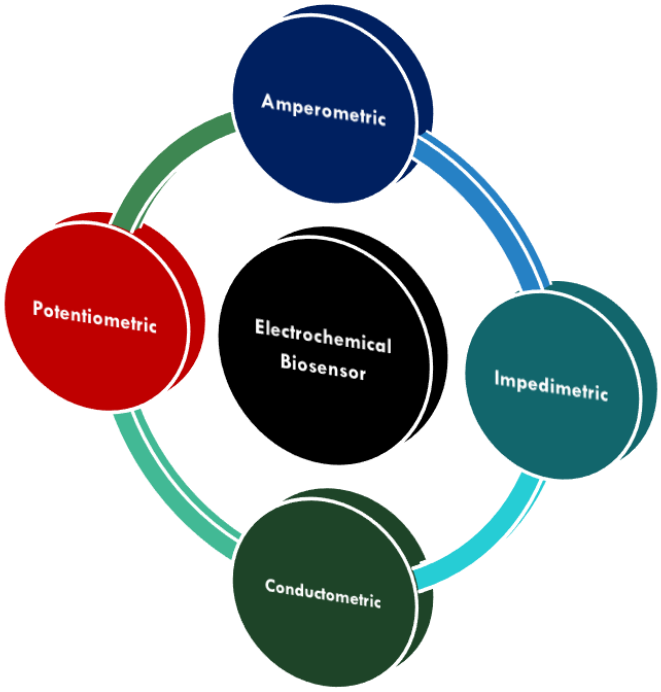


Figure 5 Types of electrochemical biosensors

2.2.2.2.1 Impedimetric biosensors

Electrochemical impedance spectroscopy (EIS) is a powerful technique to assess the interfacial properties changes of the built biosensor [18]. The technique was first described in 1975 by Lorenz and Schulze [8,15,19]. EIS has played significant role in the development of biosensor over the last few decades [18,20] and it is still

widely used in the detection of antibody-antigen interactions [20,21,22], enzyme reactions [18,23] and DNA hybridization [24]. EIS is also used to monitor the different steps during the biosensor fabrication. Changes on the electrode surface, due to chemical or biological modification during biosensor preparation, or to analyte binding to the immobilized bioreceptor, alter the charge transfer resistance of the conductive or semi-conductive electrode such that it allows the analysis of interfacial changes occurring at the surface of the electrodes [8]. Usually for an impedance measurement, a fixed AC amplitude of 5-10 mV is scanned over frequencies typically ranging from 100kHz to 100mHz. This results in a current to flow through the biosensor and the in-phase and out-of-phase current responses are determined and the resistive and capacitive component of the impedance are obtained respectively [13].

2.2.2.2.2 Amperometric biosensors

Amperometric technique is the most commonly used signal transduction method in electrochemical biosensor. In this, the change in current is measured when a constant potential is applied overtime [20]. If the current is measured over a set potential range then the technique is known as voltammetry which is useful for assessing the different fabrication steps. Voltammetric techniques are useful for low level quantitation when the potential is applied by pulses (e.g. differential pulse voltammetry, square wave voltammetry) [13]. In amperometric biosensors the current is generated due to the electrochemical oxidation or reduction of species at the working electrode. When the analyte is an electroactive species, the current measured is directly proportional to its concentration. In recent years, the amperometric approach has been widely used in detection of antibiotic residues in food substances because of its low limit of detection (LOD) [25,26], mainly due to minimal charging current when a fixed potential is applied, that minimizes the background noise that adversely affects the LOD. Many reports have been published where amperometric technique is used for the detection of antigens and nucleic acid in disease diagnosis [20,27,28] and pesticides in environmental analysis [20,29]. Although amperometric technique is simple and offers low LOD it has few drawbacks such as the interference due to the electroactive species that hampers the effective analysis of samples, and may generate a false current reading [20,30].

2.2.2.2.3 Potentiometric biosensors

Potentiometric biosensors measure the potential signal obtained upon the conversion of a biorecognition process. Basically, it measures the charge potential accumulated on the working electrode when the flow of current is negligible [15,31,32]. Usually to measure the electromotive force (EMF) across the reference electrode, a high-impedance voltmeter is used. The potentiometric biosensors generally use enzymes as the biorecognition element that catalyzes the reaction that forms the ion for which the electrode is designed to sense [13].

2.2.2.2.4 Conductometric biosensors

Conductometric devices monitor the changes in the electrical conductivity of the electrolyte solution or any medium during a chemical reaction. Conductometric biosensors are based on the relationship between conductance and a biological recognition process. Conductometric biosensors are extensively used in clinical analysis, food-borne pathogen detection and environmental related studies [33,34].

2.3 Electrode Materials and Surface Modifications for electrochemical biosensor

The performance of a biosensor mainly depends on the choice of the bioreceptor, surface modifications and transduction mechanism. Thus, one of the key steps in the development of a biosensor is the modification of the electrode surface to further immobilize the bioreceptor. Depending on the type of biosensors used different materials are used to fulfill the requirements. Graphite, glassy carbon and indium tin oxide (ITO) and metals such as gold and platinum [35,36,37] have been used to prepare electrodes with good electrical conductivity as transducers of electrochemical biosensors.

Screen-printed electrodes (SPEs) are increasingly used in the preparation of many electrochemical biosensors, mainly due to their simple design, low-cost fabrication and disposability. Due to high reproducibility and ease of use, they are extensively used as platforms in enzyme sensors, immunosensors and DNA sensors for the detection of drugs, food pathogens and many other [25,26,38,39].

Different strategies are also used to functionalize the working electrodes such as self-assembly of monolayer (SAM) of thiols and disulphides on Au electrodes and electro-grafting of diazonium salts, e.g. using 4-aminobenzoic acid, on both carbon and gold electrodes. These functionalization strategies introduce functional groups on the electrode surface to facilitate coupling of the bio-recognition element to the working electrode [40].

The electrode surface can be modified with a wide variety of materials to improve the biosensor performance. The use of nanoscale materials in the design of electrochemical biosensors is a rapidly expanding area. Nanoscale materials such as nanotubes, nanowires, nanoparticles and nanosheets have been extensively incorporated in electrode construction. This is attributed to their remarkable characteristics such as high surface-to-volume ratio and excellent electrical properties that have led to the development of sensing platforms with outstanding performance. Carbon nanotubes have been used to provide significantly large surface area, better electrical conductivity and good chemical stability [41]. The use of gold nanoparticles [42] and magnetic particles functionalized with various receptors [25,26,43] has been widely used in developing electrochemical biosensors in clinical analysis.

2.3.1 Advantage of using magnetic particles

In many chemical analysis, the components of the sample may significantly affect the way in which the experiment has to be conducted. These components other than the target molecule of interest, are called matrix. Matrices can hamper the quality of results obtained. In this direction, the use of magnetic beads to capture the analyte from the sample and discard the other components provides many advantages such as improved sensitivity, reduced analysis time, preconcentration of the analyte and more importantly reduced matrix effects.

2.4 Electrochemical biosensors in drug detection

Electrochemical biosensors have been extensively used in various areas of research. They provide several advantages over the existing methods for analyte detection such as their easy miniaturization, robustness, low detection limits and

sample volume. Over the past few years they have been increasingly used in the detection of drug molecules in biological fluids.
Electrochemical biosensors using disposable screen-printed electrodes have become the basis for the detection of various drugs such as methamphetamine (Meth) and THC [38,39]. However, to fulfill the stringent specifications required to test illicit drugs, further developments in existing analytical techniques and new approaches are necessary. This thesis focusses on developing an amperometric biosensor for the detection of THC in saliva.

3. Experimental Details

3.1 Materials

Gold (DRP-220BT) and carbon screen printed electrodes (DRP-110) were purchased from Dropsens, mercaptopropionic acid, mercaptoethanol, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC), N-hydroxysuccinimidehydrochloride (NHS), ethanolamine (> 98%), polyethylene glycol, bovine serum albumin (BSA), phosphate-buffered saline (PBS), sulphuric acid (98%), potassium ferricyanide ($K_3[Fe(CN)_6]$), potassium ferrocyanide ($K_4[Fe(CN)_6] \cdot 3H_2O$), sodium carbonate ($Na_2CO_3$), sodium bicarbonate ($NaHCO_3$), tween 20, 2-(N-Morpholino) ethanesulfonic acid (MES), hydrogen peroxide (H_2O_2), 3,3',5,5'-tetramethylbenzidine (TMB), α-naphthyl phosphate (α-NP), hydroquinone (HQ) and protein G were purchased form Sigma Aldrich. Tetrahydrocannabinol labelled with horseradish peroxidase (THC-HRP) and THC with bovine serum albumin (THC-BSA) were purchased from Fitzgerald and Pyxis laboratories Inc. respectively.

The protein G and carboxylic-modified magnetic beads (Dynabeads M-270 carboxylic acid and Dynabeads Protein G) were ordered from Thermo-Fisher Scientific and along with the three clones of mouse monoclonal antibody anti-THC [catalog #100-11-391 (Ab1), 100-11-393 (Ab2), and 100-11-395 (Ab3)] were purchased from Omega biological Inc. (USA). Tetrahydrocannabinol (THC) was kindly provided by Flinders University (SA). Instruments used were CHI

electrochemical analyzer/workstation (model 600C series) and Dropsens potentiostat.

