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Thin Film Based Biosensors for Point of Care Diagnosis of Cortisol

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THIN FILM BASED BIOSENSORS FOR POINT OF CARE DIAGNOSIS OF CORTISOL

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Syed Khalid Pasha

2018

To: Dean John L. Volakis
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This dissertation, written by Syed Khalid Pasha, and entitled Thin Film Based Biosensors for Point of Care Diagnosis of Cortisol, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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DEDICATION

I want to dedicate this work to my family who stood by me through all times.

I also dedicate this work to all my friends and colleagues who believed in me.

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ABSTRACT OF THE DISSERTATION

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Miami, Florida

Professor Shekhar Bhansali, Major Professor

In this research, the design and development strategies for thin film-based biosensors are explored to achieve rapid, sensitive, selective, and label-free detection of cortisol in the physiological range [0.05µg/dL(saliva) to 25µg/dL(blood)]. Cortisol is a steroid hormone which is related to the physiological stress. Increased cortisol levels have been associated with stress-related diseases, including chronic fatigue syndrome, irritable bowel syndrome and post-traumatic stress disorder. Therefore, accurate measurement of cortisol in saliva, serum, plasma, urine, sweat, and hair, is gaining increasing clinical significance to predict multiple physical and mental diseases.

Thin film based electrochemical immunosensors were fabricated using a self-assembled monolayer (SAM) functionalized by specific antibodies to detect cortisol in the presence of a redox probe. The fabricated electrochemical cortisol immunosensors were able to detect cortisol in human saliva samples at 10 pM level sensitivities. The results of the sensor were validated using the standard Enzyme Linked Immuno Sorbent Assay (ELISA) technique. To improve the signal amplification and label-free cortisol detection, copper nanoparticles were on the screen-printed carbon electrodes (SPCE) for the fabrication of electrochemical

cortisol immunosensor. This SPCE-based sensor showed a sensitivity of 4.21μA/M and limit of detection 6.6nM.

However, both SAM and SPCE based immunosensors exhibited a shortcoming of thermal stability due to the biological nature of antibodies at room temperature. To overcome this challenge, an antibody-free sensing platform based on molecular imprinted polymer (MIP) was developed. The design of this cortisol sensor depends on the selection of an appropriate polymer-base, wherein the sensing is dependent on the cortisol molecule's adsorption on the polymer substrate. A poly-pyrrole, a conducting polymer, was selected to fabricate MIP using electrochemical polymerization of pyrrole. The MIP based sensor detected cortisol in the physiological range at room temperature. The performance of this sensor was tested on saliva samples and the results were validated by the conventional ELISA technique. This MIP based cortisol sensor is cost-effective, easy to fabricate, temperature stable, and reusable.

Aiming to perform cortisol sensing at point-of-care (POC), an Extended Gate Field Effect Transistor (EGFET) was integrated with a developed MIP cortisol sensor. The as developed MIP-EGFET sensor was used to detect the cortisol concentration in the range of 10 pM to 100 nM. Some of the salient features of the developed sensor are its ability to provide a direct readout and a simpler electronic system. These attributes enable the POC device development of the reduced form factor.

A sensing strategy consisting of integrating MIP cortisol sensor with an extended gate field effect transistor (EGFET) configuration was demonstrated to perform cortisol sensing at point-of-care (POC). The MIP-EGFET configuration protects the active device from the liquid environment and enables the relatively simple, label-free, portable and direct measurement system for cortisol sensing.

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LIST OF ACRONYMS

$Ag/AgO_{x:}$ Silver/SilverOxide

CdSe: Cadmium Selenide

DNA : DeOxyribo Nucleic Acid

DTSP : Dithiobis (succinimidyl propionate)

EA : Ethanolamine

EC : Electrochemical

EIS : Electrochemical Impedance Spectroscopy

ELISA: Enzyme Linked Immuno Sorption Assay

FET : Field Effect Transistor

ISFET : ion Sensitive Field Effect Transistor

JFET: junction Field Effect Transistor

MIP: Molecular Imprinted Polymers

MOSFET : Metal Oxide Field Effect Transistor

*p*NPP : p-nitrophenyl phosphate

POC: Point of Care

PTSD: Post Traumatic Stress Disorder

QCM: Quartz Crystall Microbalance

RIA: Radio Immuno Assay

SPR: Surface Plasmon Resonance

TFT : Thin Film Transistor

VLSI: Very Large Scale Integrated Circuits

Chapter 1: INTRODUCTION

1.1 Motivation

Everyday experiences, events and lifestyle have been known to cause psychological stress, leading to adverse health outcomes. Although the human body reacts to stress in different ways, a typical response is the release of hormones and neurotransmitters. Prolonged exposure to stress causes the activation of signaling pathways from the brain that leads to the release of cortisol from the adrenal cortex. Various signaling pathways are affected by psychological stress. Cortisol “a steroid hormone” has been widely used for estimation of stress and exposure to chemicals. Cortisol secretion levels have been correlated to stress levels in patients suffering from stress disorders such as Post Traumatic Stress Disorder (PTSD), Cushing's syndrome, insomnia and burnout. Cortisol has also been used as a marker for determining the effect of exposure to external chemicals, such as the effects of organophosphates on the central nervous system, which alter the endocrine system, leading to an imbalance in cortisol secretion. The secretion of cortisol in individuals depends on the circadian rhythm and the environment. Hence its variations during the day at Point of Care (POC) are essential for personalized diagnosis and treatment. The detection of cortisol is based on immunoassays like chromatography, Enzyme Linked Immuno Sorbent Assay (ELISA) and Radioactive Immune Assay (RIA). These systems are bulky, expensive, require labels to operate and do not provide quick results. In contrast, Electrochemical (EC)/electrical (FET, chemiresistive, etc.) sensors demonstrate better sensing performance, can be made label-free, micro-fabricated, can provide faster results and are suitable for miniaturization. Due to their advantages

over optical, spectroscopic and mechanical sensors, electrochemical/electrical sensors were extensively explored for application towards POC systems [2-8].

Electrochemical cortisol sensing platforms reported in this research have the potential to be integrated into a POC system for online continuous monitoring of cortisol as a function of one's environment.

POC sensing systems consist of biosensors and bio-analytical devices. These systems are crucial for detection of a targeted analyte outside controlled environments of diagnostic labs and hospitals. These devices have a major impact on the applications of personal care, health, food, and environmental monitoring. An ideal POC biosensor device allows real-time, rapid, label-free, and multiplexed detection with high selectivity and sensitivity [8].

However, there are several challenges in implementing POC sensors. The requirement of redox media for electrochemical sensing adds to design complication for POC devices. The lack of robustness due to the temperature sensitivity of detection molecules (antibodies, enzymes) pose logistical challenges involving increased costs associated with storage at temperatures below zero. The complexity of the electronic system associated with these devices also prevents them from being miniaturized for POC application. To address the above challenges in thin film-based detection of cortisol, this research was performed with the following specific aims in mind.

1.2 Specific Aims

1.2.1 Specific Aim 1: Electrochemical immunosensors on a gold thin film based Inter Digitated Electrodes (IDEs) and their validation against ELISA using Human saliva samples

The first activity to be undertaken in this research was the fabrication of electrochemical immunosensors on Au thin film-based IDEs. The Au surface was functionalized using SAM of DTSP molecules. Cortisol specific Antibodies were then immobilized on this functionalized surface, and non-binding sites blocked using Ethanolamine (EA). These sensors were calibrated and used for detection of cortisol in human saliva samples. The same samples were tested for cortisol using the standard ELISA process. The results obtained using both procedures were compared to validate the electrochemical immunosensing protocol.

1.2.2 Specific Aim 2: Nano enabled signal amplification in SAM based electrochemical cortisol Immunosensors.

Electrochemical sensors have a limitation where a redox label is required for sensing. In order to address the issue of label-free sensing, SPCE were modified electrochemically with Cu/CuO nanoparticles. The nano-modified SPCE were characterized by SEM, XRD and by CV. These nano-enabled SPCE were then used to fabricate SAM based Immunosensors using cortisol specific antibodies. The nano-enabled immunosensors were then used to detect the different concentration of cortisol in a test sample.

1.2.3 Specific Aim 3: Biomimetic sensors for label-free, temperature stable and repeatable measurements of cortisol

MIPs were explored to address the challenges associated with previously fabricated immunosensors (temperature stability, label-free sensing, repeatability and direct output of sensing data). MIP thin film were formed on an SPCE surface using electro-polymerization of pyrrole. The substrates were then optimized and characterized.

The MIP modified electrodes were then be used for electrochemical sensing and demonstrate cortisol detection. The fabricated sensors overcame some of the challenges associated with the ECIS described above. These sensors do not use expensive antibodies. Hence, the fabrication costs associated with them are meager. Repeatable sensing of cortisol was also demonstrated with the same MIP based electrode to allow for potential wearable applications.

1.2.4 Specific Aim 4: Integration of EGFET with MIP based substrate for Sensing Cortisol- A direct approach.

The electrochemical sensors have limitations with regards to label-free sensing and providing a direct readout of the sensing results. To address these limitations, a much simpler approach to sensing was explored. The MIP based electrodes were integrated with a FET to give an EGFET configuration. The EGFET-MIP sensors thus fabricated allowed label-free detection of cortisol and provided direct readout by modulating drain current as a function of cortisol concentration. This approach also allows for less complexity regarding auxiliary instrumentation which is required for miniaturized devices.

1.3 Dissertation Organization

In this Dissertation, we report the development of biosensors for the detection of cortisol which has potential application towards the point of care use. In Chapter 1, the motivation for this research and specific aims are discussed. In Chapter 2, cortisol and its importance in the body are presented. Also discussed are the state of the art techniques available for the detection of cortisol. In Chapter 3, the fabrication and characterization of electrochemical immunosensors based on the gold thin film is presented. The results related to the validation of these sensors against standard ELISA technique were also discussed in this chapter. Chapter 4 reports the development of a nano-modified electrochemical immunosensor for achieving label-free sensing of cortisol. Chapter 5 and 6 present the design, fabrication, validation, and application of Molecular Imprinted Polymer based sensors in electrochemical and electronic (EGFET) configuration. These chapters address the issues associated with the devices reported in the previous chapters. The summary of the research undertaken, and future prospects are discussed in Chapter 7.

Chapter 2: BACKGROUND AND LITERATURE REVIEW

2.1 Biosensing

Medicine and healthcare have been evolving ever since the dawn of humanity. They are based on a system of observation and treatment of symptoms. The only tools available for diagnosis in early times were the sense of smell, sight, touch, and hearing. Patients were treated based on how their symptoms responded to a medicine. It was the case until the late nineteenth century. The evolution of scientific tools such as microscopes, stethoscopes, etc. helped in the diagnosis of diseases. During the 20th century, the development of chemical analysis tools such as gas chromatography [9], paper chromatography, mass spectroscopy, etc. enabled researchers to estimate the amount of targeted chemicals in a healthy and diseased specimen. The development of chemical analysis techniques allowed health care providers to form their diagnosis better and treat the patients accordingly. The detection of biochemical markers and their association with certain diseases paved the way for modern medicine and health care. The improved accuracy of the diagnosis saved countless lives. However, the bulky nature of the detecting equipment, the sample collections techniques, elaborate sample preparation and longer times to detection have made these diagnostic techniques expensive and limited them to labs [10-17].

Efforts are being made to make the detection of biomolecules fast, easy and cheap. One of the pioneering works in the development of such a system was by Leland C Clark in 1965[18]. He pioneered the development of an oxygen sensor. This sensor was later modified to detect glucose molecules in a drop of blood. This

technology resulted in a portable glucose meter still being used by millions of diabetics across the world to monitor their blood glucose levels every day.

However, there are very few biosensing systems available in the markets that come close to achieving a cheap, portable and easy to use system. The conventional techniques of diagnosis involve sample collection, shipping to the diagnostic lab, and involve elaborate sample preparation techniques before analysis. Despite being expensive, cumbersome and time-consuming, these techniques are still a viable option for quantification of biomolecules.

The proposed market share of personalized healthcare monitoring systems is estimated globally to be more than $ 20 billion [19]. In order to bridge the high supply gap, researchers focused on the development of POC sensors. Several modes of biosensing were explored (optical electrochemical, electrical, etc.) to achieve easy, miniaturized and cost-effective devices.

Today most of the biosensors used are affinity-based. That means they are based on the interaction between molecules that are complementary to each other. Some of these complementary interactions are antibody-antigen coupling, aptamer-protein, DNA hybridization, etc.

According to the World health organization, world over, chronic diseases are a leading cause of death and disability. Diseases such as cardiovascular, obstructive cardiopulmonary, diabetes and cancer, etc. are significant concerns that adversely affect the lives of millions across the globe [20]. These diseases are linked by common and preventable lifestyle choice risk factors such as unhealthy diet, physical inactivity, chronic stress, etc. Among these factors, chronic stress affects the functioning of the body and inhibits normal functioning of the immune system. The

estimation of Stress is, therefore, a necessary tool that can help provide proper diagnostics and healthcare to patients. This research will focus on the detection of cortisol as the primary biomarker for detection of stress.

2.2 Why Cortisol?

Cortisol is a steroid hormone produced in the body as a response to stressor events (physical and psychological). As a steroid hormone, cortisol is produced as part of the body's stress response. It is secreted by the hypothalamic-pituitary-adrenal (HPA) system. Normal levels of cortisol secretion follow a circadian rhythm and fluctuate significantly through a 24-hour cycle with cortisol levels highest during daybreak (30 minutes after awakening) and progressively lower as the day progresses to a minimum during night sleep [21, 22] (Figure 1).

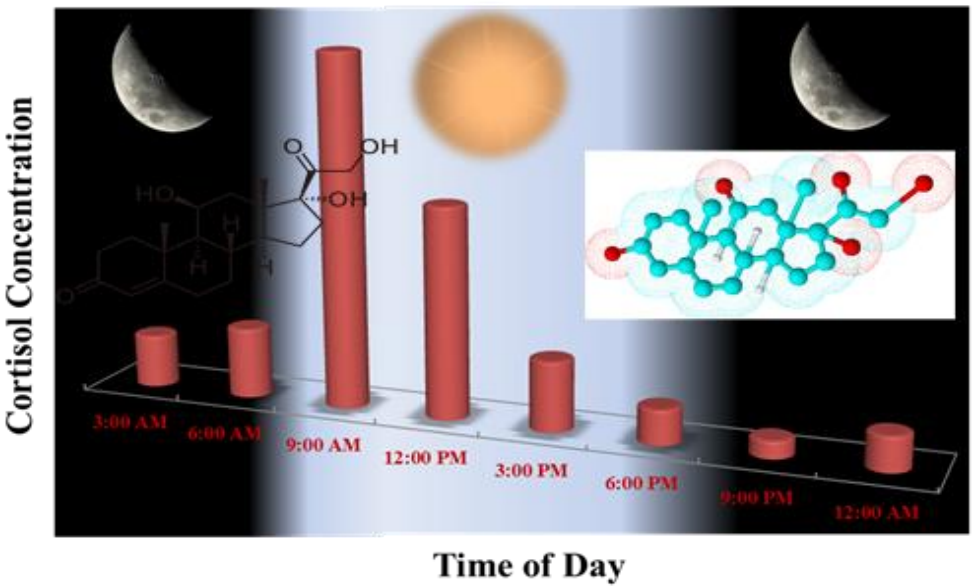

Figure 1: Chemical structure of cortisol and typical diurnal variation of cortisol levels over a 24-hour cycle [21,22].

Apart from the day-night cycle, several controllable factors can affect cortisol levels such as eating patterns and physical activity. While the abnormal increase in cortisol levels inhibits inflammation, depresses the immune system, increases fatty

and amino acid levels in blood [23], it is essential in maintaining a healthy metabolic rate and help in the daily functioning of many bodily functions [24-26]. Excess cortisol levels in the body have been shown to be associated with the development of Cushing's disease with the symptoms of obesity, fatigue and bone fragility [23]. On the other hand, a decrease in cortisol levels leads to Addison's disease which manifests in the form of weight loss, fatigue, darkening of skin folds and scars [27]. The most dominant effect on cortisol variation comes from psychological/emotional stress, which is why cortisol is popularly called the "Stress-hormone" [28].

Increasing level of psychological stress due to competitive living style is becoming a serious concern in the everyday schedule. Also, life-threatening diseases like heart attack, depression, and brain pain are the health challenges faced by most developed countries [29]. The accurate and precise detection of psychological stress is thus gaining attention for personalized health monitoring and diagnostics. The stress cycles (Figure 2) in human find its ways into the nervous system and upsets the chemistry of the entire body [30]. Cortisol abnormalities are often a good indicator of chronic conditions such as Addison's disease, Cushing's syndrome, and adrenal insufficiencies [23-26, 29]. Hence, real-time and continuous monitoring of cortisol levels is required to obtain valuable information that could assist doctors in better diagnosis and treatment of cortisol related conditions. In clinical practice, total cortisol, which is the sum of free and protein-bound fractions, is measured. However, free cortisol is the biologically active fraction [31] which is responsible for all cortisol related activities in the body. Hence, to diagnose and properly treat cortisol-related conditions, regular estimation of free cortisol is required. The state-of-the-art in cortisol detection is mostly limited to laboratory-based techniques such as chromatography [32-34], RIA [35], ECLIA [16, 36-39], ELISA [12, 40-42], SPR [43-

45], QCM [46] etc. Detection of 24-hour cortisol levels is currently a cumbersome process, which either involves admitting the patient for the time of the study [47] or the patient depositing samples (blood, saliva, sweat or urine) into vials at specified intervals during the 24-hour period and shipping of them to a diagnostic laboratory [48]. The typical turnaround time is 3 to 8 days and is still not a true representation of cortisol levels in stressful environments. At best they provide a momentary data that is difficult to relate to the stressor events at the patient end. There is currently a need to investigate cortisol sensing platforms that can be deployed at point-of-care (POC).

Figure 2: Stress cycle and its effect on human nervous system

2.2.1 Secretion of Cortisol.

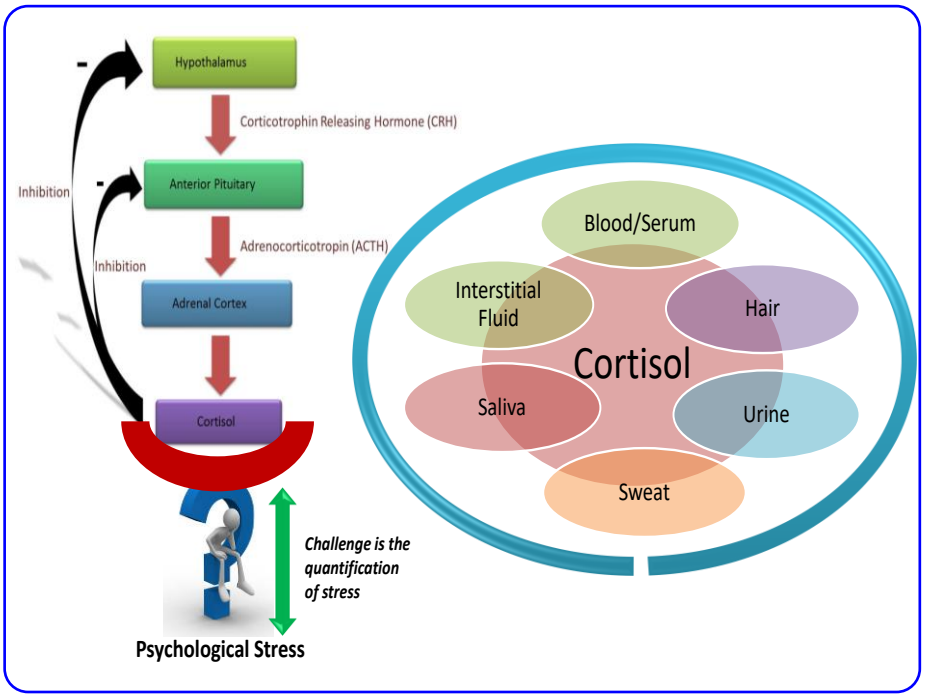

Figure 3: Cortisol secretion regulated by the HPA axis and various bio-fluids used for cortisol estimation

Cortisol is secreted from the adrenal glands located above the kidneys. Cortisol is the product of the Hypothalamic-Pituitary-Adrenal (HPA) axis, which is the main component of the human body's adaptive system to maintain regulated physiological processes under changing environmental factors. The HPA axis is a complex signaling system between the hypothalamus in the brain, the pituitary glands and the adrenal glands [24]. A typical response to an environmental trigger is initiated by the hypothalamus that releases a hormone called the CRH (Corticotrophin Releasing Hormone) that travels to the pituitary glands. Specialized cells that work synergistically with the pituitary glands release ACTH (Adrenocorticotrophic Hormone) into the bloodstream that travels to the adrenal cortex. The adrenal cortex responds by increasing the production of cortisol. Since the adrenal glands have no visible innervation, it can be inferred that ACTH is the sole stimulant for initiating

cortisol production. The adrenal glands do not store cortisol, but they aid in the production of cortisol. Cholesterol undergoes multiple catalyzed oxidation reactions to result in the formation of cortisol. This entire process takes place in a time space of a few minutes. The HPA axis is a negative feedback system, where Cortisol plays a critical rule in the homeostasis of the HPA axis (Figure 3). Moderate homeostatic alterations in the HPA axis is beneficial for the physiological and psychological development of the human body, sustained and prolonged exposure to environmental triggers such as stress leads to abnormal levels of cortisol in the circulatory system [24]. Secreted cortisol finds its way into the circulatory system and can be found in detectable quantities in several biofluids. In this section, an assessment of the advantages and disadvantages of using various biofluids such as urine, blood, sweat, interstitial fluid (ISF) and saliva for the detection of cortisol is presented.

2.2.1.1 Urine

Cortisol level in urine is measured over a 24-hour period and is referred to as the 24-hour urinary free cortisol (UFC) test. Only free cortisol, which is the active form of cortisol in the human body, is found in urine and is hence a relevant biofluid for the detection of cortisol. The excretion of hormones, salts and other waste chemicals through urine is a well-characterized process with good knowledge about the concentration of these waste substances under normal function. Observation of extreme variation in concentrations of hormones such as cortisol can be used to diagnose abnormalities in the adrenal function. Measurement of cortisol in urine typically requires the collection of all the urine generated over a 24-hour period. The normal range for cortisol in urine is 10 to 100 μg/24hrs [49]. A review of the reported literature for urinary cortisol assays has been presented by Brossaud et. Al. [49]

While the 24-hour urinary free cortisol test provides a means for a non-invasive, painless method of obtaining body fluid for cortisol measurement, it also poses several drawbacks with respect to convenience and reliability. Since the collection of the urine is spanned over a 24-hour period, sample collection becomes an inconvenient process where the patient needs to carry the special urine collection container all day long or must remain confined to a location for the 24-hour period. The container also needs to be stored under refrigeration from the time of collection until it is delivered to a diagnostic lab for testing. Also, several factors such as pregnancy and medication such as diuretics can alter the concentration of cortisol in urine making it a less reliable biofluid for cortisol detection. These several additional factors have severely limited the use of urine to cases where patients are admitted to the hospital for long-term treatments. The requirement of 24-hour sample collection renders urine unfit for real-time detection.

2.2.1.2 Interstitial Fluid (ISF)

ISF is an extracellular fluid that surrounds the cells in the human body. In composition, it is similar to blood plasma. Metabolites and proteins move into ISF as they move from capillaries to cells. In general, small to moderate sized molecules, including glucose, ethanol, and cortisol are found in ISF in similar proportion as in blood. Thus, periodic calibration using blood sampling is not required to obtain the concentration of these metabolites from ISF. ISF is present just below the skin, but the low permeability of the epidermal keratinized layer (the stratum corneum) blocks the permeation of the fluid through the skin. However, obtaining ISF for detection of a target biomarker could require an invasive approach as it is not readily accessible. Several approaches have been reported in the literature to obtain ISF in a minimally invasive, painless process. Venugopal et al. [50] have reported the construction of an

ISF harvesting system that utilizes a low-energy laser to create micropores in the stratum corneum (the uppermost layer of dead cells).

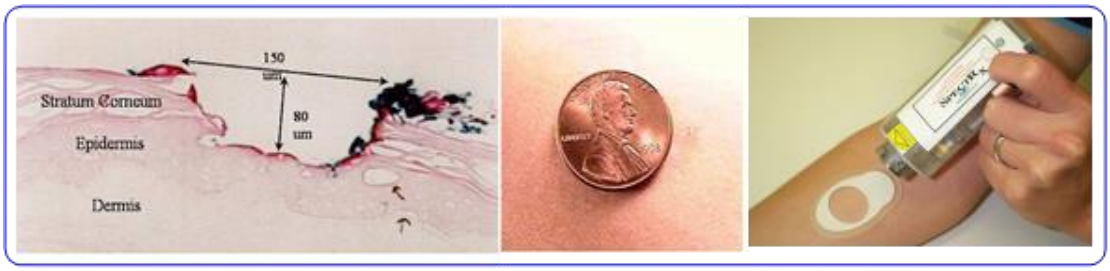

Figure 4: Left to right- Cross-section of micropore generated through laser ablation; Four pores relative to the size of a penny; Handheld laser source used to create micropores [51].

The diameter of the micropores is approximately equal to that of a human hair. The micropores only penetrate the stratum corneum and, hence, this procedure is virtually painless. ISF is drawn through these micropores continuously by application of a small amount of vacuum pressure (Figure 4). Harvesting of ISF using this set-up is reported at a rate of 10µl/hr. Coupled with an electrochemical detection system, Venugopal et al. [51] have reported the detection of cortisol using the same microporation set-up. Cortisol levels in ISF were found to be 3 – 4 times larger than that in saliva, which makes ISF an attractive biofluid for detection of cortisol. While this set-up may provide a means to access ISF for cortisol detection, the low harvesting rate (10µl/hr) would limit its applicability for obtaining instantaneous cortisol values in a point of care setting. Micromachined microneedles for transdermal application are another option to harvest ISF [52, 53]. Microneedles have been used extensively for transdermal drug delivery [53, 54]. Mukerjee et al., have reported the design, fabrication, and testing of a hollow microneedle array containing fluidic microchannels for the transdermal extraction of ISF from human skin [55]. Wang et al. [56] have also reported the fabrication of glass microneedles for ISF extraction for glucose monitoring. These approaches show promise for creating painless, minimally

invasive methods for extraction of ISF. Microneedles based transdermal ISF extraction may find suitable application in wearable biosensing system, where there is a critical need to continuously sample body fluids such as ISF at a low sampling rate. However, concerns regarding biocompatibility and biodegradation of the microneedles, protection from infection due to the usage of needles and other sterility issues will need to be carefully addressed for successful implementation.

2.2.1.3 Sweat

Sweat analysis is a well-established method for certain diagnosis of conditions such as cystic fibrosis and drug abuse. Sweat patches could be used as effective non-invasive tools to collect biofluids [57]. Cortisol in sweat has been measured by Russell et al., [58] with cortisol concentrations ranging from 141.7 ng/mL (daytime) to 8.16 ng/mL. It is hypothesized that there exists a strong correlation between cortisol levels in sweat and hair due the path that cortisol traverses from serum, sebum, and sweat to the hair. However, very little is known about cortisol in sweat, and several inherent drawbacks exist in obtaining reliable samples to measure cortisol in sweat accurately. Perspiration is dependent on several factors such as weather conditions (humidity, temperature), geographic conditions, physical activity levels, ambient temperature or conditioned temperature and genetic make of the patient. Development of microfluidic sweat collection cloth has been proposed as further advancement in sweat-based cortisol detection where sweat will be collected using the cloth patches and analyzed using an integrated device [59].

2.2.1.4 Blood

More than 90% of cortisol in blood is in an inactive state being bound to corticosteroid binding globulin (CBG) and serum albumin. Only 10% of total cortisol

is in a biologically active state to participate in cortisol-initiated processes. Typically assays for measuring cortisol in blood involves measuring the total cortisol (bound + free) and then the active fraction, called the Cortisol Free Index (CFI) is deduced using the Coolen's equation [60]. Blood sampling for cortisol detection has been the oldest form of biofluid sampling. The nominal value for cortisol in blood varies from 25µg/dL (9AM) to 2µg/dL (midnight). Blood, however, is afflicted by many drawbacks, which has made it a last choice sampling fluid. Sampling blood required attention from medical staff and specialized, sterile equipment with an ever-existing concern for infections. While cortisol is an unstable molecule at room temperature, its presence in plasma requires special handling and storage condition as it is considered a biohazard. Since sampling blood requires puncture of veins, a painful procedure, the stress experienced by patients prior to and during sampling may elevate cortisol levels [26]. Although the typical response time for cortisol spiking is 10 to 15 minutes in humans, prior knowledge of venipuncture can initiate the stress response induced cortisol spiking. Also, the costs associated with staff, equipment, handling, and storage makes blood-based assay a shunned option.

2.2.1.5 Saliva

Over the last few years, saliva has gained considerable attention as a biofluid for analysis and detection of cortisol concentrations. This has come about mainly due to inherent advantages associated with saliva. First and foremost, a well-documented and studied the strong correlation between salivary and blood cortisol levels [61, 62]. Also, of high importance is the fact that cortisol in saliva exists entirely in the free state unlike in blood (90% bound), resulting in detection of the relevant (biologically active) form of cortisol. This is mainly due to the filtering of the CBG and Albumin-bound cortisol during the capillary exchange and other intracellular mechanisms at the

salivary ducts. Harvesting sample for analysis is almost entirely non-invasive with little or no discomfort to the specimen providing the sample [63]. The last few years have seen the establishment of standardized procedures for the collection of saliva, which has led to lesser variability in analyzed results. The samples can be harvested with minimum efforts that have facilitated patients to collect their own samples at home. The advantages associated with salivary techniques have led to saliva being on the verge of becoming the preferred source of body fluid for detection of cortisol. Also, the ease of sample collection, handling, and storage has heightened its prospects for applying in point of care sensors for real-time and continuous detection of cortisol.

However, there are certain aspects that may adversely affect the prospects of saliva-based cortisol detection. Since only the active component of cortisol (free cortisol) is present in saliva, the concentration of cortisol is much lower than that of blood. Moreover, the room temperature instability of salivary cortisol possesses the problem of storage during on-site detection, the nominal values for cortisol in saliva during the diurnal cycle varies from 0.5µg/dL to 0.05 µg/dL, which requires high sensitivity assays with low detection limits for efficient detection of cortisol concentration in saliva. Strict compliance with the standard operating procedure for saliva collection can lead to erroneous results. Sometimes, the presence of blood due to oral lesions may lead to elevated levels of cortisol and cause erroneous results. Salivary cortisol assays have been reported in the literature extensively for characterizing the circadian rhythm [64], Cushing's syndrome [65], Addison's disease [66], adrenal abnormalities [67] and stress-related disorders [68].

The detailed explanation of cortisol detection in various bio-fluids using different techniques with recent advancements with further research & development at POC detection is explained in the next section.

2.2.2 Different methods of Cortisol detection

Among the label-free technologies for biomarkers detection, salivary electrochemical immunosensing has emerged as the most promising technologies for POC cortisol detection systems. Electrochemical immunosensing is based on the principle of measuring the changes in electrical properties of a conductive material due to the adsorption of an analyte on the surface functionalized with antibodies [8, 69, 70]. The electrical change is attributed to the change in the concentration of the electroactive redox species at the electrode. The mature processing capability of the micro-fabrication industry has allowed the building of microelectrodes that are highly sensitive and provide low detection limits. The simplicity of electronic circuitry for electrochemical detection and cheap volume manufacturing has driven efforts to bring electrochemical immunosensing up to speed with other immunosensing techniques [71, 72].

In recent years there have been many reports of electrochemical sensing of cortisol using electrochemical impedance spectroscopy [73] and cyclic voltammetry (CV) [74] based electrochemical cortisol immunosensor. Immunosensor has also been integrated with microfluidic systems for POC sensing [74, 75]. These reports demonstrate a workable cortisol sensing system using commercial cortisol sample. However, their application in the field is still lacking.

2.2.3 Optical/ radiological immunosensing

Amongst one of the first biosensing techniques, RIA holds a special place. It is an in vitro assay measuring accurately, the amount of antigen presents in a sample. The technique was introduced by Berson and Yalow in the ’60s [76]. They performed

an assay to determine the concentration of insulin in plasma. The technique relies on the specificity of antibodies to antigens. A chemical label is tagged to either an antigen whose concentration needs to be detected or to the antibodies. The label reacts as a part of the assay and produces a signal that can be measured in the solution. In the case of RIA, such a label is a radioactive compound, the activity of which is measured using a radiation counter. Other labels cause a change in color/ fluorescence of the solution. This change is dependent on the concentration of the target to be detected. These techniques are standardized and available commercially [16, 32, 33, 35-39]. They show good sensitivity in the order of a few picograms in a solution. However, the accompanying instrumentation, elaborate sample procedure and extensive labor involved in processing the samples make it a costly affair. Among all the available labeled immunoassays, ELISA has been found to be the most sensitive and versatile and today, is considered the gold standard in protein concentration determination. ELISA is typically performed in a sandwich format, where an enzymatic substrate is added to the secondary antibody to amplify the colorimetric or fluorescent signal, thereby providing high sensitivity. Detection of cortisol using ELISA is used widely [12, 41] and often has been the technique used to validate results obtained from newer techniques being developed for cortisol detection [42]. Manenschijn et al., collected hair samples of 195 healthy individuals, 9 hypercortisolemic and one hypocortisolemic patient to estimate cortisol using a salivary ELISA kit [40]. A positive correlation between hair cortisol and both waist circumference ($r = 0.392$, $p = 0.007$) and waist-to-hip ratio (WHR) ($r = 0.425$, $p = 0.003$) was found and no correlations were found between hair cortisol levels and blood pressure or age. While ELISA is the most widely used technique in research

labs and industry, the method is limited by the need for large sample and reagent volumes and complexity arising from multiple assay steps and large incubation times.

2.3 Electrochemical Immunosensors

Among the immunosensors, electrochemical immunosensors have been explored for detection of cortisol in recent times. In electrochemistry, the chemical changes can be related to the flow of electrons that give rise to oxidative or reductive species. Electrochemical immunosensing is based on the principle of measuring the changes in electrical properties of a conductive material due to the adsorption of an analyte on the surface functionalized with antibodies [8, 69, 77].

The change in the electrical charge due to the change in concentration of electro-active species at the electrode surface caused by the change of analyte concentration is utilized for sensing. The processing capabilities of the microelectronics industry have allowed fabrication of microelectrodes that provide high sensitivity and low detection limits.

The use of electrochemical immunosensing has advantages over conventional techniques. Fast response times (from sampling to result), ease of fabrication, bulk manufacture ability, small sample volumes and miniaturized supporting electronics makes them a suitable candidate for Point of care detection of cortisol. [8, 78]. Sun et al., reported cortisol antibodies immobilized on micro-fabricated Au electrodes for immune-electrochemical sensing using alkaline phosphatase (AP) enzyme for determination of salivary cortisol [79]. During the biochemical reaction, *p*-nitrophenol (*p*NP) generated via reaction between AP enzyme attached to the cortisol and antibodies in *p*-nitrophenyl phosphate (*p*NPP) solution *p*NP was detected using CV at

room temperature (Fig. 11). The fabricated immunosensor exhibited a detection limit of cortisol in saliva as 0.27 ng/mL with an incubation time of 10 min.

Dithiobis (succinimidyl propionate) (DTSP) self-assembled monolayer (SAM) modified interdigitated μ-electrodes (IDEs) were used for the immobilization of Cortisol specific monoclonal antibody (C-Mab) to detect cortisol using electrochemical impedance (EIS) technique [80]. EIS immunosensor exhibited the sensitivity of 2.855 kΩ/(pg/mL) and found to detect cortisol in the range of 0.36 pg/mL to 0.36 ng/mL nM in saliva. This group has further used the same immunosensing strategy to detect cortisol in ISF in vitro [73, 81]. EA/C-Mab/DTSP/Au based biosensor can accurately detect cortisol in the range of 0.36 pg/mL to 0.36 ng/mL with the detection limit of 0.36 pg/mL. The performance of this EIS based immunosensor was validated using ELISA. Authors purposed the feasibility of using impedance-based biosensor as a disposable cortisol detector, capable of working with complex bodily fluids (e.g., saliva and ISF) at point-of-care.

2.3.1 Cyclic Voltammetry

One of the most common electrochemical tools that are used for such biosensing is CV [82]. It is a powerful technique that is used for analysis of oxidized and reduced species. Using an electrochemical cell that typically comprises of working, reference and counter electrode, antibodies and antigen binding is estimated as a function of the redox current in the presence of a conducting media. For taking a CV scan, a varying potential is applied to a 3-electrode system and the response current measured. The positive peak in the voltammogram is known as the oxidation peak and the negative peak is the reduction peak. The oxidation and reduction responses can be explained using the Nernst equations. This equation relates the

potential of an electrochemical cell (E) to the standard potential of a species (E0) and the relative activities16 of the oxidized (Ox) and reduced (Red) analyte in the system at equilibrium.

$$E = E^0 + \frac{RT}{nF} \ln \frac{(Ox)}{(Red)} \qquad \text{Equation 1}$$

Where R is the universal gas constant, F is the Faraday's constant, n is the number of electrons and T is the temperature.

While looking at a one-electron reduction system, the Nernst equation can be utilized in such a way as to replace the activities with their concentrations (more easily determined by experimentation). The standard potential can be substituted with formal potential $E^{0'}$ and n is equal to one.

$$E = E^{0\prime} + 2.3026 \frac{RT}{F} log_{10} \frac{[Fc+]}{[Fc]} \qquad \text{Equation 2}$$

This equation is an excellent model to predict the change in the system with respect to change in the concentration of the chemical species or the electrode potential. Most of the CV system utilizes a working electrode, a reference electrode and a counter electrode in an electrochemical cell. One can link the change in oxidation and reduction potentials to the electron transfer at the surface of the working electrode (Figure 5). This phenomenon can be utilized for biosensing where the surface of the electrode is functionalized with detecting molecules not limited to antibody-enzyme, and aptamers.