3.2 Biosensor preparation

Surface functionalization is one of the important steps in the development of an electrochemical biosensor to further immobilize the bioreceptor (antibodies in our case). We explored various sensing approaches based on different sensing platforms for the detection of THC. Three immunosensing strategies are envisaged:

1. Direct detection of THC using EIS. Direct detection of THC binding to the anti-THC antibodies immobilized onto the electrode transducer was attempted. This involved non-oriented immobilization of antibodies onto the electrode surface.
2. Indirect amperometric detection of THC in a competitive immunoassay. Protein G was used for the oriented capture of the antibody onto the electrode surface and a competitive immunoassay was performed THC-HRP as tracer. Indirect detection of THC was performed through the enzyme HRP used as label.
3. Indirect amperometric detection of THC in a competitive magnetic immunoassay. Anti-THC antibody-functionalized magnetic particles (using both carboxylic- and protein G-modified particles to achieve non-oriented and oriented antibody immobilization respectively) were used for their competitive binding to free THC in solution and a tracer (THC-HRP).

In the above-mentioned strategies, the biosensor preparation and the mode of transduction was different. So, three different methodologies were used for the preparation of the biosensor.

3.2.1 Methodologies

A. <u>Methodology used in strategy 1</u>

In this case, the Au electrode were modified to incorporate carboxy groups on the surface, which will be later used to bind the antibodies through the N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide/N hydroxysuccinimidehydrochloride (EDC/NHS) cross-linking reaction. Au electrode were modified by self-assembled monolayer (SAM) of 3-mercaptopropionic acid (1mM), by keeping it for overnight incubation at 4°C. The carboxylic acid groups available on the surface upon functionalization were then reacted with 0.1M EDC to form an O-acylisourea intermediate that in the presence of 0.4M NHS (for 30minute) creates an amine-reactive intermediate, which is considerably more stable and that allows the conjugation of the antibodies through the amine-region. 1M ethanolamine was added to react with the rest of the activated carboxylic group and incubated at room temperature for 1 hour. Lastly, the surface was blocked with poly (ethylene glycol) (PEG) to prevent unspecific adsorption (1 hour). And the sensor was incubated with different concentrations of THC-BSA for 1 hour at room temperature. EIS measurement was carried out at each step.

B. Methodology used in strategy 2

Here, again similar procedure was followed till the addition of 0.1M EDC/0.4M NHS as mentioned in the above methodology. After the addition of EDC/ NHS, 10µg/ml of protein G was immobilized on the surface of the electrode for 1hour. Protein G selectively binds to the Fc region of the antibody and thus allows a controlled orientation of the antibody. 1M ethanolamine was added to react with the rest of the activated carboxylic group for 1 hour at room temperature and then the surface was blocked with bovine serum albumin (BSA) to prevent unspecific adsorption for 1 hour. Followed by the immobilization of antibody (Anti THC) overnight at 4°C. Lastly, a competitive assay between labelled THC with the HRP enzyme (i.e. THC-HRP) and unlabeled THC was done by keeping the concentration of labelled analyte constant and changing the concentration of the unlabeled one. After the incubation of THC-HRP/THC at room temperature (1hour), amperometric measurement was carried out in a stirred solution containing 0.25M H_2O_2 as enzyme substrate and 1mM hydroquinone HQ (used as redox mediator), applying a constant potential of -0.2V.

The Au SPE incubated with THC/THC-HRP was immersed in the electrochemical cell containing 10ml PBS and 1mM HQ (prepared daily before the amperometric measurement) and the amperometric response were recorded on addition of 40µL of 0.25mM H_2O_2 until the saturation current was reached. Dropesens potentiostat was used to measure the amperometric responses.

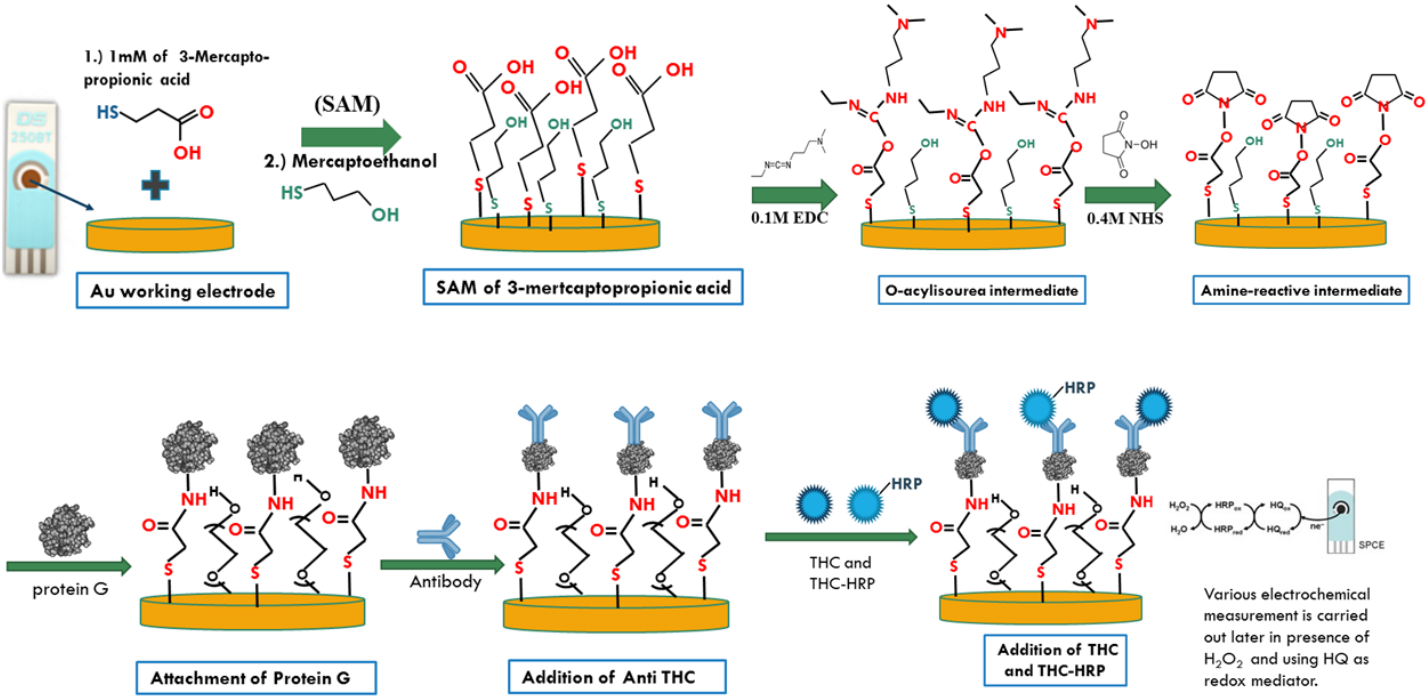


Figure 6. Various steps involved in biosensor modifications

C. <u>Methodology used in strategy 3</u>

In this methodology, carboxylic acid coated magnetic beads (size = 2.5µm) were used. The 5µL of magnetic beads suspension was collected in 1.5mL tubes and the carboxylic group on the surface of magnetic beads were activated through 50mg/ml EDC/NHS cross-linking reaction. The magnetic beads were suspended in EDC/NHS mixture for 30minutes. After washing the beads twice with MES buffer, 10µg/ml Protein G was added that allowed a controlled orientation of the antibody (incubated at room temperature for 1hour). Followed by the immobilization of 25µg/ml antibody (Anti THC) at room temperature for 1 hour. And lastly a competitive assay between labelled THC (i.e. THC-HRP) and unlabeled THC was carried out. The antibody THC-modified magnetic bead was suspended in a 100µL solution containing

THC/THC-HRP for 1 hour, after which the tube was placed on a magnetic separator, supernatant removed and the beads were washed twice using PBS-Tween (PBST) and re-suspended in 50 µL PBS to carry out the measurement. The magnetic beads were washed in the similar way as mentioned above after subsequent additions of EDC/NHS, protein G and antibody using PBST.

After the incubation of THC-HRP/THC, the modified 50 µL magnetic bead suspension was transferred onto the carbon SPE, which was assembled on a magnet holder as shown in Fig.6.b.The magnet holder-SPE with magnetic beads captured on its working electrode was immersed in the electrochemical cell containing 10ml PBS and 1mM HQ (prepared daily before the amperometric measurement) and the amperometric response were recorded in a stirred solution (constant stirring was achieved using a magnetic stirrer, kept at 800rpm), on addition of 40µL of 0.25mM H_2O_2 as enzyme substrate until the saturation current was reached (shown in Fig.6.c). A constant potential of -0.2V was applied. Dropesens potentiostat was used to measure the amperometric responses.

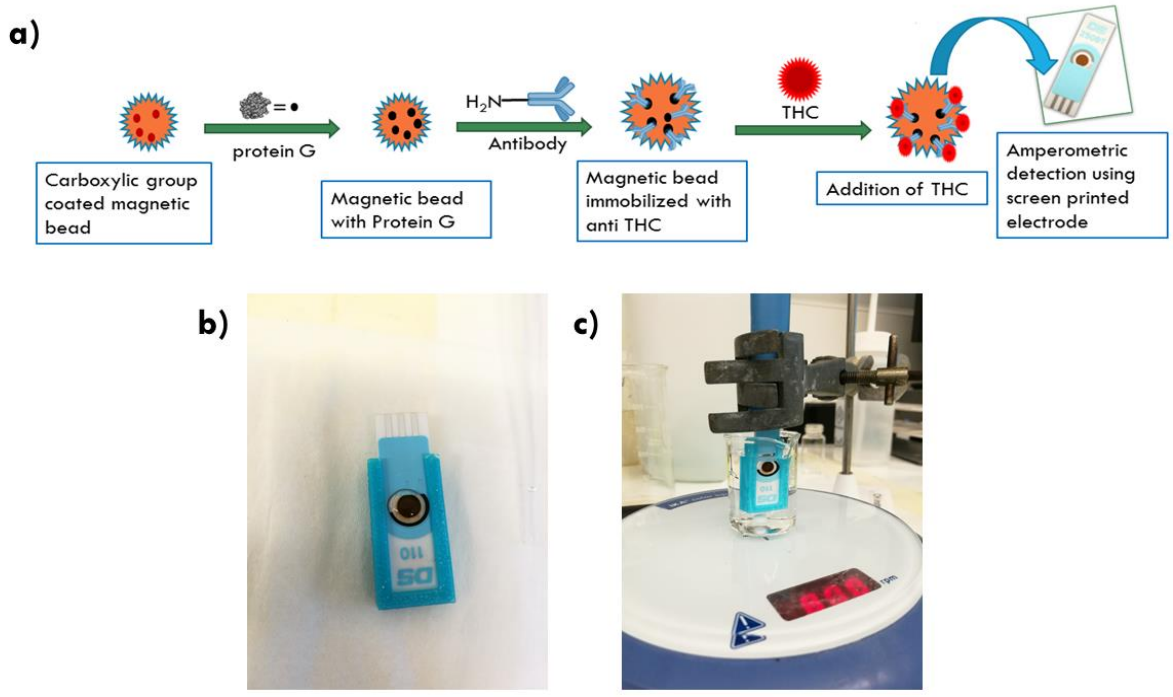


Figure 7. a) Modification of magnetic beads b) Magnetic beads on carbon screen printed electrode (SPE) c) SPE along with the magnet holder in electrochemical cell