The interactions between the antigen and the detecting moieties result in a change in the redox current [82]. We explored interdigitated microelectrodes of gold as a substrate for a CV based immunosensor. The developed sensor was used to detect the various concentrations of cortisol. The above sensor was further used to detect cortisol in human saliva samples and was benchmarked against optical immunosensing technique (ELISA) [83].

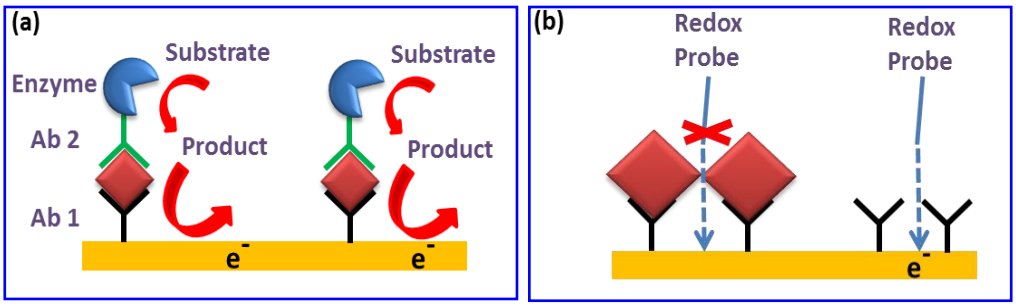

Figure 5: Schematic of strategies used for electrochemical immunosensing

2.4 Limitations of Electrochemical Immunosensors

Electrochemical immunosensors utilize biomolecules (antibodies and enzymes) for the detection of analytes. However, this causes the sensor to be unstable at room temperature and a constant need for storing available sensors in the refrigerator. This prevents Point of Care application of electrochemical sensors.

Though label-free sensing techniques have been devised, they have not seen production yet. Another important factor while considering electrochemical sensing is the complexity of accompanying instrumentation. There have been efforts for making cheap and portable electronics that can fully realize the potential of electrochemical sensors for point of care.

2.5 Label-free Detection

To overcome the tedious processes, cost and shortcomings of the current detection methods, the focus has shifted to developing label-free immunosensing techniques with high sensitivity, lower detection limits and broader detection range [84]. One such technique that demonstrates label-free detection is based on Surface Plasma Resonance (SPR) [44].

SPR works on the principle of oscillation of valence electrons in a conducting substrate irradiated with light. SPR is highly sensitive to adsorption of molecules onto the substrate, where the resonance curves shift to higher wavelengths with adsorption of molecules onto the surface. SPR has shown promise as a method to quantitatively measure the capture of the analyte on substrates coated with antibodies. The associated detection optics and electronic for SPR measurement can be reduced to miniaturized form factors and has hence attracted efforts to create point-of-care immunosensors [43]. More recently, detection of cortisol in saliva [45] and other biofluids using SPR has been reported in the literature. On the same lines as SPR, another technique gaining ground for immune-sensing is based on the resonance property of quartz crystal microbalance (QCM) [46]. Cortisol antibody was layered onto the Au gold electrodes of a 10 MHz piezoelectric crystal to fabricate a piezoelectric crystal immunosensor for the detection of cortisol [85]. Piezoelectric crystal was pre-cleaned, protein-A and immunosensors detected cortisol at concentration range 3.628 μg/mL. The proposed sensor includes elements of simplicity, short analysis time, cost-effectiveness and selectivity. However, there is a scope for improvement with respect to its detection limit.

Label-free sensing has also been demonstrated using Nanomaterials while doing CV measurements. Kaushik et all used Nanoparticles of Ag/AgO_x core-shell nanoparticles in a polymer matrix of Polyaniline to make an immunosensor for cortisol. This work tries to explore the fabrication and utilization of Copper oxide nanoparticles on a screen-printed carbon electrode for sensing purpose. The modified SPCE was used for making a cortisol immunosensor to demonstrate Label-free sensing. The fabricated sensor showed a sensitivity of 421 uA/M and had a limit of detection as 6.6 nM cortisol.

2.6 Reusable Sensors- MIPs

In nature, antibodies have receptor sites that can bind to specific antigens and function as an ultimate sensor. However, replication of such a sensing system by artificial means has been quite challenging. The use of antibodies in biosensors has been indispensable due to their sensitive and selective properties. However, their production requires significant effort and cost. This system can be mimicked using molecularly imprinted polymers at much less cost and effort. MIP are synthetic polymers having highly specific recognition sites selective towards the target analyte. The MIP-based biomimetic sensors have a considerable impact owing to their potential application. They have advantages such as low cost, easy storage, and applicability in harsh conditions. They also have long lifetimes as compared to enzymes, antibodies, and proteins which require temperature and pH-controlled environments for storage. Another attractive feature of MIP based biosensor is its reversible binding with the target analyte which makes it highly reusable where frequent/continuous monitoring is required. MIPs can be described as analogs to the antibody-antigen mechanism. Their operation can be best described as a lock and key

mechanism that allows them to bind to target molecules selectively. The schematic below (Figure 6) shows the fabrication scheme of a cortisol specific MIP sensor. Several production methods such as bulk polymerization, precipitation polymerization, and emulsion have been demonstrated for the fabrication of MIPs. However, controlled production and transfer of MIP to a transducer surface have been challenging. This research will explore an electrochemical technique for the manufacture of MIP. Electrochemical MIP fabrication enables control over thickness, morphology, and reproducibility.

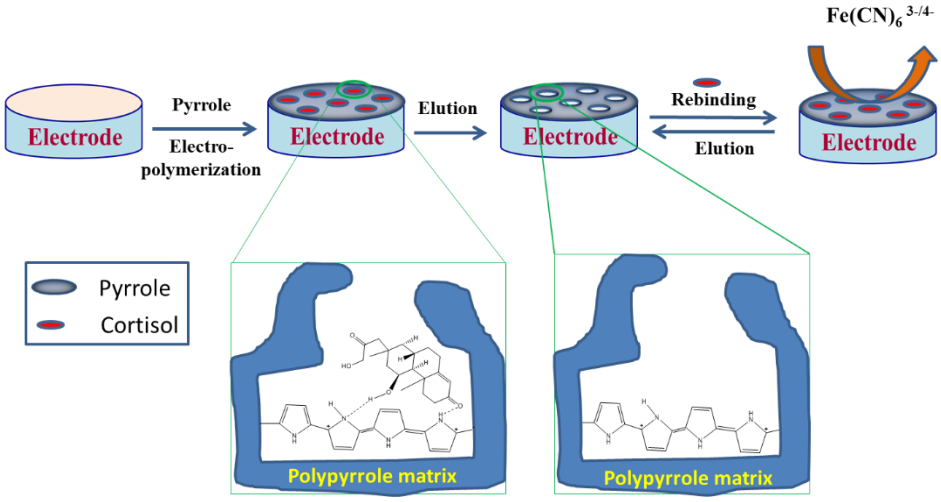

Figure 6: Schematic for fabrication of cortisol MIP biosensor on SPCE surface.

2.7 The Field Effect Transistor

The FET was first proposed in the 1930s by Lilienfield [86] and developed for practical use in 1960 by Kahng and Atalla [87]. The rapid growth and expansion of the modern semiconductor industry can be attributed to the development of metal-oxide-semiconductor-field-effect transistor (MOSFET) or the FET as it is known in short. The FET has been an essential device for VLSI circuits and has been used in

logic/ semiconductor memory devices as well. This has primarily been enabled due to the miniaturization, cost reduction, and longer lifetimes offered by switching to ICs as compared to Vacuum tubes. The integration of millions of complex active and passive devices on a single silicon wafer has enabled a new and wide array of applications. Ushering in a technological revolution that has changed the way we communicate control and process data in a matter of few decades [88].

The basic structure of a FET is composed of a semiconducting channel between two conductor pads known as source and drain. The channel is sandwiched between the substrate and an overlying oxide layer. The oxide layer is coated with a conductive layer to form what is known as a gate (Figure 7).

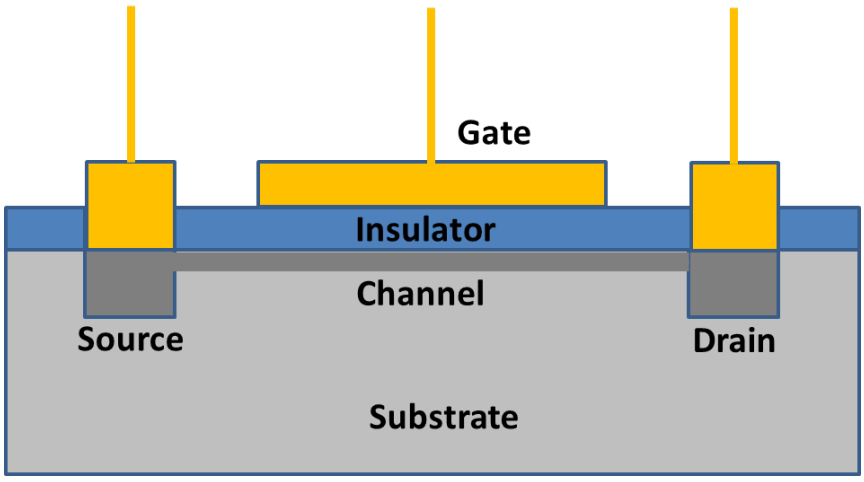

Figure 7: A typical top gated Si thin film transistor

Application of voltage on the gate controls the amount of current that flows between the source and drain pads (due to the application of a source to drain voltage). This mechanism allows the FET to be used as a variable resistor and a switch. The growth of semiconductor industry led to the growth of thin film fabrication industry as well, leading to the facilitation of the development of FETs made entirely of thin films of metals/ semiconductors/ insulators. These thin film transistors (TFTs) were first produced in 1965 [89]. They were made for scanning

image sensors and were CdSe TFTs. The fabrication of TFTs on insulating substrates helped in reduction of parasitic losses that were associated with the silicon substrate. The patent by Edgar [86] describes the first thin film transistor. Under the normal operating condition, a typical TFT, by the application of a voltage bias to the gate electrode induces the accumulation of the electrons (in case of an n-type semiconductor) in the semiconductor channel.

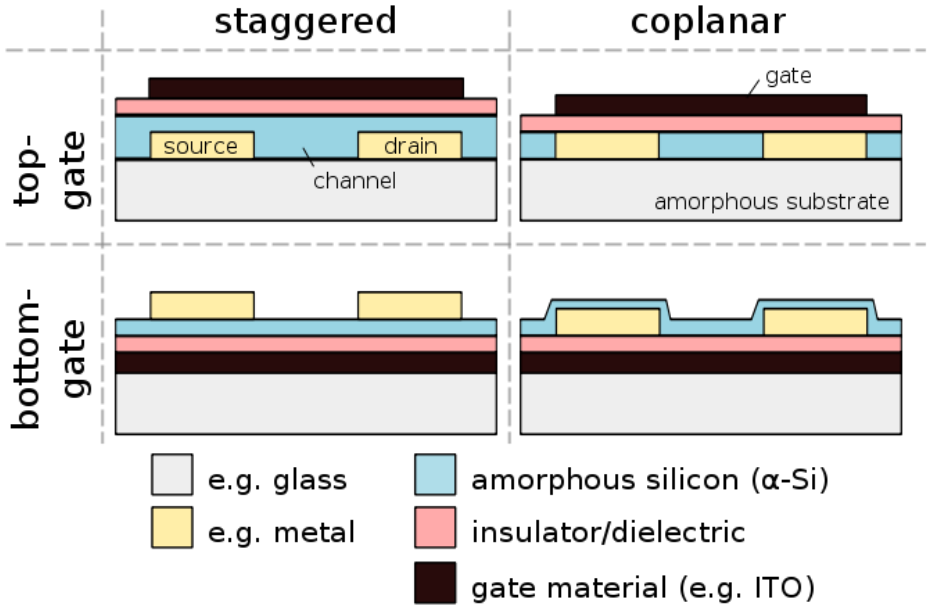

Figure 8: Different configurations of a TFT [1]

A simultaneous application of a Positive voltage to the drain results in a flow of electrons from source to drain. The initial development of MOSFETs was much rapid than TFTs, however, with the emerging technology and applications of TFTs towards thin, flexible, large area electronics, sensing, smart labels, lighting, etc. this field has rapidly caught up. Several configurations have been reported and developed for TFT applications. These can be classified according to the device structure. There are four types of FET structure – coplanar (top gated), coplanar (bottom gated), staggered (top gated) and inverted staggered (bottom gated). The position of the gate, source and drain electrodes with respect to the insulator and semiconductor layer

(Figure 8) defines these differences. In a coplanar structure, all three electrodes are on one side of the semiconductor layer. However, in a staggered structure, the gate electrode is on one side of the semiconducting layer while the source and drain electrodes are on the other side [90].

2.7.1 ISFET as a biosensor

Another form of FET that is commonly used in sensing applications is Ion Sensitive FET or ISFET. First invented by Bergveld [91]. It is used for measuring ion concentrations in a solution. The figure below shows the typical ISFET configuration. It consists of a source and drain pads with a semiconducting channel encompassing them. The channel is typically covered with a gate insulator such as SiO_2, Si_3N_4, Al_2O_3, etc. The channel and the gate insulator are then surrounded with a well to enclose the solution under test. The gating action is achieved with the help of an external electrode suspended in the solution. The solution acts as a gate electrode. Presence of an ion layer between the substrate and the oxide layer results in a voltage between them. The hydrolysis of OH bonds on the surface of the gate insulator varies as the pH of the solution varies. Resulting in a change in the drain current. The external electrode provides a reference potential against which the drain current can be calibrated. Today ISFET based sensing devices have been used for various applications. Rothberg et al. have shown an IC device for DNA Sequencing. The device contains 1.2 million wells with ISFETs in them. They were able to sequence three bacterial and a human genome using this device. Their device showed proton sensitivity of 58mV/pH. Kiani Sasakia et al. [92] reported Graphene-based electrolyte gated FET. They claimed that a label-free biosensor to measure enzyme activity was possible using ISFETs, their device had sample volumes as low as 20 microliters.

EGFET have been claimed to be an extension of traditional ISFET architecture by Viera et al. [93]. They claim to have improved upon the ISFET design by isolating the chemical environment from the FET and used this scheme to detect Glucose with a detection limit of 0.027mM. Still, in another study, the sensor chips have been integrated with a microfluidic channel to monitor potassium and pH levels [94].

2.7.2 Extended Gate FET

This configuration of FET is widely used for the fabrication of biosensors. It is derived from the ISFET configuration of the FET [95].

In this configuration, the gate of the FET is connected externally to a functionalized substrate. The substrate is often bound by a well to hold a specific amount of liquid. An external electrode is dipped in the liquid. This electrode is connected to an external supply that provides a voltage to the gate through the externally connected substrate. The substrate serves as an ion sensitive site. This configuration allows the active FET device to be isolated from the harsh chemical environment and allows the device to function stably. Also, this configuration can lead to a simple plug and play architecture enabling the user to discard the sensing substrate when it is saturated or becomes nonfunctional.

This research will explore the use of MIP based sensing substrate along with a commercially available FET for use in an EGFET configuration. The use of cortisol specific MIP on an SPCE will allow the sensor to provide direct sensor readout (in the form of drain current concerning cortisol concentration). The above configuration will also address the challenges of label-free and repeatable sensing.

2.8 Conclusion

Several techniques allow sensitive detection of cortisol in a sample. However, most of the state of art techniques suffer from limitations that prevent their widespread use. This chapter serves to introduce the reader to some of the common cortisol detection methods and them with their main advantages and limitations. Some techniques are introduced in the latter part of the chapter (CV, MIP, EGFET) that can address the issues of the earlier recorded methods. The subsequent chapter will record the developments and validation of a thin film based electrochemical immunosensor for the detection of cortisol.

Chapter 3: ELECTROCHEMICAL IMMUNOSENSING OF SALIVARY CORTISOL.

3.1 Abstract

This chapter will discuss the development and validation of Au thin film based electrochemical immunosensors for detection of cortisol. A self-assembled monolayer of DTSP was functionalized on a thin film Au based IDEs. Cortisol specific antibodies were then immobilized, and the non-binding sites were blocked using EA on the SAM layer. The sensors were characterized at each layer using CV. The fabricated immunosensors were characterized using different cortisol concentration. These sensors were then validated by detecting cortisol in human saliva samples and comparing the results with standard ELISA process. The fabricated sensors had a detection range from 10 pg/mL to 100 ng/mL and a detection limit of 10 pg/mL. These sensors performed well as compared to the ELISA results.

3.2 Introduction

Among the label-free technologies for biomarkers detection, salivary electrochemical immunosensing has emerged as the most promising technologies for POC cortisol detection systems. The cortisol concentration in saliva is lower than total serum cortisol by a factor of 10, and the correlation between serum and salivary markers is substantial [77] [96]. Salivary cortisol concentrations accurately reflect the serum concentration at various times of day (morning, evening and around midnight) [37]. Moreover, the salivary cortisol contains only free cortisol making protocol easier [25]. Electrochemical immunosensing is based on the principle of measuring the changes in electrical properties of a conductive material due to the adsorption of an

analyte on the surface functionalized with antibodies [4, 8, 69]. The electrical change is attributed to the change in the concentration of the electroactive redox species at the electrode. The mature processing capability of the micro-fabrication industry has allowed the building of microelectrodes that are highly sensitive and provide low detection limits. The simplicity of electronic circuitry for electrochemical detection and low-cost volume manufacturing has driven efforts to bring electrochemical immunosensing up to speed with other immunosensing techniques [71, 72].

In recent years there have been many reports of electrochemical sensing of cortisol using electrochemical impedance spectroscopy [80] and CV [97]. SAM [73, 81], Au nanowires [98], Au-PANI nanocomposite [99], and AgO@Ag-PANI nanocomposite[4] based electrochemical cortisol immunosensor were reported. Immunosensor were also integrated with microfluidic systems for POC sensing [74, 75].

These reports demonstrate a workable cortisol sensing system using commercial cortisol sample. However, their application for field use is not explored yet. In this paper, we report on the ability of the sensor to detect cortisol in saliva samples of farm workers. IDEs functionalized with DTSP-SAM followed by covalent immobilization of Anti-Cab were used to detect the concentration of cortisol present in the saliva samples. The results obtained from the electrochemical detection were validated through ELISA protocol currently in use.

This work aimed to establish a cortisol sensing protocol using saliva samples collected from human subjects and its validation with the standard ELISA technique, which was not reported earlier. This protocol will be used to understand the behavioral change of farm workers on exposure to pesticides.

3.3 Materials and Methods

3.3.1 Saliva sample Collection

Saliva samples were derived from a biologically-relevant sample of two female agricultural workers of reproductive age who engage in shift-work. Evidence indicates that cortisol levels vary significantly among female shift workers [70, 100-102] and that stress associated with agricultural work is associated with cortisol level variation [103-107], as is exposure to cortisol [108]. Farmworkers were 24 and 39 years of age and were recruited based on the following criteria: be at least 18 years of age, and work in agricultural fieldwork, work early morning shifts, and have exposure to pesticides. These samples were drawn from another study measuring the effects of a behavioral study to assess pesticide exposures and stress response. Recruitment, analyses and other procedures for this study were approved by the Human Subjects Boards at the Penn State University and the Florida International University.

Samples were collected at awakening, before their first work break, at their lunch break (before eating), upon arrival from work and before bedtime. Samples were stored by participants' at freezing (-20^{o}C) until the project team transferred them and stored at -80^{o}C in the field lab. The standard protocol requires the storage saliva samples to at -20^{o}C or lower for the preservation of natural and biological properties over extended periods and during transportation. Saliva samples were collected by each farmworker using a Salivette (Sarstedt Inc., Rommelsdorf, Germany), which consists of a small cotton roll inside a centrifuge tube. Each farmworker took the Salivette home after receiving written and oral instruction on how to collect saliva. Participants collected a saliva sample by chewing on the cotton roll for 60 seconds.

The collected saliva samples were stored at -20^{o}C to maintain its biological characteristics. Before the sensing, the saliva samples were brought to room temperature and centrifuged at 3500 rpm for 15 min to extract saliva from salivettes. The separated saliva was then pipetted out and kept at -20^{o}C when not in use. These samples were further used to detect cortisol using electrochemical immunosensors and ELISA.

3.3.2 Immunosensor Fabrication

3.3.2.1 Electrochemical Cleaning of IDEs

The IDEs (chamber volume ~5 µL, electrode width, and electrode gap 10 µm) were procured from Micrux Technologies (Fig 1). The IDEs were electrochemically cleaned prior to DTSP-SAM modification through performing CV, Autolab Potentiostat/Galvanostat (Eco Chemie, Netherlands)] using 5 µL of 0.1 M H_2SO_4 as a function of varying scanning cycles at a scan rate of 50 mV/S. The parameters of the IDE cleaning process were optimized using CV techniques in 5 µL PBS (pH 7.4) containing 5 mM $K_4[Fe(CN)_6]^{3-/4-}$ at a scan rate of 50 mV/s (Fig 2A). The magnitude of the current response obtained was found to increase with increasing cleaning scanning cycles (5-10). The higher current response was observed in the case of IDEs cleaned using 9 cycles and higher. However, the electrode started to etch after 9 scanning cycles. Thus, IDEs were cleaned using 7 scanning cycles in 5 µL, 0.1M H_2SO_4. All IDEs were cleaned using the same process and exhibited identical current response (within ~ 2% variation, Fig 2B) without morphological damage.

3.3.2.2 Fabrication of DTSP-SAM onto IDEs and Electrochemical Cortisol Immunosensing

DTSP, Sodium Borohydride ($NaBH_4$), monoclonal anti-cortisol antibody (Anti-C_{ab}), Cortisol and all other chemicals were purchased from Sigma-Aldrich and were used without any further purification. For fabrication of DTSP-SAM, the IDEs were immersed in 2 mg/mL solution of DTSP in acetone for 2h. During the SAM fabrication, DTSP was reduced using $NaBH_4$ (10 mg/mL in DI water). After SAM modification, all DTSP/IDEs were rinsed with acetone and then by DI water to remove unbound DTSP particles. Anti-C_{ab} (5 µL) was covalently immobilized via a facile reaction between the amino group of antibodies and reactive succinimidyl group of the DTSP SAM surface. The DTSP-SAM/IDE were incubated with Anit-C_{ab} for 2hrs followed by carefully washing with PBS (pH 7.4, 10 mM) to remove any unbound molecules [74]. Phosphate buffer saline (PBS) solution (10mM, pH 7.4) was prepared by dissolving 1 PBS tablet in 200 mL of de-ionized (DI) water and used to prepare the Anti-C_{ab} (1mg/mL) and cortisol solutions. The non-binding sites of Anti-C_{ab}/DTSP/IDEs bioelectrode were blocked using 5 µL of EA. The incubation time was 10 min. EA/Anti-C_{ab}/DTSP/IDEs were washed with PBS and stored in a refrigerator at 4^{o}C when not in use. The fabrication of SAM onto IDEs and immunosensor development is shown in Figure 1A.

3.3.2.3 Measurement of Cortisol

The fabricated EA/Anti-C_{ab}/DTSP-SAM/IDEs immunoelectrodes were used to detect the various concentration of cortisol using CV techniques in 5µL PBS (pH 7.4) containing 5 mM $[Fe\ (CN)_6]^{3-/4-}$ as redox moieties. The pH of 7.4 was chosen for electrochemical response studies of immunosensor, as this is the recommended pH for biomolecules to presume higher biological activity [73]. The electrochemical and

immuno-chemical reaction on IDEs surface is shown Figure 1B. The fabricated immunoelectrodes were used to detect cortisol in saliva samples of two specimens collected at different time intervals. 5 µl of saliva sample was placed on the electrochemical immunosensor. The saliva sample was incubated for 30 minutes on the sensor to ensure proper binding. Immunoelectrodes were then washed using PBS ~10 mL to remove unbound saliva.

Cortisol Enzyme Immunoassay Kit was procured form Arbor Assays, MI and a standard protocol were adopted to detect saliva cortisol. In Brief, 50 µl of 4X diluted saliva was used for the detection of cortisol.

3.4 Results and Discussions

3.4.1 Electrochemical Characterization

CV technique was used to optimize the step-wise fabrication of electrochemical immunosensor which is utilized to detect cortisol. Figure 9 shows the results of the CV studies for IDEs (a), DTSP-SAM/IDEs (b), Anti-Cab/DTSP-SAM/IDEs immunoelectrode (c) and EA/Anti-Cab/DTSP-SAM/IDEs (d) in 5µL PBS (pH 7.4) containing 5 mM Fe(II)/Fe(III) at 50 mV/s in a potential range from -0.6 to 0.6 V. CV of IDEs exhibited a well-defined oxidation and reduction peaks of redox moieties present in the electrolyte. The magnitude of electrochemical current response decreases after modifying IDEs by DTSP-SAM. This suggests that SAM hinders the electron transport from Fe(II)/Fe(III) to IDEs due to strong Thiol bonding between DTSP-SAM and Au of IDEs which indicates successful DTSP-SAM fabrication. After the immobilization of Anti-$C_{ab,}$ the magnitude of current from DTSP-SAM/IDEs further decreases due to the covalent binding between Anti-C_{ab} and DTSP

via amide bond formation. The magnitude of current from EA/Anti-C_{ab}/DTSP-SAM/IDEs immunoelectrode was lower than Anti-C_{ab}/DTSP-SAM/IDEs immunoelectrode. This reduction of current is a clear indication that the non-conducting EA blocks the non-binding sites on immunoelectrode and reduce the electron transport from the electrolyte to IDEs.

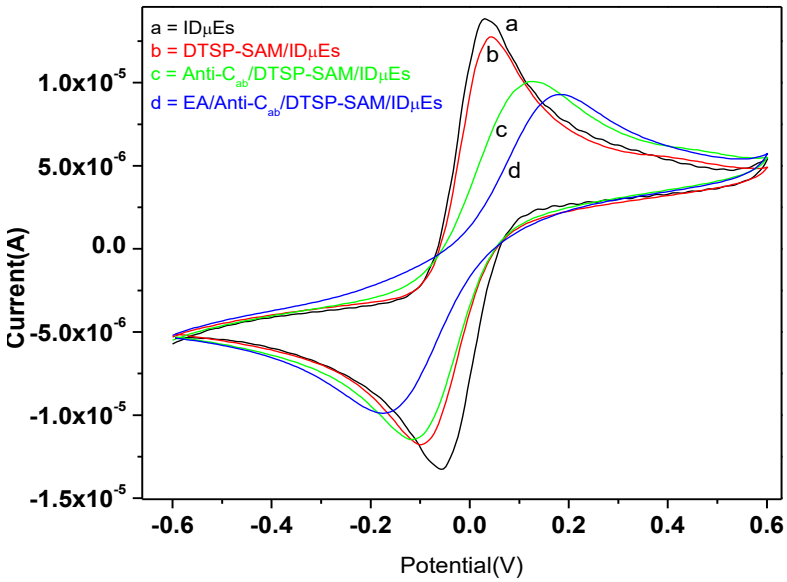

Figure 9: CV studies of IDEs (a) DTSP-SAM modified IDEs (b), Anti-Cab immobilized DTSP-SAM/IDEs electrode (c), and EA immobilized Anti-Cab/DTSP-SAM/IDEs immunoelectrode (d) using 5 µL PBS (pH 7.4) containing 5 mM [Fe(CV)6]3-/4- at 50 mV/s.

The CV studies of electrodes and immuno-electrodes have been performed as a function of scan rate (10-100 mV/s). A linear relationship between the magnitude of oxidation/reduction current and the square root of scan rate (data not shown) has been observed for all electrodes. This confirms that the assay is a linear-diffusion-driven process. It obeys Randles-Sevcik equation $\boldsymbol{i_p = (2.69x10^5)\, n^{2/3}ACD^{1/2}v^{1/2}}$, where n is the number of transferred electrons, A is the active area of the electrode, C is the bulk concentration of the redox species, D is the diffusion coefficient, and *v* is the scan rate[109]. The magnitude of the current response is also linearly related to differences

in potentials revealing the facile electron transport from the electrode to the IDE surface.

3.4.2 Electrochemical Response Studies of Cortisol

Electrochemical response studies of developed DTSP-SAM-modified IDEs immunosensor as a function of cortisol concentration (10 pg/mL to 100 ng/mL) was performed using CV technique in 5 µL PBS (pH 7.4) containing 5mM [Fe(CN)6]3-/4- with a potential range of -0.6 to 0.6 V at a scan rate of 0.5mV/s. Prior to electrochemical measurement, 5 µL of each cortisol sample was incubated on the immunosensor surface for 30 mins for complete binding of Anti-Cab with standard cortisol solution. A washing step using 30 µL of PBS was performed to remove the unbound cortisol from the sensor surface. All the CV measurements were performed in triplets. The result of CV studies indicates that the magnitude of current response decreases on the addition of cortisol due to the formation of insulating immuno-complex between Anti-Cab and cortisol which further inhibit the electron transport from the electrolyte to IDEs surface (Figure 10).

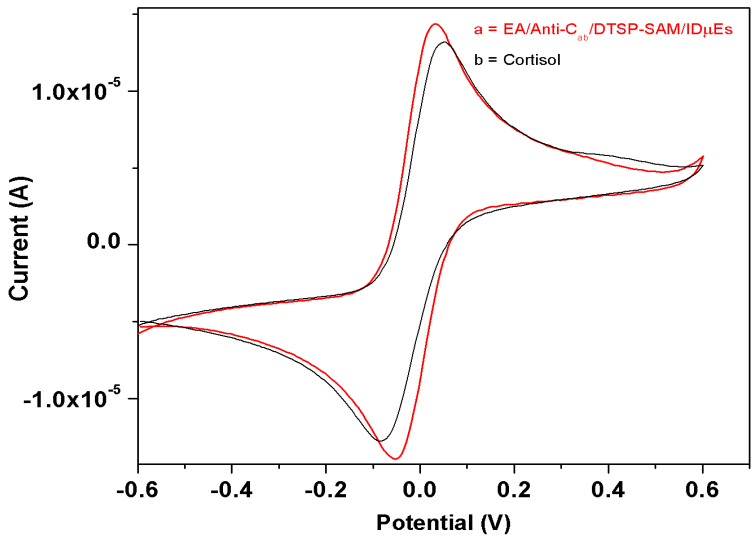

Figure 10: Electrochemical response of EA/Anti-C_{ab}/DTSP-SAM/IDEs immunoelectrode after addition of standard cortisol solution

A calibration curve (Figure 11) plotted between the magnitude of current response and logarithm of cortisol concentration was found to be linearly dependent and follow the equation **[Y = 1.5 × 10^{-5} + 6× 10^{-6} log (Cortisol concentration); R = 0.99].** The fabricated EA/Anti-C_{ab}/DTSP-SAM/IDEs immunosensor exhibited linearity in the range of 0.1 to 100 ng/mL, a detection limit of 10 pg/mL, a sensitivity of 6 μA/(pg/mL) with the regression coefficient of 0.99 and standard deviation of 0.6 μA.

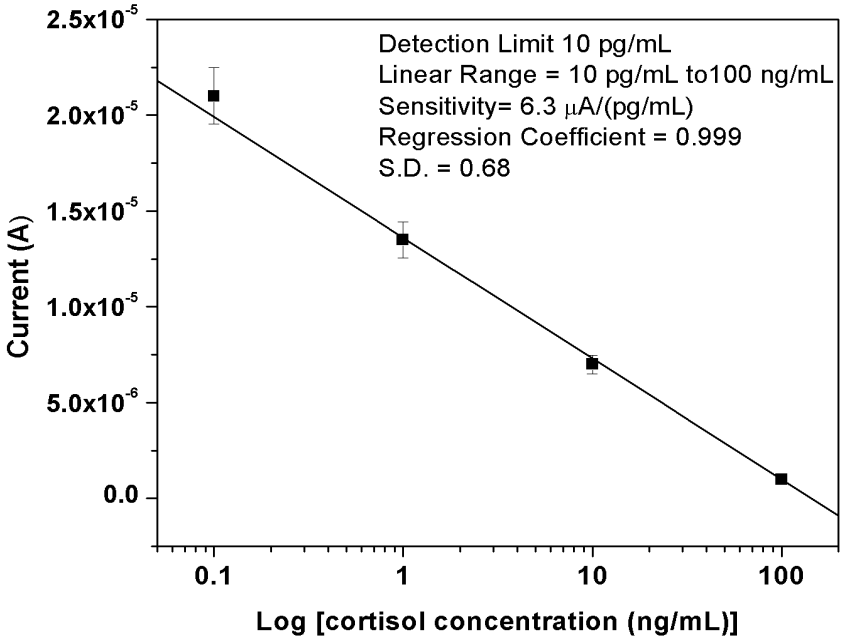

Figure 11: Calibration curve plotted between the magnitudes of current response obtained and logarithm of cortisol concentration (10 pg/mL to 100 ng/mL)

The confirmation of Anti-C_{ab} onto DTSP-SAM/IDEs electrode and its affinity with cortisol was understood through the association constant (*K*a, 3 × 10^{12} L mol^{-1}) between Anti-C_{ab} and cortisol, calculated using Lineweaver–Burke-like plot. *Ka* value greater than 10^{11} is accepted as a demonstration of high affinity [110]. Figure 12 shows the results of the CV studies of EA/Anti-C_{ab}/DTSP-SAM/IDEs immuno-electrode with respects to interferents such as PSA (100 pg/mL), NSE (100 pg/mL), EGFR (100 pg/mL) and BSA+Cortisol (100 pg/mL) with respect to cortisol (100 pg/mL). The interferents were dispensed on the immunosensor (EA/AntiC-

Abs/DTSP-SAM/μIDEs), and their electrochemical response was measured. The change in electrochemical response of the interferents was compared to that of the immunosensor, and it was found to be of the order of 1-2%. As compared to the change in the response of Cortisol signal, which was in the order of 13-14 %, the effects of interferents can be conveniently neglected. This demonstrates that the effect of interferents on EA/Anti-C_{ab}/DTSP-SAM/IDEs immunosensor is minimal and the antibodies are selective to the cortisol molecule. A CV study was also carried out to study the shelf life of the EA/Anti-C_{ab}/DTSP-SAM/IDEs immunoelectrode at intervals of 1 week (data not shown). The results of the study showed electrodes were stable for 28 days and beyond that time the electrochemical response reduced.

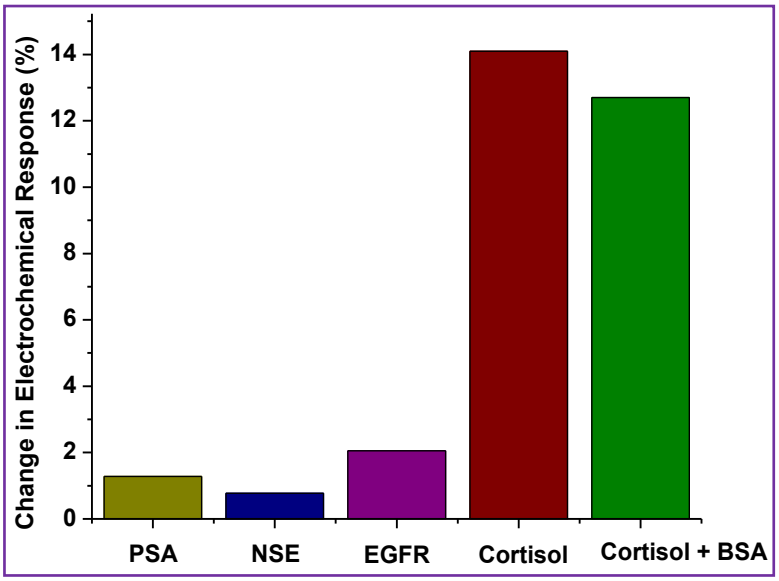

Figure 12: CV studies of EA/Anti-Cab/DTSP-SAM/IDEs immuno-electrode with respects to interferents such as PSA (100 pg/mL), NSE (100pg/mL), EGFR (100pg/mL) and BSA+Cortisol (100 pg/mL) with respect to cortisol (100 pg/mL)

3.4.3 Electrochemical Immunosensing of Saliva Cortisol

The IDEs based electrochemical immunosensor for cortisol sensing was utilized for testing of cortisol concentrations in saliva samples. 05 μL of the saliva sample was incubated on the electrochemical immunosensor for a period of 30 mins.

A washing step using 30 µL of PBS was performed to remove the saliva and any unbound cortisol from the sensor surface. 5 µl of Ferri Ferro solution was then used to perform CV. The electrochemical response studies of EA/Anti-C_{ab}/DTSP-SAM/IDEs immunoelectrodes carried out using CV in 5 µL of PBS containing 5mM Fe(II)/ Fe(III) as the redox probe at a scan rate of 50 mV/s. A very significant change in current response of immunosensor is observed on adding each saliva samples. Cortisol concentration was read using the calibration curve developed in electrochemical response studies (section 3.2). All samples were measured in triplicates, and the average was computed to validate with the ELISA technique as presented in Table 1 and Table 2.