3.3 Detection Techniques

3.3.1 Enzyme linked immuno-sorbent assay (ELISA)

Immunoassay techniques are one of the most popular biochemical test that determines the presence or concentration of a molecule of interest from a mixture of macromolecules. ELISA is an immunoassay test that uses enzymes as label for the detection of a molecule of interest, in a liquid sample. It exploits the ability of antibodies to recognize antigen in a given sample. Horse radish peroxidase (HRP), alkaline phosphatase (ALP), β-galactosidase are few of the enzymes that are immensely used as labels. In our case ALP and HRP were used, which showed characteristic color change in presence of their substrate p-nitrophenyl phosphate (p-NPP) or 3,3',5,5'-Tetramethylbenzidine (TMB) respectively. ELISA can be of different types, basically- sandwich and competitive ELISA.

In sandwich ELISA, as the name suggests, the antigen is sandwiched between a pair of antibodies namely the capture antibody and detection antibody i.e. the labeled secondary antibody. This is highly sensitive. If the antigen is small or has one epitope, then competitive ELISA is a very useful strategy. The competitive assay can be of two type -direct and indirect competitive assay. Direct competitive ELISA involves the competition between labelled and unlabelled antigen to attach the binding site of antibody. The amount of labeled antigen attached to antibody is measured. Higher the amount of labelled antigen attached stronger the signal would be, representing a competition where the labelled antigen competes off the unlabelled one. In Indirect competitive assay, the labelled antibody competes for its binding to antigen added in solution and immobilized antigen.

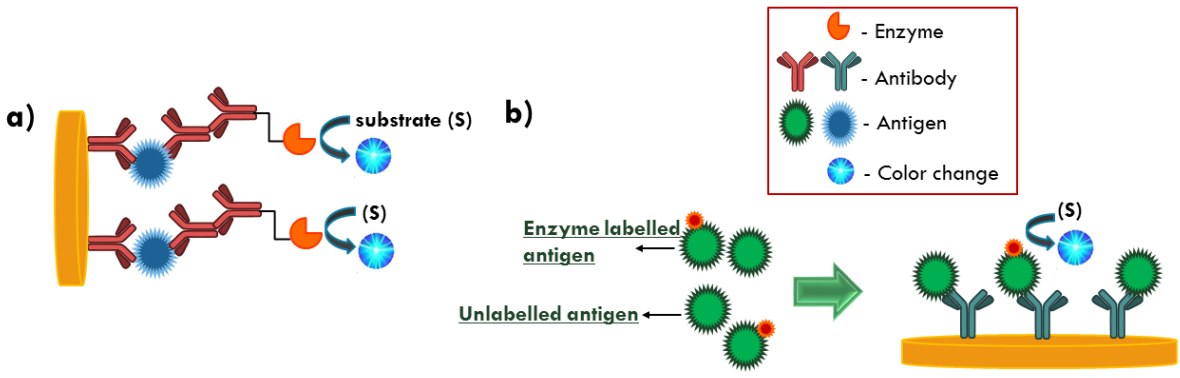


Figure 8 Diagrammatic representation of a) Sandwich ELISA and d) Competitive ELISA

3.3.2 Amperometry

Amperometry has gained considerable popularity since the 1970s and since then has been significantly used in the development of biosensors. As mentioned earlier, it is the most commonly used signal transduction method in electrochemical biosensors. It basically measures the steady-state current as a function of time when a constant potential is applied to the working electrode. The current is produced due to the electrochemical oxidation or reduction of electroactive species. The magnitude of the current depends on the concentration of the oxidized or reduced species and this relation is given by the Cottrell equation:

$$I(t) = \frac{nFAC_0\sqrt{D}}{\sqrt{\pi t}}$$

Where,

I = diffusion current t = time

F = Faraday constant (96 487 C/mol) n = number of electrons involved

D = diffusion coefficient C_0 = concentration of electroactive species.

A = area of the electrode

THC is indirectly detected through a competitive assay which involves THC-HRP. HRP catalyzes the enzymatic reaction of the substrate hydrogen peroxide (H_2O_2)

$$Donor\ molecule + H_2O_2 \xrightarrow{HRP} 2H_2O + oxidized\ donor$$

The hydrogen peroxide undergoes electrochemical reduction and since current is proportional to the number of electrons generated, the magnitude of the current increases on addition of H_2O_2 [15]. So, in the absence of H_2O_2 the current generated is very weak and is termed as zero current. This is shown in the Fig.8. In our case, the activity of different amount of HRP has been measured based on H_2O_2 detection.

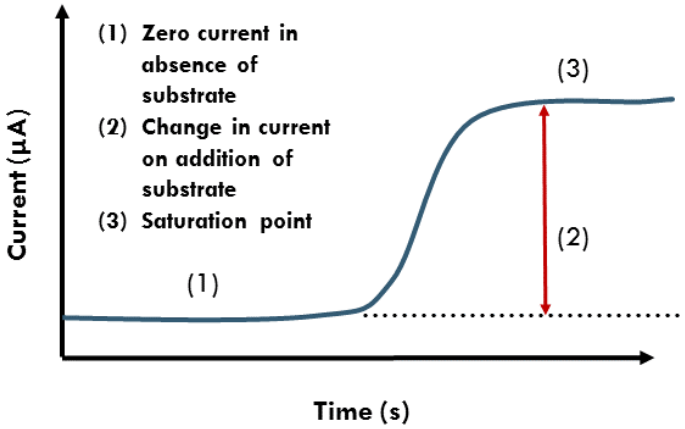


Figure 9. Amperometric plot

3.3.3 Electrochemical Impedance Spectroscopy (EIS)

Electrochemical impedance spectroscopy (EIS) was the technique used to characterize various stages of the biosensor modifications. EIS provides the information of any interfacial changes occurring at the surface of the electrode so, it was used both to check the biosensor preparation and as detection technique.

An impedance spectrum was recorded before and after incubation with THC in phosphate buffer saline buffer (PBS) containing 2 mM of $[Fe(CN)6]^{3-/4-}$ by applying a fixed potential of +0.12 V and scanning from 100 kHz to 100 mHz with a fixed AC amplitude of 10 mV.

$$Z(j\omega) = Z_{re}(\omega) + jZ_{im}(\omega)$$

Where, $Z(j\omega)$ = complex impedance and $\omega = 2\pi f$

$Z_{re}(\omega)$ = real component

$jZ_{im}(\omega)$ = imaginary component

The Nyquist plots obtained include the real and imaginary component of impedance. The complex impedance is the sum of the real and imaginary components of impedance. The Impedance spectrum obtained from the Nyquist plot is fitted to the Randles circuit.

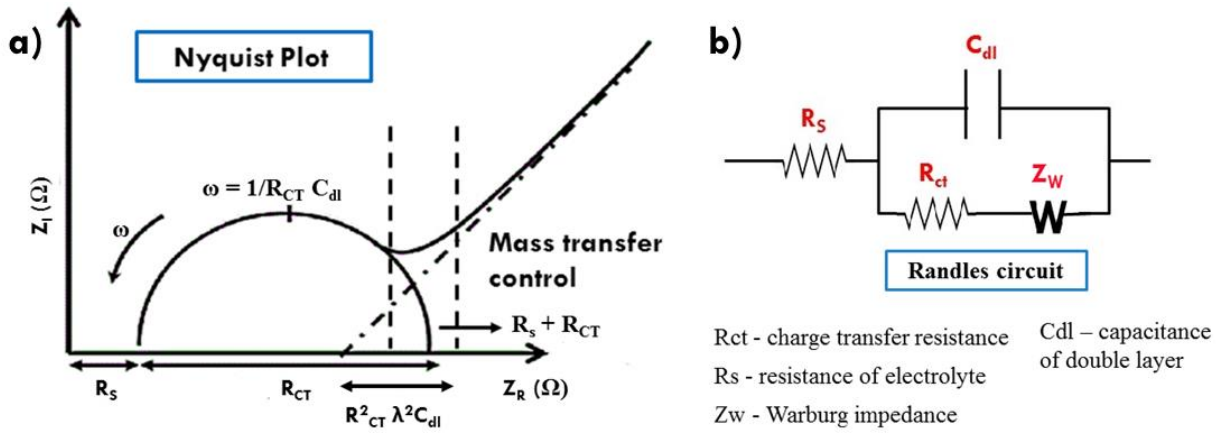


Figure 10.a) Impedance spectra in the form of Nyquist plot b) Randles circuit

The randles circuit, corresponds to the more basic equivalent electric circuit involving the charge transfer resistance (Rct) between the electrolyte and surface of the electrode, resistance of the solution (Rs), Warburg impedance (Zw) arising due to diffusion of charges from the solution to the electrode surface and a constant phase element (CPE). The sensitivity of a sensor is evaluated in terms of the variation of Rct. EIS offers several advantages such as high signal to noise ratio, lower assay cost etc. but should be carefully designed to reduce non-specific binding of interferences.

4. Results and Discussion

4.1 Study of Anti-THC affinity

Enzyme-linked immunosorbent assay (ELISA) provides useful information regarding the affinity of antibody towards antigen. To study the affinity of three different clones of mouse anti-THC antibody i.e. Ab1, Ab2, and Ab3 towards THC, ELISA was performed. The competition between THC free in solution and immobilized THC conjugated with BSA (THC-BSA) for their binding to anti-THC added in solution at a fixed concentration was carried out. THC-BSA is used to facilitate its absorption on the wells of the microplate. 96 wells microplate was coated with two different concentrations of THC-BSA i.e. at 10 ng/ml and 100 ng/ml prepared in 0.05 M

carbonate buffer (15 mM Na_2CO_3 and 35 mM $NaHCO_3$, pH 9.6) and kept for an overnight incubation at 4°C. After the incubation, the microplate was washed thrice with PBST (PBS with 0.05% tween) and later blocked with 1% BSA by incubating it for 1 hour at 37°C. The microplate was again washed thrice with PBST and then incubated with THC/anti-THC mixture for 1hour at 37°C. The THC antibody (Ab1, Ab2, Ab3 with a dilution of 1:2500 in PBS) competed for its binding to THC added in solution (ranging from 0 ng/ml to 500 ng/ml) and immobilized THC-BSA. Anti-mouse alkaline phosphatase (ALP) labelled IgG (with a dilution of 1:30000 in PBS) was used as the secondary/detecting antibody. Lastly, 100µL/well of the substrate p-nitrophenyl phosphate (p-NPP) was added and incubated for 30 min. The absorbance spectra was measured at a wavelength of 405 nm.

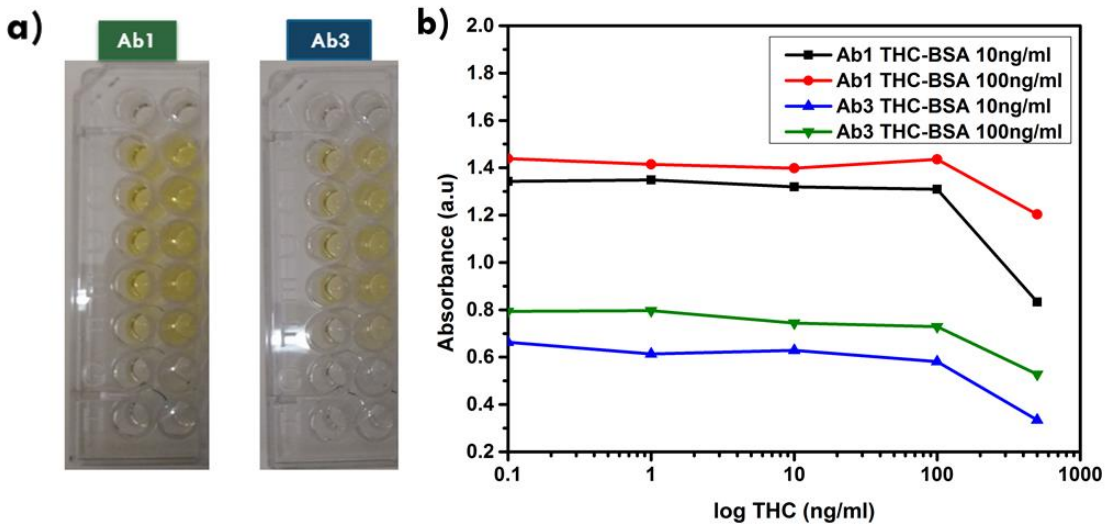


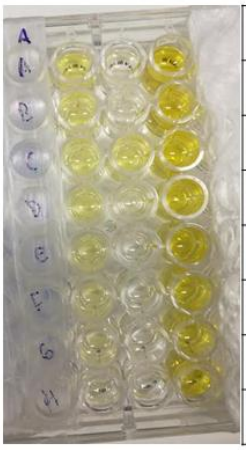
Figure 11.a) Picture showing color change in 96 well microplate for Ab1 and Ab3 on increasing the concentration of THC b) Plots of Absorbance vs THC concentration for Ab1 and Ab3 using two concentrations of THC-BSA

The results from the immunoassay (ELISA) are shown in the Fig.11. It was found that, out of three antibodies only Ab1 and Ab3 showed affinity towards THC while no color change was observed in case of Ab2. Fig.11 b. shows the plot of absorbance vs THC concentration at 405nm for two antibodies- Ab1 and Ab3 respectively. As it can be seen, on increasing the concentration of THC in the sample Ab1 shows better response compared to Ab2 when competed with 0ng/ml and 100 ng/ml of THC-BSA. The difference between the two THC-BSA shows that 10 ng/ml is the best option.

Based on this data, Ab1 was selected as the capture antibody for performing methodologies 1 and 2.

The affinity of the antibodies (viz Ab1, Ab2 and Ab3) were again checked, this time to carry out the magnetic particle based sensing. Antibodies (1µg/ml) prepared in 0.05 M carbonate buffer (pH 9.6) were incubated overnight at 4°C on the 96 well microplate. The microplate was washed thrice using PBST and later blocked with 1%BSA by incubating it for 1 hour at 37°C. The microplate was again washed thrice with PBST and then incubated with THC/THC-HRP mixture for 1hour at 37°C. The THC antibody (Ab1, Ab3) competed for its binding to THC added on solution (ranging from 0 ng/ml to 1500 ng/ml) and THC-HRP (dilution of 1:250 in PBS. Lastly, incubation of the microplate was incubated with 100µL/well of TMB for 30min. TMB was used as the enzymatic substrate which brought about a characteristic blue color on its addition against different concentrations of THC. In order to stop the color reaction 50 µL of 2 M H_2SO_4 was added, that turned blue to yellow. The absorbance spectra were measured at a wavelength of 450 nm keeping 630 nm as reference wavelength.

a)



A	0/Ab1	1000/Ab1	0/Ab3
B	0.001/Ab1	1500/Ab1	0.001/Ab3
C	0.01/Ab1	1000/Ab3	0.01/Ab3
D	0.1/Ab1	1500/Ab3	0.1/Ab3
E	1/Ab1	0/C1	1/Ab3
F	10/Ab1	0/C2	10/Ab3
G	100/Ab1	0/C3	100/Ab3
H	500/Ab1	0/Ab2	500/Ab3

b)

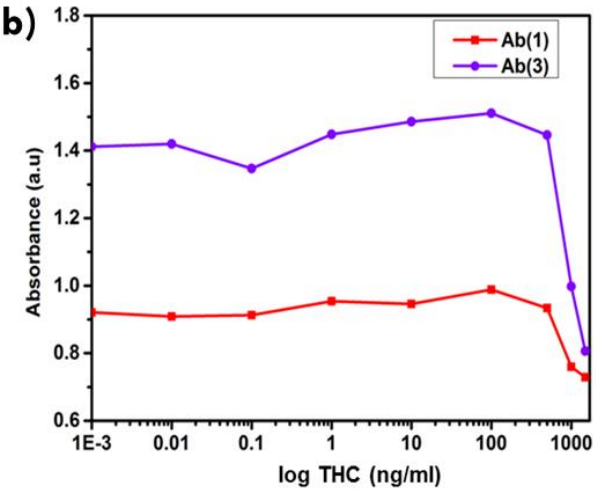


Figure 12.a) Picture showing color change in 96 well microplate for Ab1, Ab2 and Ab3 with the table depicts corresponding well with antibodies for different concentration of THC b) Plots of Absorbance vs THC concentration for Ab1 and Ab3

As shown in the Fig.12. b., the sensitivity was better for Ab3 when compared with Ab1, as slope of Ab3 seems to be higher whereas the slope for Ab1 saturates at higher THC concentrations. Hence, Ab3 was used as the antibody in the methodology 3.

4.2 Impedimetric biosensors based on strategy 1

To study THC sensing using methodology 1, electrochemical impedance spectroscopy (EIS) was performed. The Au working electrode of SPCE was modified with SAM of 3-mercaptopropionic acid for anchoring antibody onto the surface of electrode. EIS was also used to monitor the different steps during biosensor fabrication (Fig.13.a), starting from the bare electrode to the actual THC sensing. From the Nyquist plot in Fig.13.b, it can be seen how the charge transfer resistance increases after incubation with higher concentrations of THC which can be attributed to the size THC. Fig.13.c shows the response to the sensor prepared with non-specific antibody which was taken as control.

The response of the sensor can be evaluated in terms of the variation of the charge transfer resistance calculated from the Randles circuit fitting, which is shown in Fig.13.d. As can be seen, there is an increase towards increasing concentrations of THC, until its saturation at the highest evaluated concentration, which could be attributed to complete binding of antigen to antibody. To further confirm those results, an analogous biosensor prepared employing an unspecific antibody towards THC was also prepared. As can be seen, showing no response.

The reproducibility of this methodology was checked for different concentrations of THC but the results were difficult to reproduce. And since THC being a small molecule, not enough changes were produced on the interfacial surface to be detected by EIS. In response to this, a competitive assay combined with amperometry as the detection technique was employed.