Table 1: Cortisol Values as measured in saliva samples using the developed immunosensor

Sample	Saliva Collection Time and Date	Measured Electrochemical Response (µA)	Cortisol Concentration (Obtained Concn. x 2.2) (nMol/L)	Final Cortisol Concentration (ng/dL)
A	3/26/2013 (9:47AM)	7.16	3.63	131.78
	3/27/2013 (10:00AM)	7.38	3.81	138.37
	3/27/2013 (10:03PM)	12.23	0.90	32.94
	3/28/2013 (2:00AM)	8.89	1.818	65.89
B	3/26/2013 (10:13AM)	5.24	9.09	329.45
	3/27/2013 (1:42AM)	7.76	2.727	98.83
	3/27/2013 (10:13AM)	4.26	14.54	428.29
	3/28/2013 (3:02AM)	9.17	1.93	69.18

Table 2: Cortisol Values measured in saliva samples using Cortisol ELISA kit

Sample	Saliva Collection Time and Date	Measured Response (4x dilution) (%B/Bo)	Cortisol Concentration (pg/mL) 4X dilution	Final Cortisol Concentration (ng/dL)
A	3/26/2013 (9:47AM)	64.133	389	155.6
	3/27/2013 (10:00AM)	66.909	360	144
	3/27/2013 (10:03PM)	56.113	594	237.6
	3/28/2013 (2:00AM)	81.758	182	72.8
B	3/26/2013 (10:13AM)	41.705	995	398
	3/27/2013 (1:42AM)	99.559	125	50
	3/27/2013 (10:13AM)	40.956	1187	474.8
	3/28/2013 (3:02AM)	96.695	87	34.8

3.4.4 Detection of Salivary Cortisol using ELISA

Saliva samples from the same salivette were tested for cortisol using ELISA (Arbor Assays, MI). This was done to validate the results obtained from the electrochemical immunosensor. The protocol prescribed by the ELISA kit vendor was followed for cortisol detection in saliva samples. A calibration curve for cortisol measurement was established, the assay was performed in a 96-well titer plate consisting of six known standard cortisol concentrations (100, 200, 400, 800, 1600 and 3200 pg/mL) and the saliva samples. The calibration curve obtained from the measurement of the standard solutions is presented in Fig 6. Next, the saliva samples were diluted 1:4 in the provided assay buffer and 50 µL of saliva samples were utilized for each measurement. Saliva samples from each of the salivettes were tested in triplicate sets, and the average value was utilized to determine the final cortisol concentration and is presented in

Table 2. Figure 13 presents the comparison of results obtained for cortisol detection in saliva samples tested using the electrochemical immunosensor and ELISA. The trend observed for the electrochemical measurements were mostly consistent with that obtained from ELISA, except for the saliva sample from Specimen A [3/27/2013 (10:03pm)], in which the ELISA value was observed to be a high value at night time. This observed difference may be due to an experimental error of denatured saliva sample.

Since both methods have different transduction techniques to detect cortisol concentration, the results were in different magnitudes. To compare and correlate these responses, one was scaled to the other. All values of cortisol concentration obtained from the corresponding electrochemical calibration curve were adjusted by a factor of 2.2 to compare them with values obtained using ELISA assays. A good correlation was achieved between the two sets of data, thus enabling quantitative validation of the electrochemical immunosensing of Cortisol in a biologically relevant fluid such as saliva.

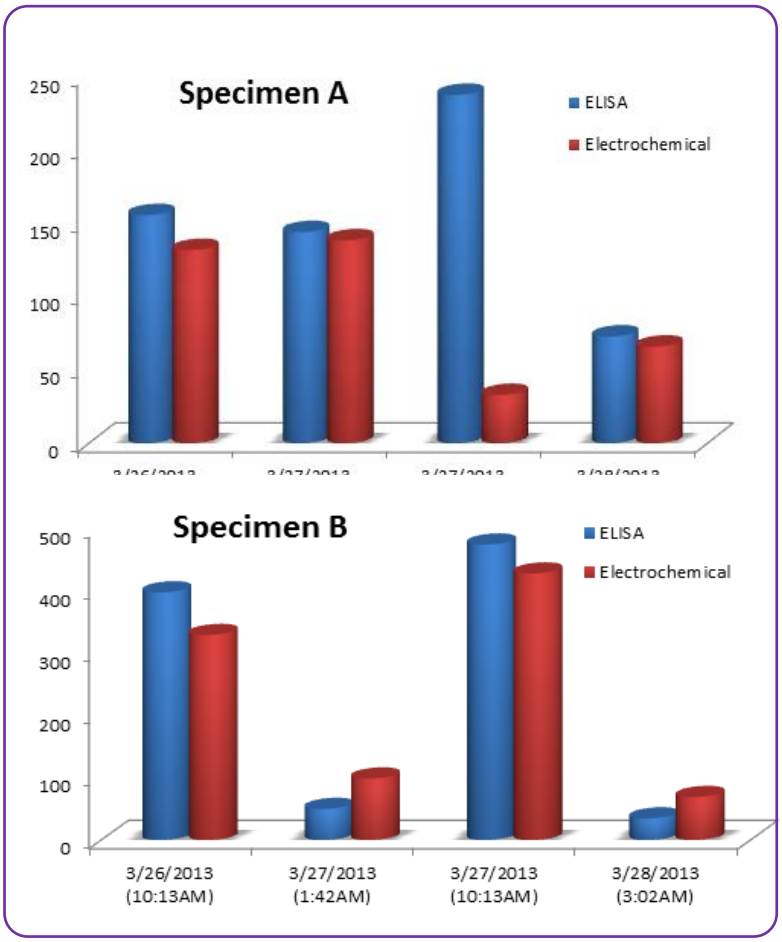

Figure 13: Comparison of cortisol concentration measured by ELISA and Electrochemical measurement on saliva samples from Specimen A & B at specific time intervals over three days.

3.5 Conclusions

The electrochemical immunosensing platform was utilized for a low-cost and label-free detection of cortisol via covalent immobilization of Anti-C_{ab} onto DTSP-SAM-modified IDEs (chamber volume 5 µL, electrode width 10 µm and electrode gap 10 µm). The fabricated electrochemical immunosensor exhibited a detection range from 10 pg/mL to 100 ng/mL, a detection limit of 10 pg/mL, and a sensitivity of 6 µA/(pg/mL) with the regression coefficient of 0.99. The sensor has been used for salivary cortisol detection in clinically relevant samples, and sensing performance has successfully validated using conventional ELISA method. The cortisol value in saliva samples of two specimens collected at different time intervals using DSTP-SAM/IDEs correlates (within 2-5%) with those obtained using ELISA. The developed sensor can reliably detect cortisol in saliva within a physiological range which can be used as a marker to understand various physical, behavioral and psychological variables which may affect the central nervous system.

Chapter 4: NANO ENABLED SIGNAL AMPLIFICATION IN SAM BASED ELECTROCHEMICAL CORTISOL IMMUNOSENSORS

4.1 Abstract

Electrochemical immunosensors function by measuring the changes in the electrical properties such as current, potential, conductance, capacitance, and impedance during an immunochemical reaction between antigen (target molecule) and the antibody [111, 112]. Although capacitive and impedimetric immunosensors can be directly utilized to investigate the antibody-antigen interaction without the need of other reagents and separation steps, their analytical sensitivity is limited in clinical applications. Amperometric immunosensors based on the measurement of currents resulting from the electrochemical oxidation or reduction of electroactive species has attracted more interests for high sensitivity and low complexity instrumentation [112]. However, amperometric immunosensors requires labeling of either antigen or antibody, since both the reaction partners are electrochemically inert. Labeling is a time-consuming process, costly and denatures the biomolecules. Amperometric immunosensors that utilize an electrolyte solution containing reversible redox species have been widely used as mediator probes instead of labeling antibodies/antigens; however, this causes electrode contamination, reagent-consumption and operation inconvenience., label-free amperometric immunosensors have been proposed to address the above issues. One possible way to achieve label-free sensing was by integrating of signal generating probe on electrode surfaces itself. This was achieved by electrochemical fabrication of Copper based nanoparticles on SPCE working surface.

4.2 Introduction

The use of nanomaterials as signal generating probes for electrochemical detection of biomarkers have been reviewed earlier [111]. However, the selection of signal generating probe is vital for electrochemical immunosensor, especially for label-free electrochemical immunosensor, since it is responsible for three major tasks: (i) being redox active and produce electrochemical signals for corresponding immunocomplex formation, (ii) increasing specific surface area of electrode to immobilize more antibodies and (iii) improving conductivity for sensitive detection of antigen. For electrochemical devices, nanoparticles, carbon nanotubes, and graphene have been used to catalyze electrochemical reactions and to improve electron transfer. Nanoparticles, especially have also been used as labels to generate an intense signal in comparison with electroactive molecules or organometallic complexes like ferrocene. Indeed, due to their high number of atoms, a large number of electrons can be exchanged through oxidation or reduction.

Group 11 metals (Cu, Ag, and Au), which are very weak chemisorbing agents, often display marked catalytic and electrocatalytic properties. Some types of copper electrodes showed an excellent electrocatalytic activity for DNA detection without observable self-poisoning. Copper electrodes undergo oxidation at unexpectedly low potentials, typically within the so-called double layer region, to form surface-bonded copper (I) hydrous oxide species.

A reagent-less and label-free voltammetric immunosensing strategy was demonstrated by employing a nanomaterial integrated electrode as a signal generating probe. Copper/copper oxide (Cu/Cu_xO) nanoparticles integrated SPCE were used to construct the immunosensing platform. The stress biomarker, cortisol was employed

as a model analyte. Cortisol specific monoclonal antibodies (C-Mab) were covalently immobilized on the surface of the Cu/Cu_xO-SPCE functionalized with DTSP-SAM. The formation of Cu/Cu_xO nanoparticles on the surface of SPCE was characterized by SEM and XRD analysis. The redox signal of the Cu/Cu_xO nanoparticles could be detected directly through CV and used as signal generation probe for the direct detection of cortisol. The formation of immunocomplexes leads to a decrease in the electrochemical redox signal of Cu/CuO nanoparticles. This was due to the increased spatial blocking of the electrode surface. The proposed immunosensing strategy allows a rapid and sensitive means of cortisol analysis with a limit of detection of about 1 pg/mL. The Cu/Cu_xO platform can be further applied for designing other label-free immunoassays.

4.3 Experimental

4.3.1 Materials and methods

SPCE from Zensor was used for the construction of biosensor. The thiol-based crosslinker, DTSP, and sodium borohydride (NaBH4) were purchased from Thermo Fisher Scientific. Copper (II) Chloride (CuCl2), Potassium chloride (KCl) and Sodium Hydroxide (NaOH), used for deposition of copper nanoparticles were purchased from Sigma Aldrich. The buffer solution used for characterization was prepared from sodium phosphate monobasic (NaH2PO4) and sodium phosphate dibasic (Na2HPO4), also purchased from Sigma Aldrich. All the chemicals were of analytical grade and were used without further purification.

4.3.2 Measurement and apparatus

CV and amperometry were employed using a potentiostat from CHI Instruments Inc. Electrochemical Instrumentation (Austin, TX, USA) to record the amperometry and voltammetry plots. The three-electrode configuration system used in this work consisted of a carbon working electrode and silver reference electrode and an external Pt mesh as a counter electrode. This was placed at the bottom of a 6 ml electrochemical cell for cleaning and deposition with constant stirring through a magnetic stirrer on a heater under different temperature variants. SEM images of the nanostructures on the sensor surface were taken using SEM 6330/JEOL FE-SEM

4.3.3 Electro-synthesis of copper nanostructures

Before carrying out the nano-assembly on to the SPCE, the working electrode surface of SPCE was electrochemically pretreated by immersing it in 0.1 M H2SO4 and cycling the potential between - 0.5 and 1.0 V at 100 mV/s. The primary purpose of this pretreatment was to remove organic ink constituents or contaminants and to increase the surface roughness of the SPCE. After pretreatment, the SPCE was washed well with deionized water and dried in air. Amperometry was used for the electro-deposition of copper nanoparticles onto the working electrode of SPCE. Cu nanoparticles were deposited onto the electrode at a constant potential, and then they were oxidized into CuO by potential cycling. Briefly, a constant potential of -0.35 V was applied to the electrode in the precursor solution of 0.1 M KCl and 0.01 M CuCl2 which had been pre-purged with N2 for 15 min. The electrode was then rinsed several times with water and dried with a flow of N2 before it was repeatedly scanned in a 0.1 M NaOH with CV under the potential range of -0.5 to 0.3V at 100 mV/s for 20 cycles, allowing the Cu nanoparticles to be oxidized into CuO nanoparticles. This was

followed by rinsing in DI water and characterization in 0.1M PBS after purging with N_2.

4.3.4 Biosensor fabrication and enzyme immobilization

Formation of a self-assembled monolayer (SAM) of DTSP on the metal nanostructure integrated SPCE followed by anti-cortisol antibody immobilization. DTSP solution (10 mg/ml) was prepared using acetone and incubated on the electrode for 2hrs for SAM formation where the enzyme was covalently immobilized with DTSP via crosslinking. DTSP solution was first reduced using NaBH4 and then dispensed on the nano-material integrated electrodes at room temperature for SAM formation. The DTSP SAM-modified electrodes were then rinsed with acetone to eliminate any unbound DTSP followed by cleaning in DI water. [12] Cortisol antibodies were then covalently attached to the SAM-modified electrode by drop-casting with 100ul of anti-cortisol antibody solutions It is from the facile reaction between the amino group of the antibody and reactive succinimidyl group of the DTSP on the SAM surface below, that the covalent binding results [13]. The non-binding sites of Ab/Cu-CuO/SPCE bioelectrode were obstructed by immobilizing 10μL of bovine serum albumin (BSA) for an hour followed by rinsing in PBS and dried with N2 at room temperature.

4.4 Results and discussion

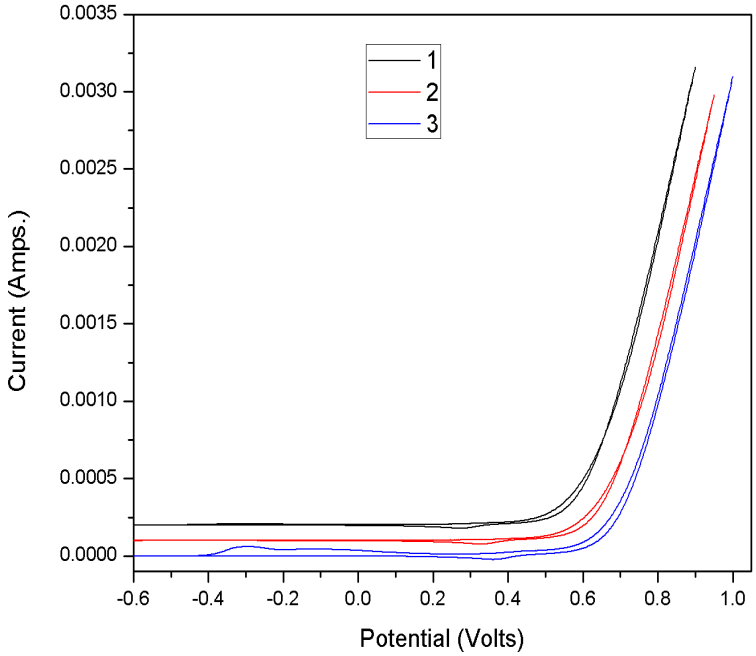

Figure 14: CV of copper nanoparticles being overoxidized for three cycles

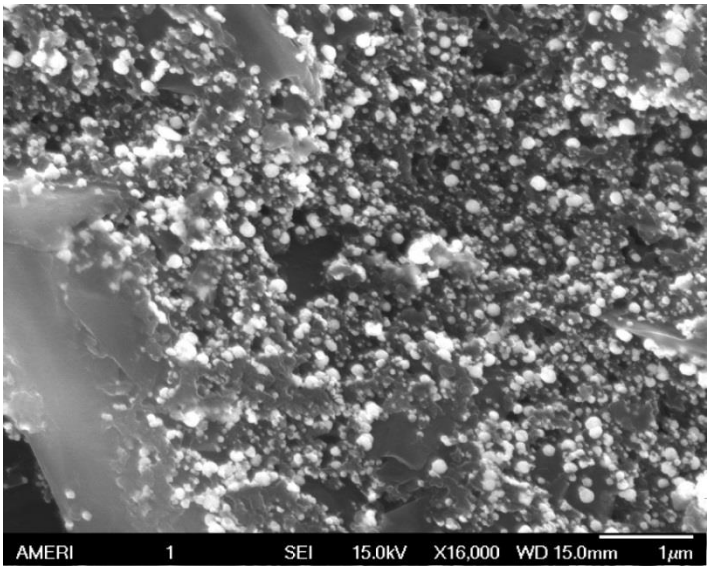

Figure 15: SEM image of the copper Oxide Nanoparticle deposited SPCE. The Particle size ranges from ~150nm.

The Copper Oxide nanoparticle modified SPCE was characterized by CV using the potentiometer form CHI instruments. The CV scans in Figure 14 show the overoxidation of copper nanoparticles to copper oxide at potentials higher than 0.5 V over three cycles. With each subsequent cycle the overoxidation potential shifts towards the higher side, and at the end of the third cycle, it is observed to be at 0.6 V.

The resulting copper oxide nanoparticles were characterized by SEM [JEOL JSM-6330F FESEM]. Figure 15 shows the SEM image of the CuO modified SPCEs showing the dispersion of nanoparticles. XRD studies of the nano-functionalized electrode surface show the presence of Copper and Copper Oxide peaks.

The SEM image in Figure 15 shows the uniform distribution of copper oxide nanoparticles on the SPCE. The estimated average particle size is ~150 nm. The XRD graph in Figure 16 shows the presence of a prominent copper peak and smaller

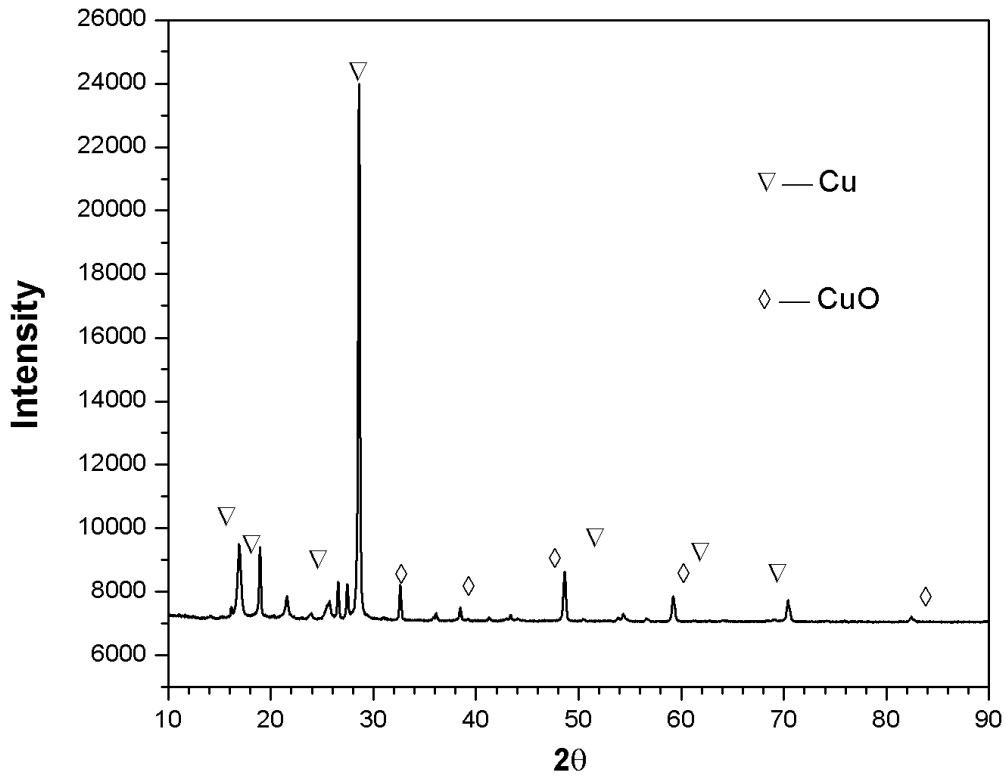

Figure 16: XRD image of Copper/ Copper oxide nanoparticles on the SPCE

copper oxide peak. This indicates the formation of a core-shell structure of a CuO/Cu nanoparticle. The core of nanoparticle consists of Cu surrounded by a shell of CuO. The fabricated immunosensors were used to detect the various concentration of cortisol using CV with PBS as the electrolyte. The CV scans for PBS shows the oxidation response current at 431 µA. After the Anti-cortisol antibody immobilization, the magnitude the oxidation current response went down to 213 µA. this indicates successful binding of the antibodies to the sensor surface. The reduction in current response can be explained due to the hindrance in electron transport caused by the insulating nature of the antibodies. The electrochemical response current of the fabricated immunosensor was studied in PBS (pH-7) as a function of cortisol concentration. Figure 18 shows the oxidation peak current of immunosensor from cortisol Concentrations of 100µM to 1pM. The decrease in the magnitude of electrochemical oxidation response can be explained due to the formation of cortisol and anti-cortisol antibody immunocomplex. This hinders the electron transport from the surface of the electrode to the electrolyte. A calibration curve of the current

response to the logarithmic cortisol concentration has been plotted in Figure 17. The curve reveals a linear correlation from 100 µM to 100 pM with a sensitivity of 4.21 µA/M and the standard deviation of 0.0094. The formula 3xSD/m was used to estimated Limit of detection, which was found to be 6.6nM

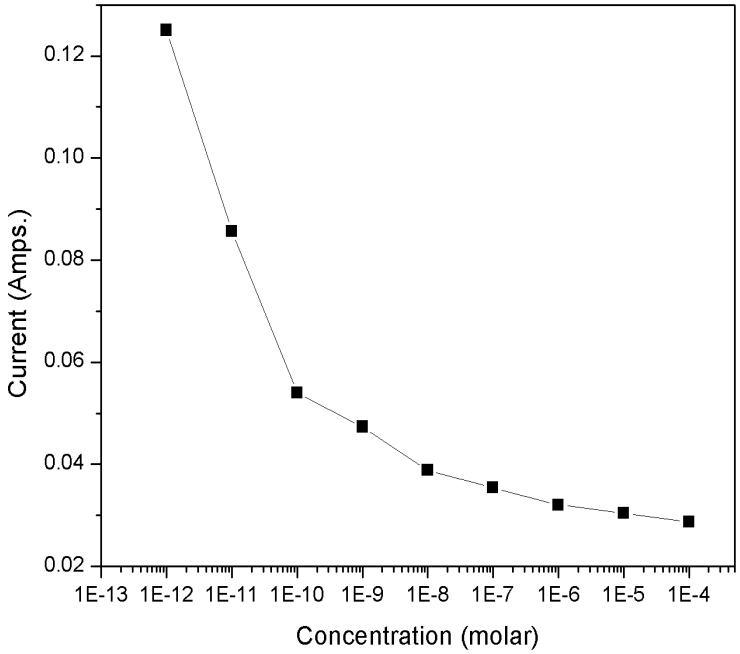

Figure 17: Concentration curve of the Cortisol immunosensor showing linear concentration from 10 M to 10 nM samples of cortisol.

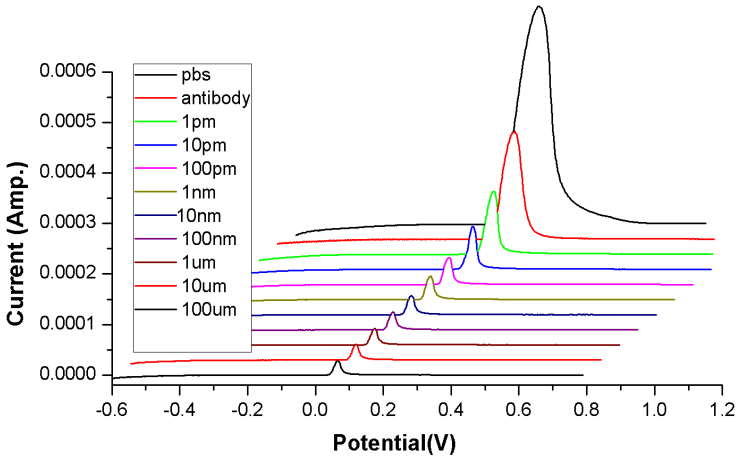

Figure 18: Different concentration of cortisol dispersed on the immunosensor and their oxidation response current.

4.5 Conclusion

A label-free immunosensor specific to cortisol using Copper/copper oxide nanoparticles was successfully fabricated on the SPCEs. The fabricated immunosensor was characterized by CV, SEM, and XRD. The sensors were used to detect the different concentration of cortisol with PBS as the electrolyte medium. The sensitivity of the fabricated sensor was determined to be 4.21 µA/M and the limit of detection 6.6 nM. Though this research enables the label-free immunosensing of cortisol, it has some inherent challenges associated. The Antibodies are temperature dependent and limit the shelf life of the sensors. The cost associated with the fabrication of immunosensors is high due to the expensive nature of antibodies. The next chapter seeks to investigate polymers for electrochemical detection of Cortisol. This new technique aims to make the sensor cost effective, temperature stable and make repeatable measurements possible with the same sensor element.

Chapter 5: BIO-MIMETIC SENSOR FOR LABEL-FREE DETECTION OF CORTISOL USING MOLECULARLY IMPRINTED POLYMERS APPROACH

5.1 Abstract

We report here, an in *situ* electro-polymerized MIP based electrochemical sensor for the detection of cortisol. Cortisol specific MIP films were prepared by electro-polymerizing pyrrole monomer on the electrode surface in the presence of cortisol as a template molecule. After eluting the cortisol through electrochemical overoxidation, cortisol specific imprinted sites created on the polymeric matrix were used to detect cortisol using a redox mediator Fe(II)/Fe(III). The imprinted matrix was characterized using CV and scanning electron microscopy (SEM). The effect of current responses on electro-polymerization cycle, monomer concentration, elution cycles (target removal), pH of the supporting electrolyte, and rebinding time was investigated and optimized. The MIP based cortisol sensor exhibited a detection limit of 1 pM L^{-1} cortisol. Moreover, the sensor showed good selectivity and reusability the sensitivity remained >90% after 7 cycles of elution/rebinding, while the sensitivity only decreased to 90% after 4 weeks of storage at room temperature. The reusable MIP sensor has been applied to detect cortisol in real samples and validated with ELISA. These features make the MIP based cortisol sensing attractive for reusable and continuous cortisol monitoring applications.

5.2 Introduction

Current, analytical methods such as ELISA and electrochemical immunosensors are widely used to detect cortisol [77, 83, 113-115]. These analytical techniques involve the use of cortisol specific antibody as a biorecognition element

and provide selective detection of cortisol. However, instability of antibodies, the need for refrigerated transport and storage, and the high cost of producing immune-reagents are the severe roadblocks to the commercialization of immunosensor for point-of-care (POC) applications [78, 116]. Therefore, the design and synthesis of artificial receptor systems as stable mimics to the conventional antibodies with high target binding affinity is in demand for diagnostic applications. One technique that is being increasingly adopted for the generation of artificial bioreceptors is molecular imprinting [117, 118].

Molecular imprinting is a technique for creating specific recognition sites in synthetic polymeric matrices that are structurally complementary to target molecules. MIPs are synthetic polymers having recognition sites selective towards the target analyte. They are versatile, stable and cost-effective substitutes for the natural antibodies [119-122]. They have a considerable impact and potential applications in biosensor commercialization owing to their number of merits such as low cost, easy storage, and applicability in harsh conditions. MIPs possess longer lifetimes over the enzymes, antibodies, and proteins [123-126]. Another attractive feature of MIP based biosensor is its reversible binding with the target analyte, which makes the MIP sensors reusable where frequent/continuous monitoring is required.

Traditional polymerization techniques such as bulk polymerization, precipitation polymerization, and emulsion have been widely used for the synthesis of MIPs. However, controlled integration of MIPs onto the transducer surface still remains a challenge. Electrochemical polymerization is an attractive method for patterning MIPs directly onto the surface of biochips. Moreover, the electropolymerization process allows us to control the thickness, morphology, and reproducibility of MIP films by tuning the experimental parameters such as the

concentration of target/monomer, polymerization cycles, and applied potential [127-130]. Various electroactive monomers, such as pyrrole, aniline, and o-phenylenediamine were explored as potential materials for synthesis MIPs by electropolymerization. Among them, polypyrrole (PPy) based MIP has received interest because of its facile electropolymerization, high conductivity, and stability in ambient conditions [131-133]. In the past years, attempts were made to synthesize MIPs for separation of cortisol [134-136]. However, no attempts were made to obtain an *in situ* fabricated MIPs for the electrochemical detection of cortisol. In this work, for the first time, we report a facile electrochemical strategy to fabricate a stable cortisol MIP sensor using PPy. One of the key aspects in designing reproducible MIP biosensor is controlled removal of template molecule from the polymer matrix. To remove the entrapped template molecule from the polymeric matrix, different strategies have been performed. The first and most straightforward approach is extraction with an appropriate solvent. In some cases, the target extraction involves temperature, microwave, or ultrasonic assisted approaches to increase the rate of extraction. However, these methods cause imprinted cavities to distort and, consequently, makes the MIPs less efficient towards rebinding and selectivity [137]. In the current work, an electrochemical elution approach was performed using CV to extract the cortisol from the polymeric matrix. The effect of electrochemical extraction cycles on the template removal was also investigated.

Moreover, we demonstrate an efficient and fast protocol for removing the template cortisol from the polymeric matrix, which improves the facile fabrication cortisol imprinted biosensor. The biosensor was then applied to detect human salivary cortisol levels, and the results were validated using the ELISA technique.

5.3 Experimental

5.3.1 Materials

Pyrrole monomer (≥98%), potassium ferricyanide ($K_3[Fe(CN)_6]$), potassium ferrocyanide ($K_4[Fe(CN)_6]$), potassium chloride was purchased from Sigma-Aldrich Co., USA. Phosphate buffer solution (PBS) (0.1 M, pH 7.2) was prepared by dissolving 1 tablet in 200 mL of de-ionized (DI) water. A stock solution of cortisol was prepared by dissolving 1 mg of cortisol in 1 mL of ethanol. Working aliquots were prepared by diluting the cortisol stock solution with PBS. A standard stock solution of $K_3[Fe(CN)_6]$)/ $K_4[Fe(CN)_6]$ (5 mM) was prepared in PBS. Cortisol enzyme immunoassay kit was procured from Arbor Assays, MI and a standard protocol were adopted to detect saliva cortisol. In Brief, 50 µl of 4X diluted saliva was used for the detection of cortisol. All other reagents were of analytical grade and solutions were prepared using double distilled water.

5.3.2 Preparation of MIP and non-imprinted polymer (NIP) modified electrodes

Prior to the electrosynthesis of MIP, the SPCE was cleaned to remove organic ink constituents/contaminants and to increase surface roughness. The pre-treatment of the SPCE was carried out by cycling the potential between -1.5 V to 1.5 V at a scan rate of 100 mV/s in 0.1 M H_2SO_4 for 10 cycles [111, 138]. The SPCE was then washed with deionized water and dried at room temperature. The electrosynthesis of MIP films was performed using CV by cycling the potential range between 0 and 0.9 V at a scan rate of 50 mV/s for 10 cycles in 0.1 M PBS containing 0.8 M pyrrole and 0.1 M KCl. 10 mM cortisol was added to the solution as a template molecule before the polymerization. As the polymer matrix grows on the working electrode, cortisol

molecules diffuse toward the electrode surface and get entrapped in the polymer matrix.

After the polymerization, polymerization, the entrapped cortisol molecules were extracted from the conducting polymer matrix to produce a surface complementary in shape and functionality to the original template, cortisol. The extraction was performed through over-oxidizing the PPy by cycling the potential range between -0.2 to 0.8 V for 40 cycles in 0.1 M PBS solution. The identical procedure (polymerization followed by overoxidation) was used to fabricate NIP electrode but without adding cortisol as a template. Modified electrodes were then dried under nitrogen flow and stored at room temperature.

5.4 Cortisol sensing using MIP

The electrochemical detection of cortisol by the MIP sensor was performed by placing 5 µL of an appropriate concentration of cortisol solutions at the working electrode, for 10 min without stirring. The sensors were washed with water to remove any material that may have been absorbed on the surface. Subsequently, the electrochemical measurements were carried out in the presence of 40 µL of 5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ solution containing 0.1 M PBS at room temperature.

5.5 Results and Discussion

5.5.1 Fabrication of cortisol MIP and NIP electrodes

Formation of cortisol specific MIP films on the electrode surface was achieved by electrochemical polymerization of pyrrole in the presence of cortisol. The polymer growth was monitored through the changes in the current response obtained during

polymerization cycles. The voltammograms obtained during electro-polymerization of pyrrole in the absence (dotted lines) and in the presence of cortisol (solid lines), from 0 V to 0.9 V at 50 mV/s using 0.1 M KCl as supporting electrolyte are compared in Fig. 1A. In both the cases, the oxidation currents were increased remarkably at each voltage sweep, suggesting a controlled growth of the polymer, suggesting the thickness of the polymer film can be controlled by the regulating the scan cycles. During the polymerization, cortisol molecules diffuse towards the electrode and bind to the PPy conducting matrix through hydrogen bonding (Scheme 1). Although the two voltammograms look similar in shape, the capacitive currents observed for the electropolymerization in the presence of cortisol (solid lines) is significantly higher than those obtained in its absence (dotted lines). The increased capacitive current of the polymer film in the presence of cortisol confirms the binding of cortisol, an electro-inactive molecule, with the polymer.

Removal of cortisol templates from the polymeric matrix is necessary to form complementary imprinted sites for the subsequent rebinding. The electrochemical over-oxidation process was used to remove cortisol molecules from the PPy matrix.

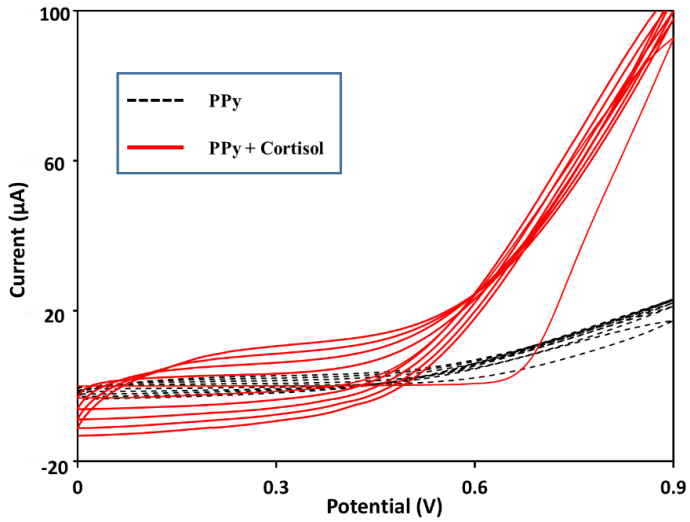

Figure 19: CV scans taken during the electropolymerization of pyrrole in the absence (dotted lines) and in the presence (solid lines) of 10 mM cortisol onto the SPCE. Scan rate: 50 mV/s.

The polymer matrix was over-oxidized by scanning the potential in a range of -0.2 V to +0.8 V for 40 cycles in 0.1 M PBS at a scan rate of 50 mV/s (Figure 19). For clarity, the initial five scans were shown in Figure 20. During the elution scans, a broad irreversible voltammetric peak was observed at ~ 0.3 V due to the overoxidation of PPy (Li and Qian, 2000). As seen in Figure 20, no cathodic peak was observed during the reverse scan, which indicates that the over-oxidation of PPy is electrochemically irreversible. The above process causes the removal of cortisol from the PPy matrix while oxygen-containing groups, such as carboxyl, are incorporated into the polymer backbone. The decrease in the current response during subsequent scan cycles is due to a decrease in conductivity of the PPy film. Since the over-oxidation was performed here in a neutral condition, the degradation of PPy matrix was avoided. The NIP fabrication process involves the same protocol as used to fabricate MIP (polymerization followed by overoxidation) but without the addition of cortisol as a template.

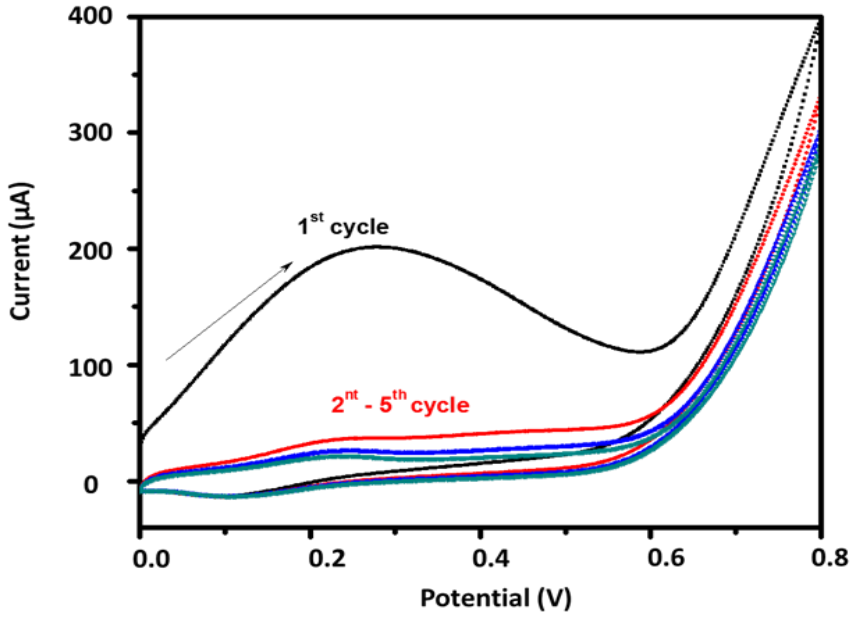

Figure 20: Recorded voltammograms during the elution of cortisol from the PPy matrix at 0.1 M PBS; scan rate of 50 mV/s.

5.5.2 Electrochemical characterization of cortisol specific MIP

The electrochemical behavior of stepwise fabrication of cortisol MIP sensor was studied in 5 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ solution containing 0.1 M PBS. The results are shown in Figure 21. The CVs of $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ redox probe was chosen as a marker to investigate the changes in the electrode behavior after each step of the sensor assembly. As shown in curve (a), well-defined reversible redox peaks for the $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ redox couple were observed with bare SPCE. The PPy modified SPCE showed an apparent increase in the peak current for the redox probe along with the increase in capacitive currents (curve b). This was attributed to the increase in active surface area by conducting PPy matrix on the SPCE surface. The permeability of the porous PPy film is also responsible for the increased electron transport kinetics. $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ is an anion; due to the electrostatic interactions, the $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ ions inserts easily into the positively charged PPy film and thus involved in rapid electron transfer kinetics. The peak current response observed for cortisol loaded PPy matrix (curve (c)) was much lower than the one observed for PPy matrix without cortisol (curve (b)). This confirms that cortisol was effectively loaded onto the PPy matrix and hinders the electrochemical response of $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$.