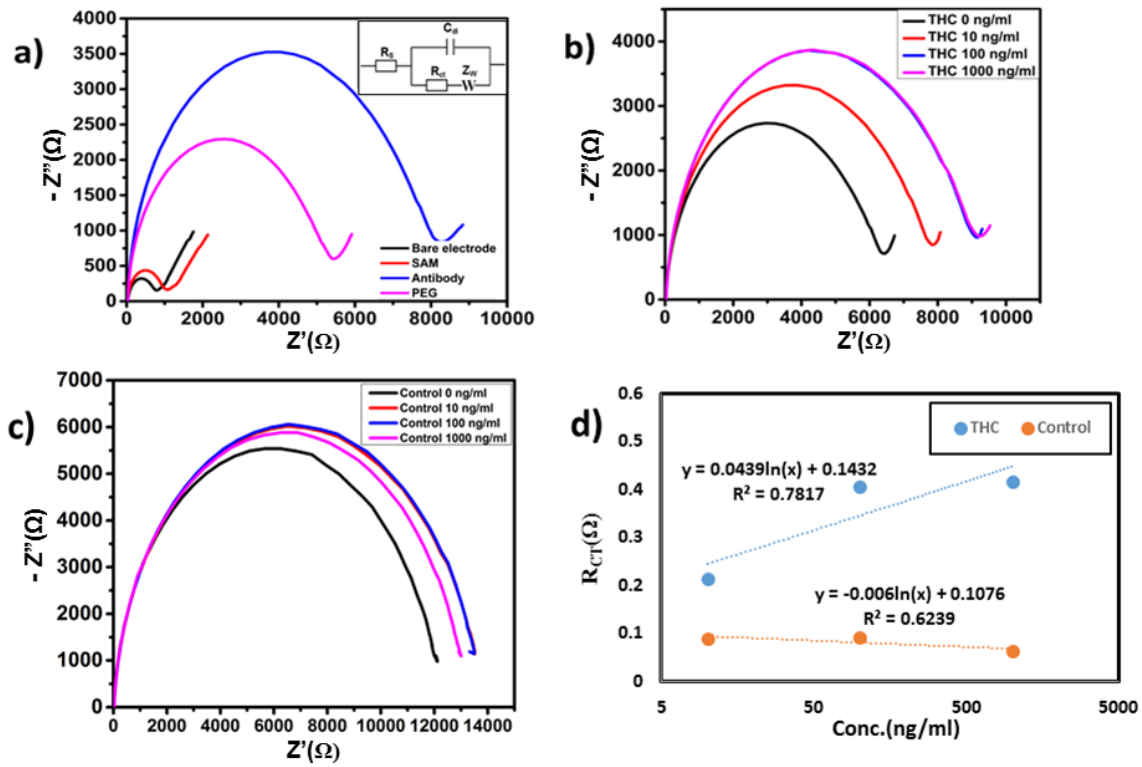


Figure 13.a) Nyquist plot corresponding to the various stages of biosensor modifications (inset shows the Randles circuit) b) Nyquist plot for anti-THC towards THC c) Nyquist plot for control d) Rct vs concentration plot for anti THC and unspecific antibody

4.3 Amperometric biosensors based on strategy 2

Strategy 2 involved indirect amperometric detection of THC in a competitive immunoassay. Protein G was used for the oriented capture of the antibody onto the electrode surface.

Amperometry was used as the mode of transduction instead of using EIS.

4.3.1 Optimization of THC-HRP

Enzyme labelled THC i.e. THC-HRP was used in this method to carry out a competitive assay. Before carrying out the competition between THC and THC-HRP, the dilution of the THC-HRP was optimized.

The Au SPE were modified to incorporate carboxy groups on the surface, which will be later used to bind the protein G through carbodiimide chemistry. After the activation with EDC/ NHS, 10 µg/ml of protein G was immobilized on the surface of the electrode for 1hour. 1M ethanolamine was added to react with the rest of the activated carboxylic group for 1 hour at room temperature after which the surface was blocked with 1% BSA for 1 hour to prevent unspecific adsorption. The immobilization of antibody (Anti THC in PBS) took place overnight at 4°C. Lastly, to optimize THC-HRP dilution, four sets of THC-HRP dilutions were prepared viz. at 1:40,1:100, 1:150 and 1:200 which were incubated at room temperature for 1 hour. An analogous biosensor employing an unspecific antibody towards THC was also prepared with 1:40 and 1:100 THC-HRP dilutions (shown in Fig.14) The modified SPEs were immersed in the electrochemical cell containing 10 ml PBS (pH 7.4) and 1 mM HQ (prepared daily before the amperometric measurements) and the amperometric response were recorded in a stirred solution (constant stirring was achieved using a magnetic stirrer, kept at 800 rpm) on addition of 40 µL of 0.25 mM H_2O_2 as enzyme substrate until the saturation of the enzyme was reached. A constant potential of -0.2 V vs the Ag pseudo reference electrode was applied.

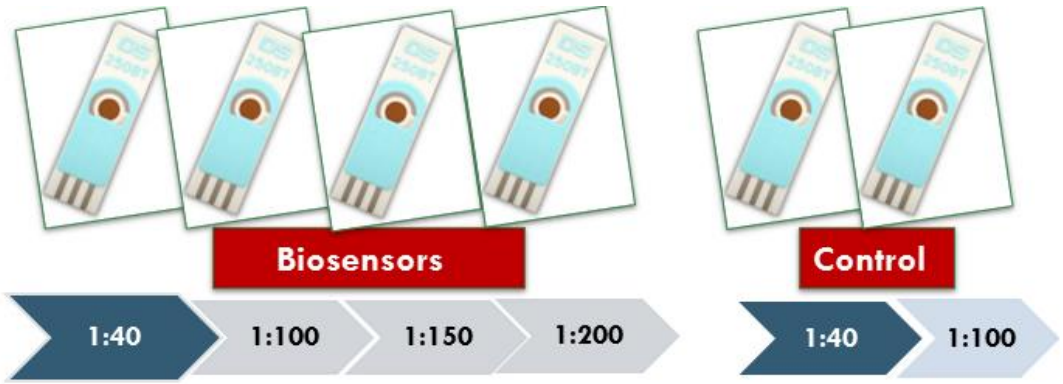


Figure 14. Biosensor and control SPEs with different THC-HRP dilutions

Fig.15, shows the plot of current intensity against four dilutions (1:40, 1:100, 1:150 and 1:200) of THC-HRP for biosensors prepared with Anti-THC and unspecific antibody.

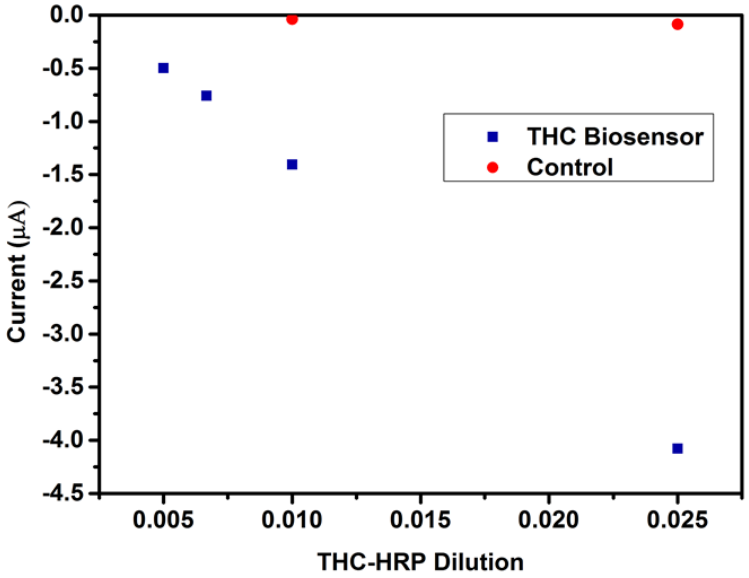
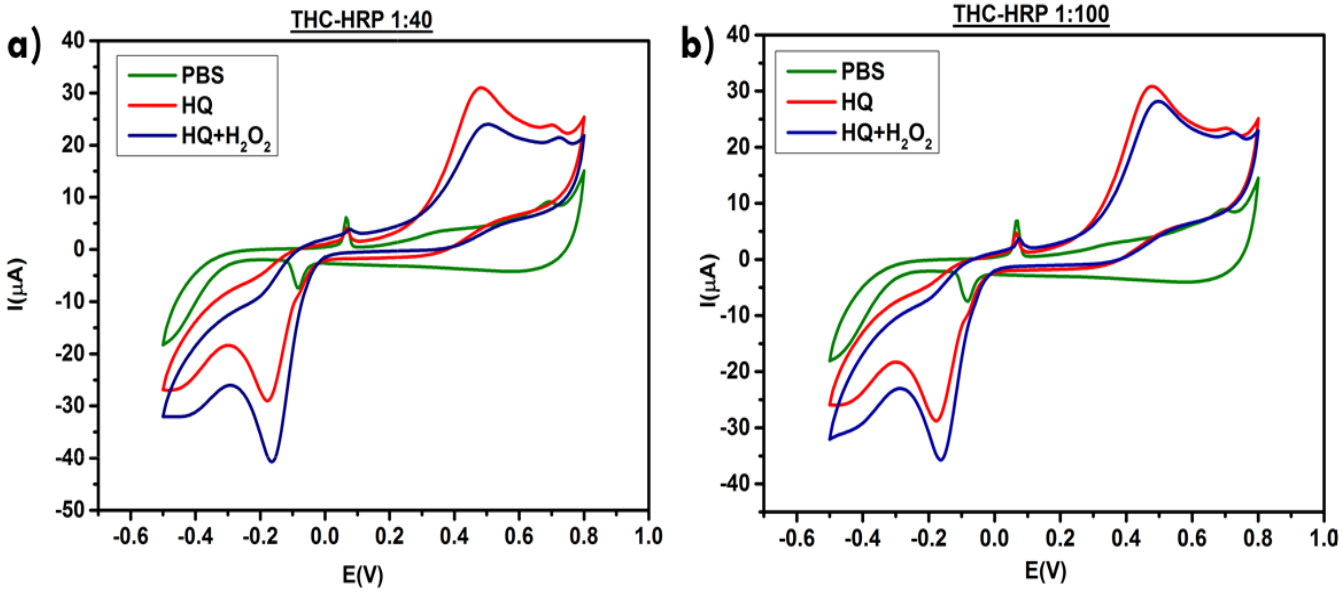


Figure 15. Plot of current intensity vs concentration of THC-HRP for THC biosensor and control (analogous sensor)

As expected, higher dilutions of THC-HRP, the magnitude of current decreases for biosensors immobilized with antibody while almost no response is observed for the control. This observation was further strengthened by the results obtained from cyclic voltammetry (CV) (shown in Fig.16). Fig.16 clearly shows that the current intensity is higher for biosensor incubated with 1:40 THC-HRP dilution which means reduction of the substrate H_2O_2 to H_2O is significantly higher compared to other dilutions.