It is interesting to note that, removal of cortisol from the PPy matrix drastically decreases the current response of the redox probe (curve (d)). The voltage induced overoxidation leads to conductivity loss in PPy due to the introduction of carbonyl and carboxyl groups in the polymer backbone. The inset of Figure 21 depicts the enlarged version of the curve d, showing the redox currents observed with the overoxidized PPy matrix.

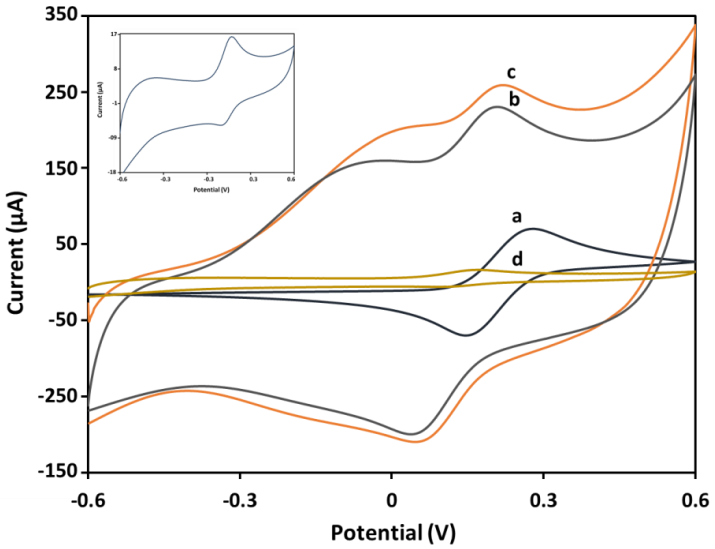

Figure 21: CV scans of 5 mM K4[Fe(CN)6]/K3[Fe(CN)6] solution at (a) bare SPCE, (b) PPy-SPCE, (c) Cortisol-PPy-SPCE and (d) MIP-PPy-SPCE; scan rate of 50 mVs-1; (Inset: Enlarged version of curve d).

5.5.3 Surface morphological characterization of cortisol MIP electrodes

Surface morphologies of MIP and NIP modified electrodes are obtained at the magnification of x30,000x and are shown in Figure 22(A) shows the SEM image of PPy coated SPCE. After electropolymerization, the surface of the SPCE is coated with a microporous PPy matrix with cauliflower-like structure constituted by micro-spherical grains. The average diameter of the grain is 343.47 nm. When pyrrole was polymerized in the presence of cortisol, an increase in grain size is noted (Figure 22C). The average diameter of the grains increased to 616.32 nm, suggesting the incorporation of cortisol to the polymeric matrix. Overoxidation of PPy matrix

leading to an increase in porosity and decrease in grain size due to the removal of cortisol from the matrix (The average diameter of the grain size is 342.83 nm).

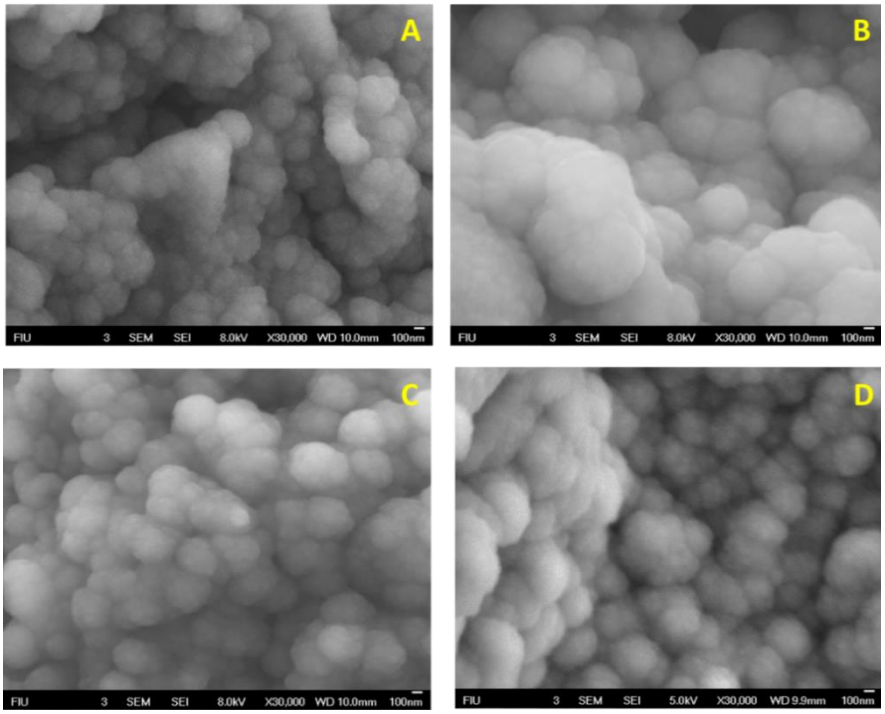

Figure 22: SEM images of (A) PPy-SPCE, (B) Cortisol-PPy-SPCE, (C) MIP-PPy-SPCE, and (D) NIP-PPy-SPCE

For comparison, the SEM image of the NIP modified electrode is given in Figure 22D. As can be seen from the SEM images, the surface of the MIP modified electrode is rough due to cavities of cortisol whereas the surface of the NIP modified electrode is smooth without the imprinting cavities. The distribution of polymer grain size is given in Figure 23.

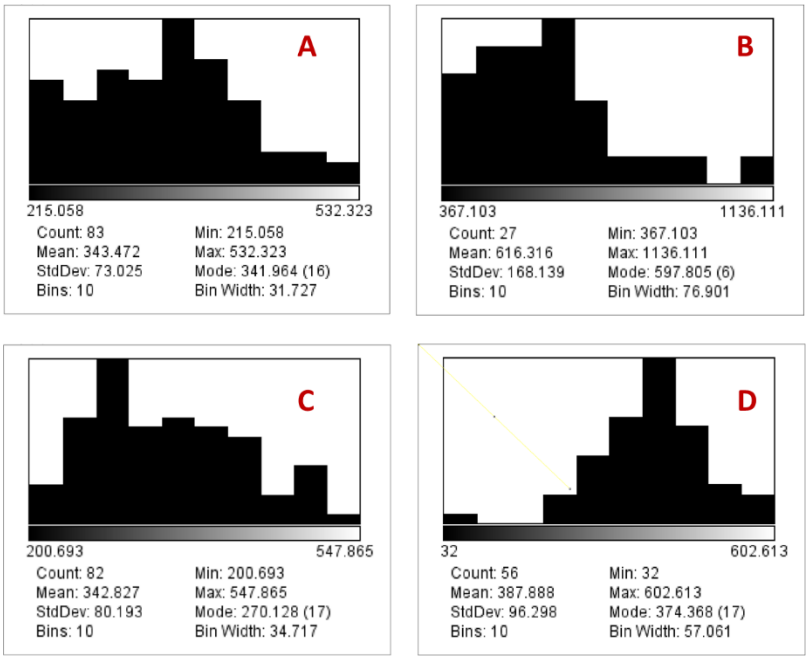

Figure 23: Polymer grain size distributions observed for (A) PPy-SPCE, (B) Cortisol-PPy-SPCE, (C) MIP-PPy-SPCE, and (d) NIP-PPy-SPCE

5.5.4 Optimization of experimental parameters

The thickness of MIP film is an important factor affecting the film's recognition ability, reproducibility and it could be controlled by tuning the concentration of monomer and a number of polymerization cycles.

5.5.5 Effect of concentration of pyrrole monomer.

The effect of monomer concentrations on the electrochemical response of the MIP sensor was investigated. The cortisol MIP electrodes were prepared in solutions of a

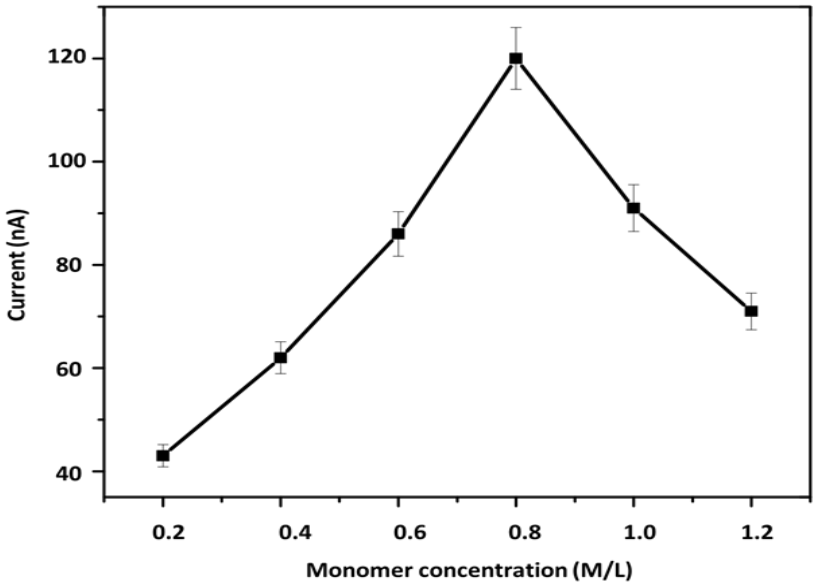

Figure 24: Effect of the monomer concentration on the response of the sensor to cortisol

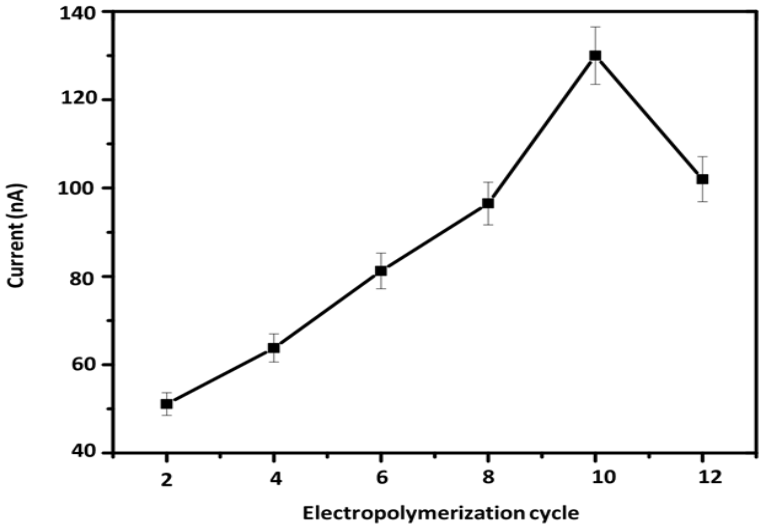

Figure 25: Effect of the different cycles of the electro polymerization of MIP

constant concentration of cortisol (10 mM) and different pyrrole concentrations in the range of 0.2 M to 1.2 M (Figure 24). The polymerization and elution of cortisol to form MIP electrodes were followed as mentioned in the previous section. The

fabricated MIP electrodes were then characterized using CV in 5 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ solution containing 0.1 M PBS. The current response of the $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ redox probe increased with increase in monomer concentration and reached a maximum value at 0.8 M. The current response then decreased with further increase in the concentration of pyrrole monomer. This behavior can be attributed to the formation of a very thick layer of MIP at higher concentration of monomer which in turn affects the electron transfer of $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ redox couple. Thus, the optimized 0.8 M pyrrole monomer concentration was chosen for the electrochemical polymerization to obtain the highest sensitivity for the determination of cortisol.

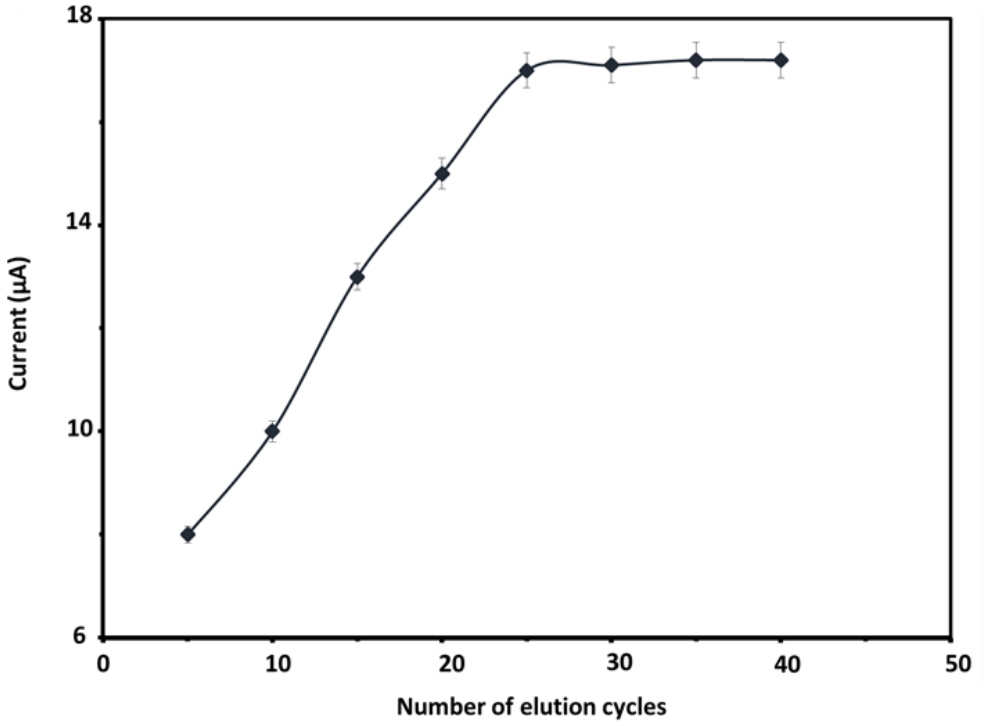

Figure 26: Effect of electrochemical extraction cycle on the current response

5.5.6 Effect of number of electropolymerization cycles and rate

Since the thickness of polymer matrix can also be tuned via controlling the number of electro-polymerization cycles, the number of scan cycles used for MIP synthesis was optimized. A series of MIP electrodes were fabricated by following the experimental procedure mentioned in section 2.3 but with a different number of polymerization cycles to find out the optimal number of polymerization cycle. The electrochemical elution of cortisol template from the polymeric matrix was performed using CV as detailed in section 3.1. As shown in Figure 25, the current response of the redox probe $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ reached maximum after the tenth cycle of polymerization, and then decreased with further increase in the number of polymerization cycles. MIP films that were formed at less than 10 scan cycles were found to be thin and less stable at the working electrode whereas higher than 10 cycles formed thicker sensing layer which hinders the electron transfer of $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ redox probe. The current response changes of cortisol on MIP modified electrode implied that the optimum polymerization cycle was to be 10.

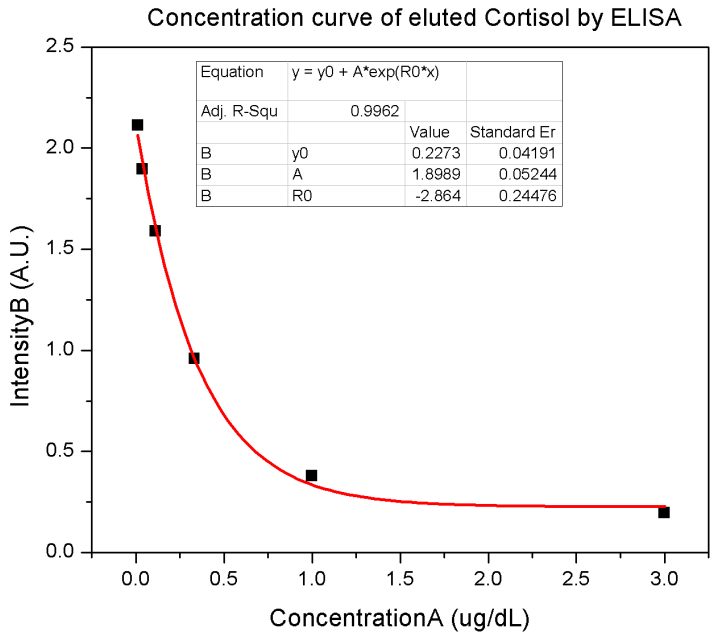

Figure 27: concentration Curve after testing the Elute using ELISA

5.5.7 Optimization of the extraction cycle

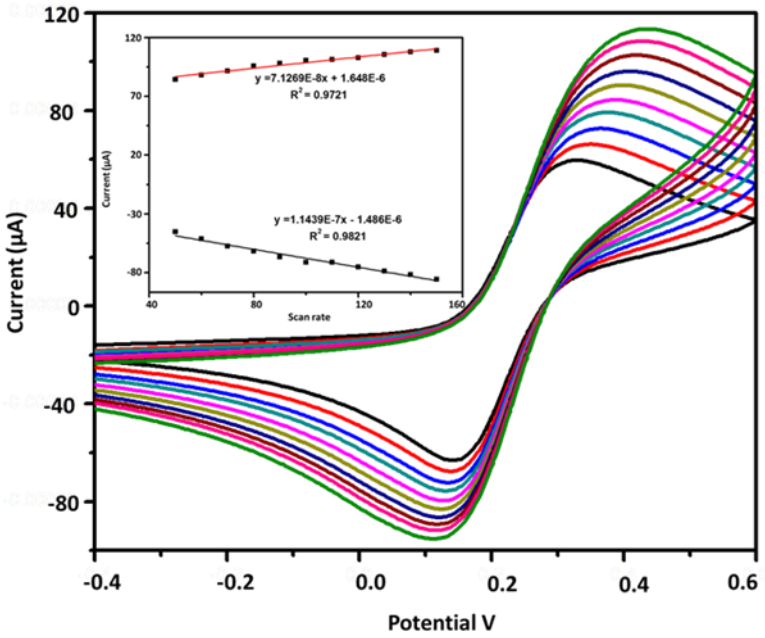

Figure 28: CV studies of the cortisol MIP sensor as a function of scan rate (50-150 mV/s), inset: magnitude of current response vs. scan rate.

Controlling and enhancing the formation of imprinting sites by regulating the removal of target molecules from the polymeric matrix is essential to fabricate reproducible MIP biosensors. An effective method is needed to regenerate the imprinting sites after the binding event to make the MIP sensors reusable. In this study, voltage induced target removal was performed to create imprinting sites. The electrochemical elution of cortisol template from the polymeric matrix was performed using CV as detailed in section 3.1. To optimize the extraction cycles, different extraction cycles (5-40) were performed on the cortisol embedded PPy modified SPCE. The MIP sensors were characterized after each cycle of target removal, using the redox probe $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$. As the number of elution cycles increases, the current response of the MIP sensor also increases indicating the controlled release of cortisol molecule. The maximum current response of the electrode is obtained at

the 25th cycle (Figure 26). Thus, the extraction cycles were optimized at 25 for complete extraction of the template from the polymeric matrix.