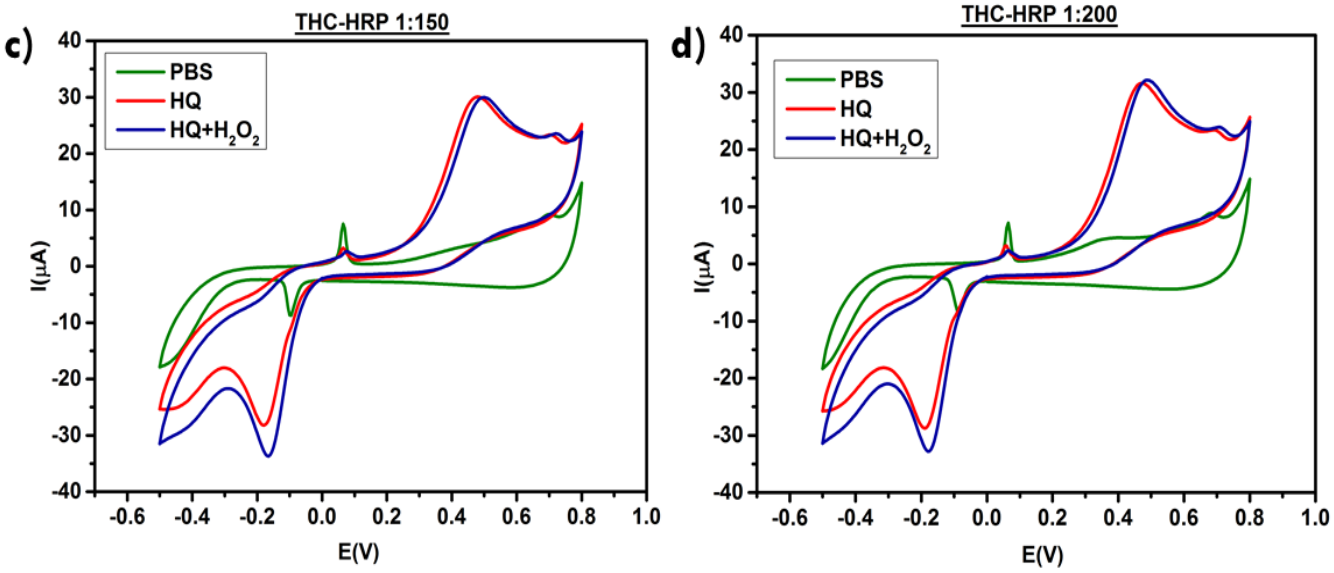


Figure 16. Cyclic voltammogram for various THC-HRP dilutions a) 1:40 b) 1:100 c) 1:150 d) 1:200

Later, a competitive assay was performed between a fixed dilution of THC-HRP (i.e. at 1:50) and with concentrations of free THC in solution ranging from 0 ng/ml to 1000 ng/ml. Another biosensor was developed where the competition was carried out with THC-HRP dilution of 1:250 and changing THC concentration (ranging from 0 ng/ml to 1000 ng/ml). The mixture of THC/THC-HRP was incubated onto the surface of Au SPEs for 1 hour at room temperature, followed by amperometric measurements (protocol as mentioned in the strategy 2). The basic purpose was to check the performance of the biosensor at two THC-HRP dilution and to see if the biosensor works at low THC-HRP dilution, which would reduce the amount of THC-HRP used in biosensor preparation and hence in a way reducing cost of the biosensor. Moreover, to find the optimal dilution of THC-HRP that provides the highest sensitivity for the competition between THC and THC-HRP.

Amperometric response (Fig.17.c) show how the current signal decreases after incubation with higher concentrations of THC, which is due to the competition. The figure also shows the response to the sensor towards the unspecified antibody which was taken as control. Besides, using 1:50 dilution of THC-HRP a better sensitivity in current signal is observed at lower THC concentration. Moreover, at higher concentration of THC-HRP , much better signal/noise ratio was observed.

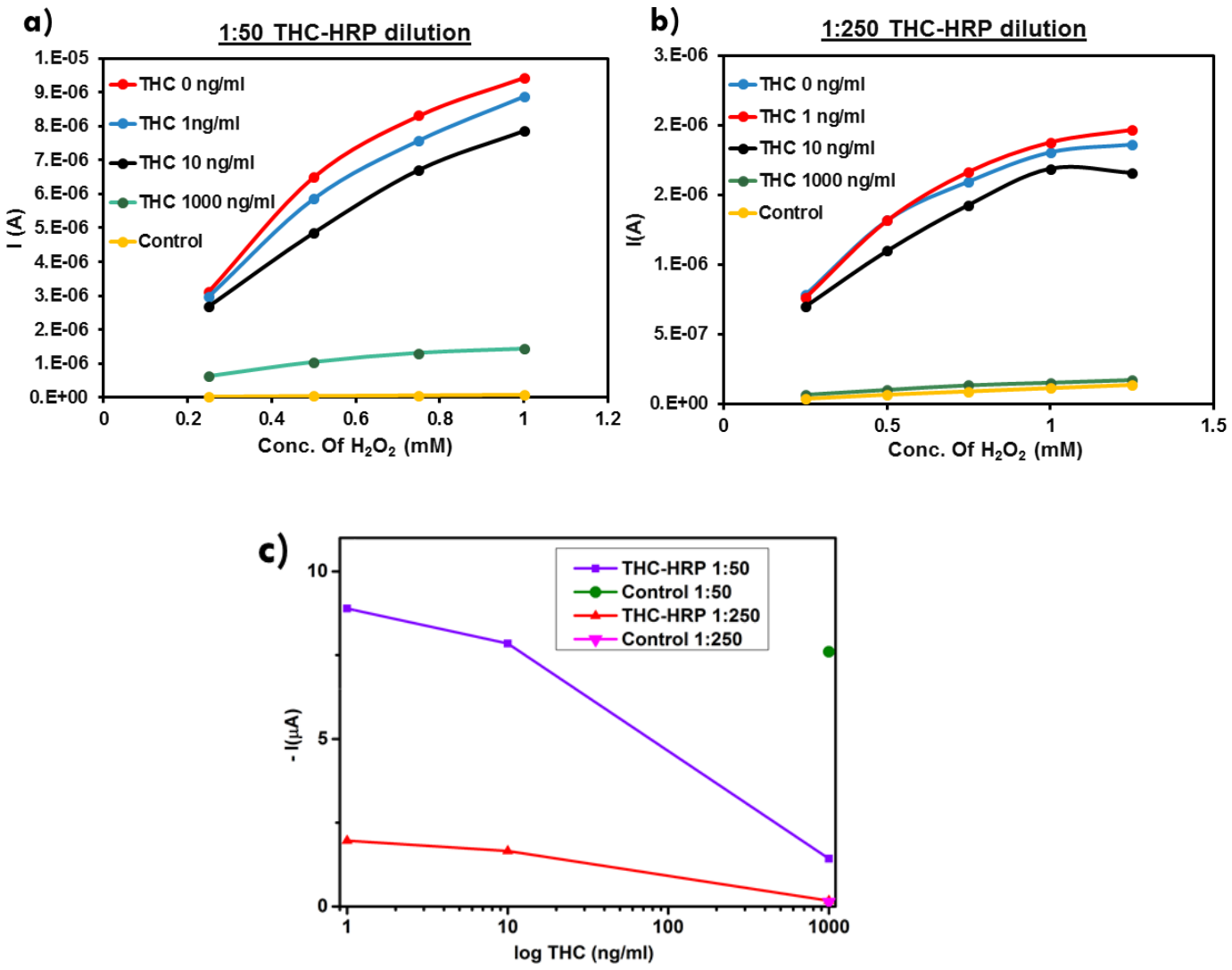


Figure 17. Plots of current intensity vs H2O2 concentration for THC concentrations at a) 1:50 b) 1:250 THC-HRP dilutions c) current vs concentration of THC plot for THC-HRP at two dilutions and with their respective control

4.3.2 Optimization of antibody concentration

After the optimization of THC-HRP, the next step was to optimize the concentration of immobilized antibody. Three different concentrations (7.5 µg/ml, 25 µg/ml and 75 µg/ml) of anti-THC were studied. Anti-THC was immobilized onto the surface of Au SPEs by means of protein G. A competitive assay was carried out, fixing the dilution of THC-HRP at 1:50 and the changing concentration of THC to 0 ng/ml and 700 ng/ml. Amperometric measurement was carried out in a stirred solution containing 10 ml PBS (pH 7.4) and 1 mM HQ and on addition of 40 µL of 0.25 mM H_2O_2 . A constant potential of -0.2 V vs the Ag pseudo reference electrode.

The plots for the amperometric measurement is shown in Fig.18. It can be seen that the competition between THC and THC-HRP is achieved at all three antibody concentrations. The unlabelled THC competes out the labelled THC on increasing -in absence of THC but it is reduced, once THC is added i.e. at 700 ng/ml confirming a competition. There is an increase in current intensity for electrode immobilized with anti-THC having concentration 25 µg/ml (shown in Fig.18.d.) This shows that more antigen binding sites are made available for THC at higher antibody concentrations. Since not much significant difference in current is observed at 25 µg/ml and 75 µg/ml, it was decided to use 25 µg/ml of anti-THC for subsequent experiments to carry out.

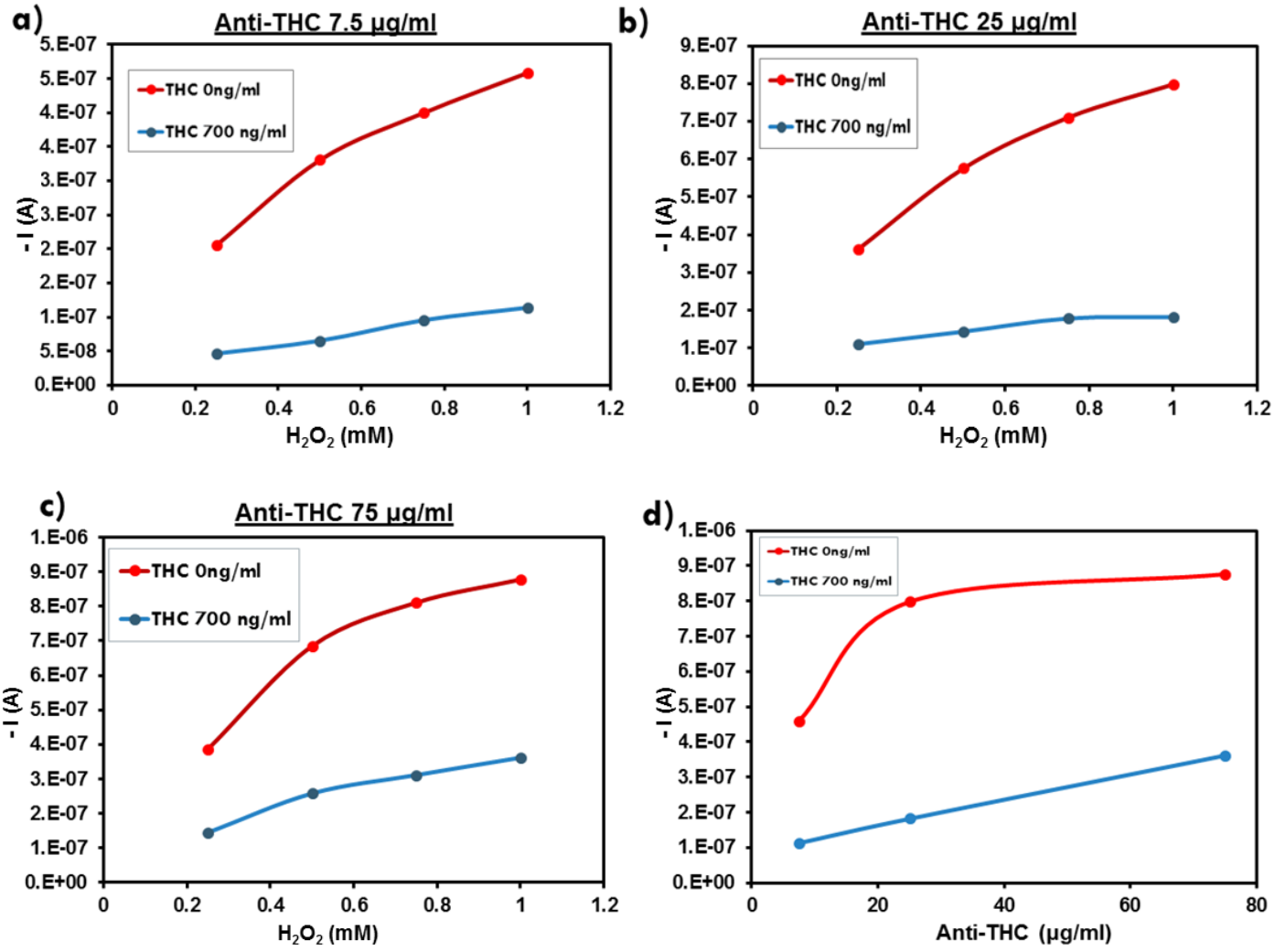


Figure 18. Plots of current intensity vs H2O2 obtained for anti-THC immobilized SPEs with concentrations a) anti-THC 7.5ug/ml b) anti-THC 25ug/ml and c) anti-THC 75ug/ml d) plots of vs anti-THC concentration for two concentration of THC

4.3.3 Study of incubation time

An efficient biosensing device offers specificity, low limit of detection and short analysis time. Short time to result for drug detection, has become extremely critical in the development of biosensors and hence plays a significant role in point-of-care testing. Conventional drug detection techniques use sophisticated and expensive instrumentations provided with short time analysis of several hours. In this direction, electrochemical biosensors can be defined to reduce the analysis time.

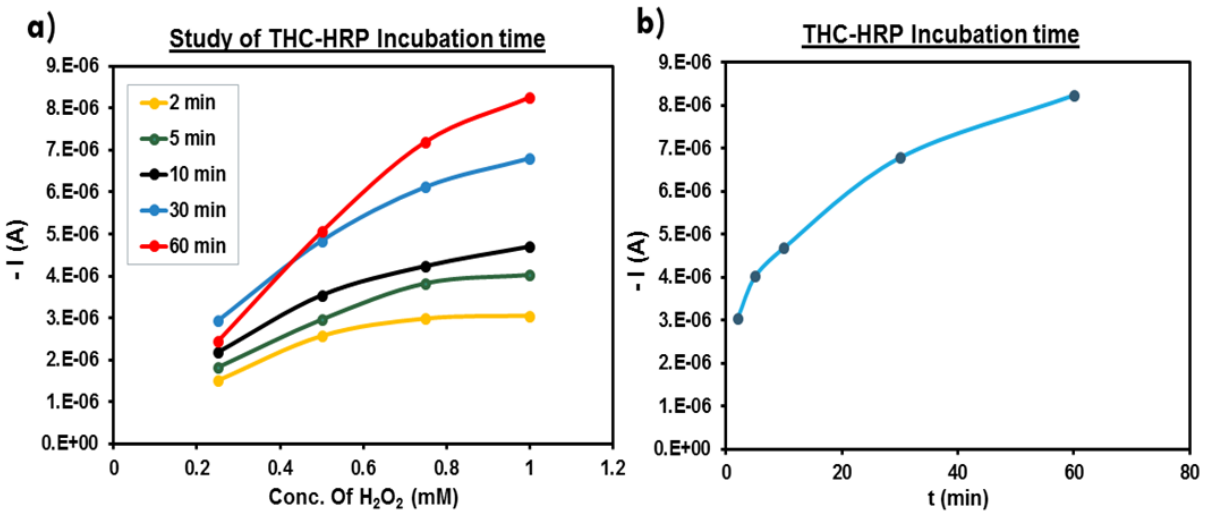


Figure 19. a) Plot of current intensity vs H2O2 concentration for different THC-HRP incubation times b) plot showing current signal against different incubation time

In order to reduce the analysis time, biosensors were prepared with a 25 µg/ml anti-THC, anchored onto the surface of Au SPEs with the help of protein G. The biosensor was then subjected to THC-HRP incubation at a dilution of 1:50 during 2 min, 5 min,10 min, 30 min, 60 min. Amperometric measurement was carried applying a constant potential of -0.2V vs the Ag pseudo reference electrode.

Amperometric responses for different incubation times viz 2min, 5min,10min, 30min and 60min are shown in the Fig.19.a. It can be seen that the current response increases with increasing the time of incubation of THC-HRP. The biosensor shows a significant response even at short incubation time of 2 min. Fig.19.b. shows the increasing current response of the biosensor when plotted against time.

Keeping the incubation time for 2 min, 5 min and 10 min, a competitive assay was carried out both in presence (700ng/ml) and absence (0ng/ml) of THC and

maintaining a fixed THC-HRP dilution of 1:50. The results showed that although the competition was higher when incubated for 10 min as compared to 2 min and 5 min (shown in Fig.20), 2 min was enough to perform the competition.

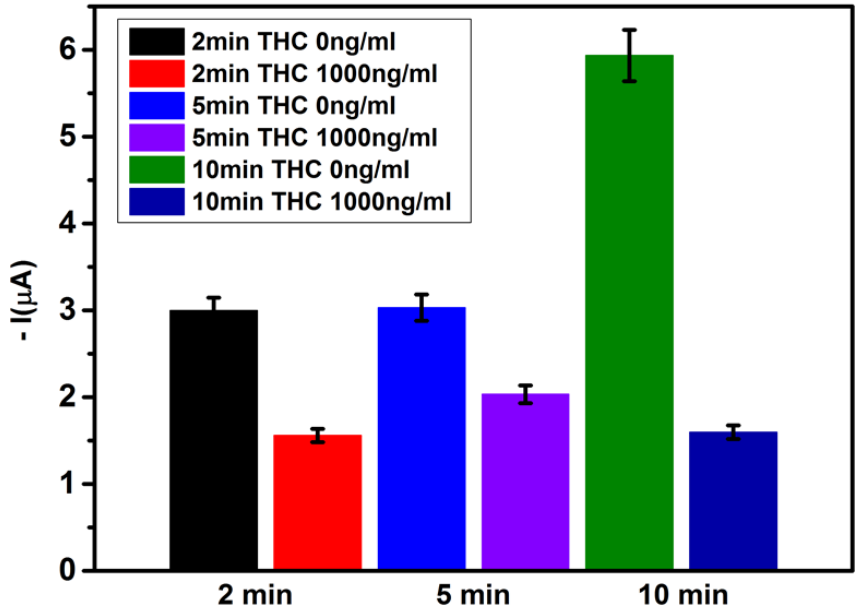


Figure 20. Current intensity obtained in the absence (0 ng/ml) and presence (1000 ng/ml) of THC for three different incubation time

4.4 Magnetic immunosensor based on strategy 3

To further increase the sensitivity and to reduce the matrix effect arising from the sample solution, a magnetic particle based sensing approach was explored. The protocol as mentioned in the methodology 3 was followed.

4.4.1 Magnetic Immunoassay

The sensitivity of Ab1 and Ab3 was also checked using amperometric measurements. The carboxylic group-coated magnetic beads having a size of 2.5 μm were used. 5 μL of magnetic beads suspension was collected in 1.5 mL tubes. The carboxylic group on the surface of magnetic beads were activated through carbodiimide chemistry. The magnetic beads were suspended in EDC/NHS mixture for 30 minutes. After washing the beads twice with MES buffer, 10 μg/ml Protein G was added that allowed a controlled orientation of the antibody (incubated at room

temperature for 1 hour). Followed by the incubation of 25 µg/ml antibody (Anti THC) at room temperature for 1 hour. A competitive assay was carried out in presence (1000 ng/ml) and absence (0 ng/ml) of THC, keeping the dilution of THC-HRP fixed at 1:50 for 1 hour at room temperature, after which the tube was placed on a magnetic separator, supernatant removed and the beads were washed twice using PBS-Tween (PBST) and re-suspended in 50 µL PBS to carry out the measurement. The magnetic beads were washed in the similar way as mentioned above after subsequent additions of protein G and antibody using PBST.

After the incubation of THC-HRP/THC, the modified 50 µL magnetic bead suspension was transferred onto the carbon SPE, which was assembled on a magnet holder (as shown in Fig.6.b.)The magnet holder-SPE with magnetic beads captured on its working electrode was immersed in the electrochemical cell containing 10 ml PBS (pH 7.4) and 1 mM HQ and the amperometric response were recorded in a stirred solution, on addition of 40 µL of 0.25 mM H_2O_2 as enzyme substrate until the saturation of the enzyme was reached (shown in Fig.6.c). A constant potential of -0.2 V vs the Ag pseudo reference electrode was applied.

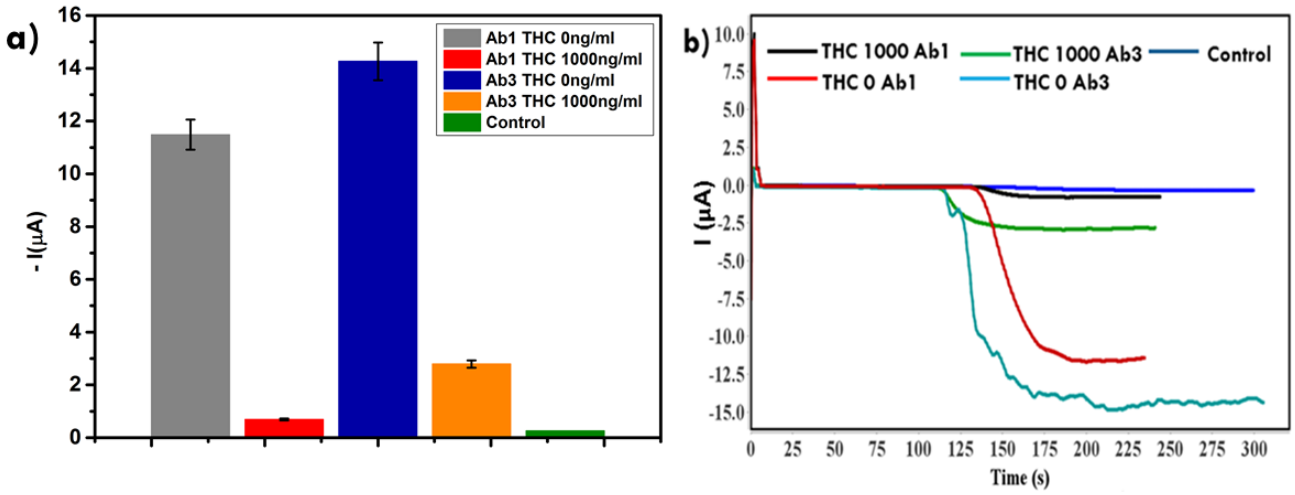


Figure 21.a) Plot showing peak current signal for magnetic immunosensor with different antibodies in 0 ng/ml and 1000 ng/ml of THC b) Amperometric responses measured for Ab1- and Ab3-modified magnetic beads in 0 ng/ml and 1000 ng/ml of THC

Fig.21.a.,clearly confirms that the difference in current response is higher for Ab3 than Ab1 in reference to the control. While no significant response was observed for control (unspecific antibody). The current difference of Ab1 both in absence and presence of THC with respect to control were 11.2 µA and 0.4 µA respectively, whereas the same

for Ab3 were 13.9 μA (absence of THC) and 2.5 μA (presence of THC). Hence, Ab3 selected as the capture antibody in the preparation of magnetic immunosensor.

4.4.2 Performance of Magnetic Immunosensor

To study the sensitivity towards different concentration of THC ranging from 0 ng/ml to 1000 ng/ml, a competitive assay was carried out fixing the THC-HRP dilution at 1:50. The mixture of THC/THC-HRP was incubated at room temperature for 5 minutes. Based on the experiment done so far, Ab3 was used for the preparation of magnetic immunosensor with an optimum concentration of 25 μg/ml. The amperometric current measured was a constant potential of -0.2V against Ag pseudo reference electrode using 40 μl of 0.25 mM H_2O_2 as substrate and HQ as redox mediator (protocol as mentioned in strategy 3). To further confirm the results, an analogous magnetic immunosensor was prepared employing an unspecific antibody towards THC.

The Fig.22.a, shows the plot of current against concentration of THC (0 ng/ml, 10 ng/ml, 100 ng/ml and 1000 ng/ml) for the magnetic biosensor and unspecific antibody (control). As expected a high degree of competition was achieved on increasing the concentration of THC (from 0 ng/ml to 1000 ng/ml) and a decreasing current signal can be observed for THC biosensor with respect to the control (shown in Fig.22.a). This shows that the THC competes out THC-HRP in the solution. And the competition takes place even at concentration as low as 10 ng/ml for the THC biosensor. The control did not show any response.

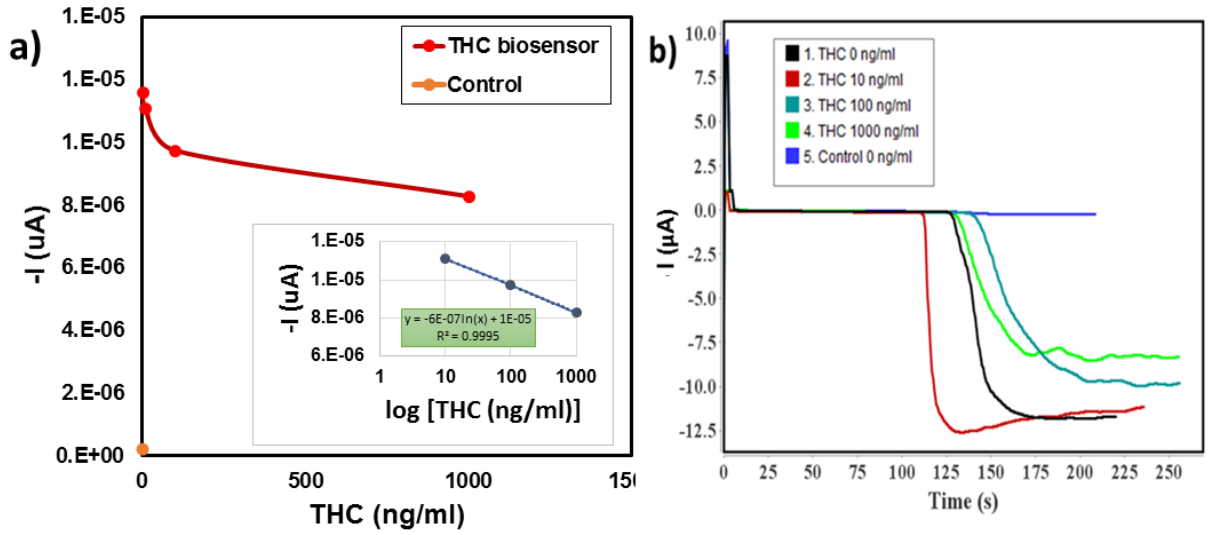


Figure22.a)) Plot of current intensity vs concentration of THC for biosensor unspecified antibody, inset shows the current intensity plotted against logarithmic THC concentration b) amperometric responses measured for different THC concentrations

5. Conclusion

The study illustrates the development of an electrochemical biosensor for the detection of an illicit drug i.e. THC, the main psychoactive component of marijuana. Three different strategies were explored for biosensor development. This has shown that electrochemical biosensing is a more feasible method in terms of short analysis time for the detection of THC. The developed magnetic particle based amperometric biosensor showed sensitivity towards THC at concentration as low as 10ng/ml. However, the detection of THC in the body fluid like saliva still needs to be investigated. The study shed light on the short time to results for the biosensor developed, as it was found that the biosensor showed a short analysis time of 2 minutes. Finally, few more works needs to be studied using magnetic beads based sensing such as the optimization of incubation time, concentration of magnetic beads, limit of detection and to evaluate the working of biosensor in real samples of saliva.

6. References

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