5.5.8 Effect of binding time and scan rate

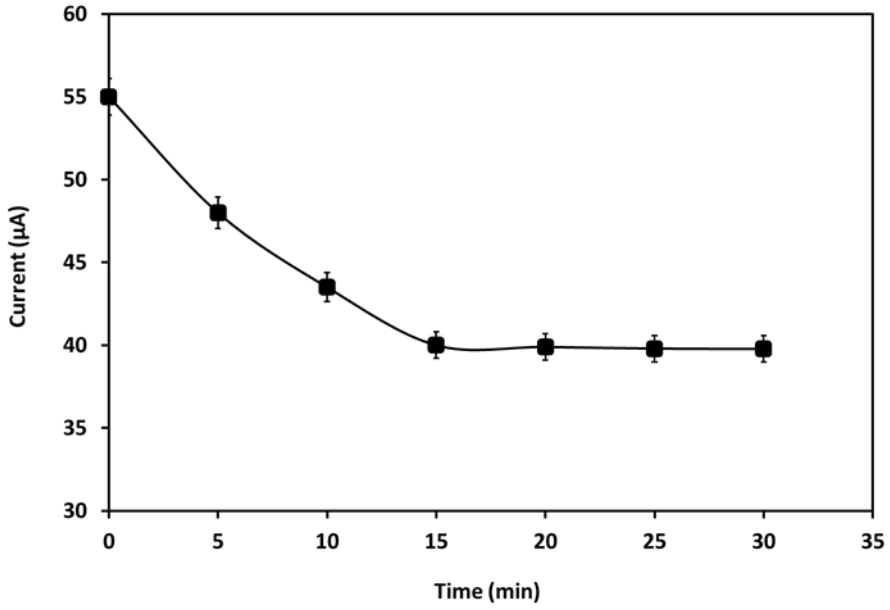

Figure 29: Effect of incubation time on current response of the cortisol MIP

The typical CV curves of the cortisol MIP sensor in $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ redox probe at different scan rates ranging from 50 to 150 50 mV/s were studied and are shown in Figure 28. As is observed in Figure 28, both the cathodic and anodic peak currents were increased with an increase in scan rate from 50 to 150 mV/s. The anodic and cathodic peak currents both are linearly proportional to the square root of the appropriate scan rate, suggesting a diffusion-controlled behavior with an electron transfer process [139].

The effect of the incubation time on the performance of the cortisol MIP sensor was also investigated (Figure 29). After the extraction of cortisol, the MIP sensors were left in contact with a standard solution of 10 pM cortisol for different

incubation times (0-30 min). With increasing incubation time, the current responses of the $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ redox probe decreases and then stabilize when the incubation time is longer than 15 min. Thus, an incubation time of 15 min is adopted in the subsequent work.

5.5.9 Effect of pH

To evaluate the influence of the pH on the performance of the cortisol MIP sensor, the sensor was tested with a series of PBS with different pH, ranging from 5.8 to 7.8 (data not shown). The experimental results show that an increase in pH from 5.8 to 7.2 resulted in an increased peak current of $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ redox probe; further increase in pH resulted in a decrease in peak current. The results showed that the maximum current response occurred at pH 7.2. The optimum pH 7.2 was hence chosen for all the experiments.

5.5.10 Calibration curve of the sensor

The typical CV scans obtained for several concentrations of cortisol in 0.1 M PBS containing five mM $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ using the cortisol MIP sensor at 50 mV/s are shown in **Error! Reference source not found.**. The figure depicts the magnitude of electrochemical current response which decreases as a function of increasing cortisol concentration when incubated for 15 min. The decrease in current response is attributed to the binding of cortisol molecules to the MIP sites that hinder electron transport of $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ redox probe. A calibration curve between the magnitude of the current response and logarithm of cortisol concentration has been plotted (inset, **Error! Reference source not found.**). Cathodic peak current observed at -0.35 V was used to plot calibration curves. The current responses to

cortisol obtained with the MIP sensor were linear from 1 pM to 10 µM (r^2=0.9925), with a detection limit of 1 pM.

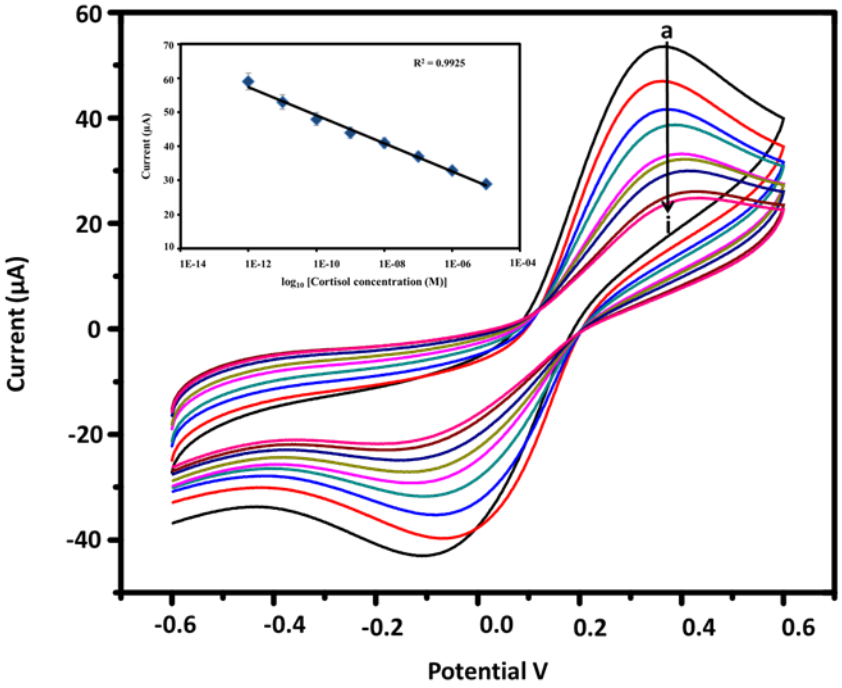

Figure 31: Electrochemical response studies of MIP-PPy-SPCE as a function of cortisol concentration (1 pM to10 uM) using five mM K4[Fe(CN)6]/K3[Fe(CN)6] solution, inset: calibration curve between the magnitude of response current and logarithm of cortisol concentration

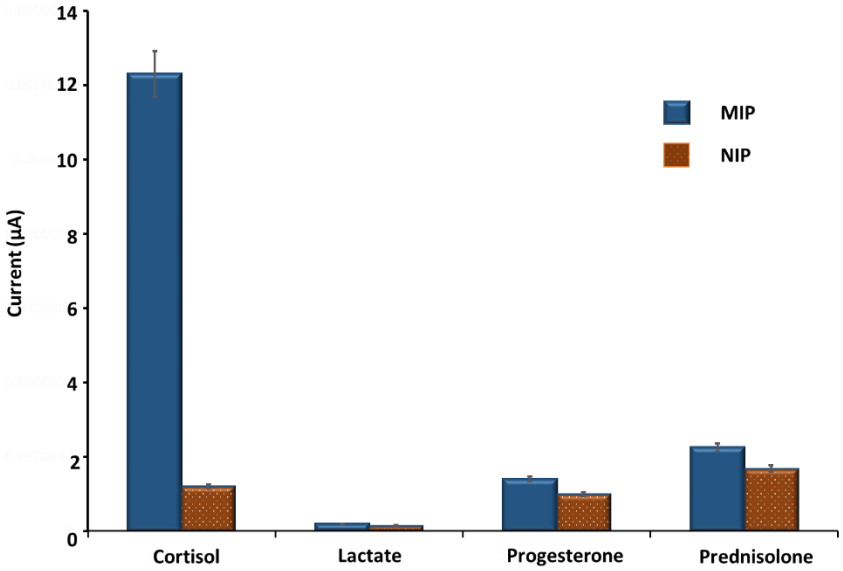

Figure 30: The change in peak current values observed at cortisol MIP (blue) and NIP (red) electrode in the presence of cortisol and other interferents individually each at 100 nM.

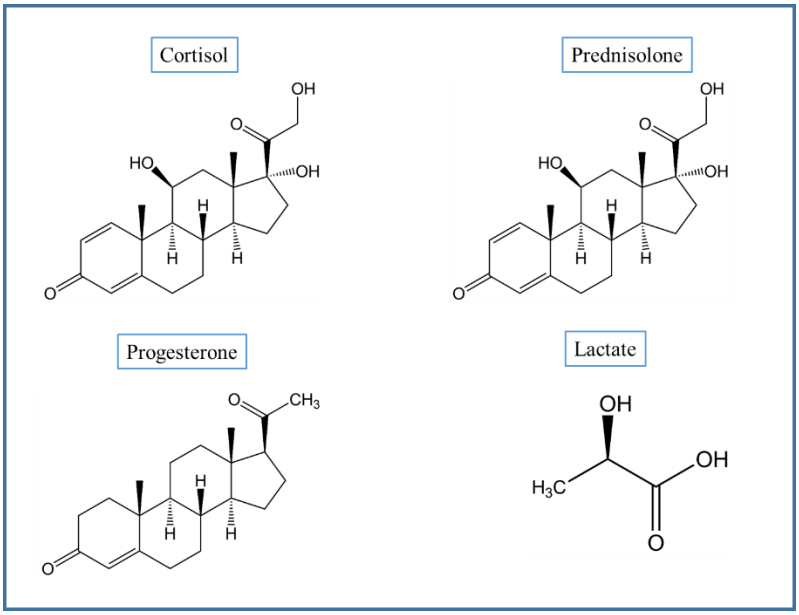

Figure 32: Molecular structure of cortisol and its structural analogs prednisolone, progesterone and lactate.

5.5.11 Selectivity, reproducibility, and repeatability

$$\beta = \frac{I_{pa}\ (MIP\ sensor)}{I_{pa}\ (NIP\ sensor)} \qquad \text{Equation: 3}$$

One of the significant limitations of currently available commercial cortisol immunoassay kits and immunosensors is their cross-reactivity and interference with the cortisol structural analogs viz. progesterone and prednisolone [140, 141]. As recently reviewed by Krasowski and his team, commercially available cortisol immunoassay kits still have cross-reactivity more than 100% with the analogous molecules, especially prednisolone [142]. So, there is a real need to develop an assay for cortisol detection which has lower cross-reactivity with the analogous. Earlier, Ramstorm and his group have evaluated the application of synthetic polymer capable

of molecular recognition of cortisol with reduced cross-reactivity [136]. The MIP prepared by this method showed only 36% cross-reactivity with the analog prednisolone. In this work, selectivity assay for cortisol MIP sensor was carried out using three interfering analytes, progesterone, prednisolone, and lactate. The change in peak current values observed at MIP and NIP electrodes in the presence of cortisol and other interferents individually each at 100 nM is shown in Figure 31.

The imprinting factor (β), a measure of the strength of interaction between the target molecule and MIP sensor was calculated according to

$$\beta = \frac{I_{pa}\ (MIP\ sensor)}{I_{pa}\ (NIP\ sensor)} \qquad \text{Equation: 3}$$

where I_{pa} is the cathodic peak current.

Table 3: Reproducibility experiments of the MIP-PPy-SPCE sensor

Cortisol MIP sensors	1	2	3	4	5	**RSD**
Current (µA)	30.11	29.61	28.24	30.39	28.59	4.16%

Table 4: Repeatability experiments of the cortisol MIP-PPy-SPCE sensor

Cortisol solutions	**1**	**2**	**3**	**4**	**5**	**RSD**
Current (µA)	28.15	29.10	29.85	30.98	31.80	4.81%

The values of β were 10.25, 1.27, 1.41, and 1.33 for cortisol, lactate, progesterone, and prednisolone respectively. From the values obtained, the MIP sensor shows higher binding capability towards cortisol (maximum β value) over other interferents. The above results revealed that the MIP sensor exhibited higher recognition selectivity toward cortisol. The cross-reactivity percentage was also calculated using the current values. It showed that lactate has very negligible

interference (1.5%) while progesterone (11.4%) and prednisolone (18.3%) have slightly higher cross-reactivity with cortisol detection. The chemical structures of cortisol and other interferents are shown in Figure 32. As can be seen from the figure, prednisolone and progesterone have chemical structures very similar to cortisol and thus interfere with cortisol measurement. However, the percentage of cross-reactivity of the present MIP sensors is much lower than the conventional immunoassay. Even this small percentage of cross-reactivity of the cortisol MIP sensors will be addressed in the future by computational modeling [143].

The controlled fabrication process provided good reproducibility for the MIP sensors. Five sensors were prepared freshly and used to detect the same concentration of cortisol (100 nM). These sensors were used to investigate their reproducibility. Based on the current responses obtained, the relative standard deviation (RSD) was calculated to be as 4.16%, confirming that the sensor had good reproducibility (Table 3). The RSD was determined by five successive measurements of a 100 nM cortisol solution using the same cortisol MIP electrode (i.e., used each time after elution process) and was estimated to be 4.81% (

Table 4). After the binding event, sensors were regenerated by electrochemical elution and were reused to detect cortisol. The experimental results demonstrated that the cortisol MIP sensor could be regenerated seven times; after that, the adhesion of the polymer to the electrode is weakened. The stability of the sensor has been examined by monitoring the current response for 10 nM cortisol solution at regular intervals (2 days) for four weeks. After four weeks, the sensor retained 90% of the current response. Suggesting, that the proposed sensor has acceptable stability.

5.5.12 Analytical applications

To further investigate the feasibility of the newly developed cortisol MIP sensor for clinical analysis, cortisol measurement in human saliva samples were performed. The protocol to detect cortisol using the sensor and ELISA is adopted from our previous work (Pasha et al., 2013). In brief, five µl of saliva samples were placed on the biosensor and were allowed to incubate for 15 min to ensure proper binding. The MIP sensors were then washed using 0.1 M PBS to remove unbound saliva. This was followed by the recording CV scans in 5 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ solution containing 0.1 M PBS. These results obtained using the MIP cortisol sensor and estimation performed using ELISA are comparable (Table 5). These results, therefore, confirm the suitability of the electrode for the real sample applications.

Table 5: Comparison of saliva cortisol estimated using ELISA and cortisol MIP sensor.

Saliva samples	**Salivary Cortisol (ELISA) (µg/dL)**	**Salivary Cortisol (MIP sensor) (nM/L)**
Sample 1	0.237	6.75
Sample 2	0.155	4.32
Sample 3	0.144	4.13
Sample 4	0.072	2.16

5.6 Conclusion

In summary, a novel electrochemical sensor for the determination of cortisol was designed for the first time using molecular imprinting technique. The cortisol

MIP sensor was created by electropolymerization of pyrrole monomer in the presence of cortisol template, followed by removal of cortisol under optimized conditions. The electrochemistry of $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ redox probe was used to characterize stepwise fabrication of sensor and detection of cortisol. The sensor response was linear to the cortisol concentration in the range 1 pM to 10 μM (r^2=0.984), with a detection limit of 1 pM. The sensor exhibited good sensitivity, short response time and regenerated seven times. Also, the sensor showed good repeatability, stability, and selectivity, and was applied to detect cortisol in real samples. Despite the advantages of this approach, the use of electrochemical sensing requires complex auxiliary electronics for detection. The output of the system is not a direct readout and has to be correlated to the concentration of the curve by complex calculations. Apart from that, the presence of electrolytic redox species as the label makes it difficult for portable use.

The next chapter will discuss the application of MIP in an EGFET configuration to address the above issues.

Chapter 6: INTEGRATION OF MIP WITH EGFET FOR DIRECT SENSING OF CORTISOL

6.1 Abstract

Electrochemical immunosensing offers a fast and effective method for detection of cortisol. However, it suffers from limitations such as difficulty in implementing label-free sensing. One of the challenges for implementing electrochemical immunosensing is the failure to provide a direct sensing readout for the detected biomolecule. In this chapter, we will address the challenges faced by the electrochemical method. The MIP sensor fabrication reported in the previous chapters will be used in an EGFET configuration to provide label-free and repeatable sensing. The proposed system is easy to implement and is cost effective. The most significant advantage of using this method is the simplicity of the sensing system as it provides a value of current that can be directly related to cortisol concentration. Another advantage of the system is that it requires a simplistic approach to auxiliary electronics. Thus, paving the way for miniaturized sensing devices.

6.2 Introduction

The first semiconductor device in the form of a transistor ushered in a revolution for modern electronics. The days of vacuum tubes were gone, and power-hungry equipment made way for sleek and efficient devices. The development of oxide semiconductor FET further allowed the rapid growth of semiconductor electronics. The MOSFET has been one of the most critical devices for VLSI circuits. This has enabled applications of electronics in control systems, data communication, storage and processing at affordable costs.

The effect of electric field on the conductivity of a conducting channel in a semiconductor of the FET defines its current carrying capability (thus the name Field Effect Transistor). MOSFETs, JFET, TFET, etc. are some of the types of FETs that are available. However, despite individual differences, all FETs share a basic underlying principle.

Two heavily doped n-type regions are created in a p-type substrate. These regions serve as source and drain for the device. A thin layer of insulator (generally silicon dioxide is deposited between the source and the drain. This insulating layer is coated with a thin film of metal to serve as a gate contact pad. Metal contacts are layered on top of the source and drain as well.

While in operation, the source is grounded, and a positive voltage applied between the source gate (Vgs). The application of a Vgs causes an accumulation of electrons between the source and drain regions. These accumulated electrons start to form a conducting channel. Vgs determine the thickness of this channel. Now the application of a drain voltage causes a current to flow between the drain and the source. The magnitude of this current is dependent on the resistance of the channel which in turn is dependent on the gate voltage.

The following equation can express the magnitude of drain current.

$$I_d = \beta(V_{gs} - V_T - \frac{1}{2}V_{ds})V_{ds}I_d \qquad \text{Equation 5}$$

Where V_T is known as the threshold voltage. The threshold voltage is the value of Vgs beyond which the narrow conduction channel starts forming.

β is the trans-conductance parameter and expressed as

$$\beta = \mu C_{ox} \frac{W}{L}$$ Equation 6

β is a function of the mobility of the electrons (μ) in the inversion layer, the gate insulator capacitance per area (C_{ox}) and the channel width to length ratio (W/L).

A closer look at the trans-conductance parameter shows the linearity of the FET device. As the Vds is increased, the current Id through the channel increases. Effectively the FET behaves as a resistor whose resistance can be calculated by the slope of the line from Vd vs. Id curves.

However, the channel width is uniform for small values of Vds. As Vds is increased, the channel becomes narrower near the drain as compared to the source; this results in the rolling of Id vs. Vd curve as shown in the figure below.

These properties of FET were first utilized by Bergveld et al. electrophysiological applications resulting in a Configuration Called ISFET. With this pioneering work, ISFET based sensors gained focus in the rapidly growing field of biosensors and were utilized in several different ways. Fast response time, high sensitivity, batch processing and the possibility of integrating on a single chip have pushed FET sensors in the forefront of sensing technology.

6.3 EGFET

The EGFET is based on the principle of an ion sensitive FET. The ISFET consists of a gate insulator, where the electrolyte can be immobilized. Instead of having a direct metal contact on the gate, an external electrode dips in the electrolyte.

In the presence of a constant external gate voltage, a change in the concentration of the ions present in the electrolyte can cause a potential change at the surface of the gate insulator. The potential change, in turn, is responsible for the modulation of the drain-source current in the FET. By using this configuration of ISFET, biosensing applications are made possible. This is possible by immobilizing bio sensitive moieties on the gate insulator surface. However, this configuration can destroy the device due to often harsh chemical nature of the electrolytes used. The EGFETS avoid this problem by isolating the chemical environment from the FET itself. The system consists of an external functionalized substrate that is connecte4d to the gate as shown in the figure below. The substrate has a well that limits the electrolyte volume. The external electrode touches the electrolyte, and a gate voltage is applied across it. The interaction between the ions present in the electrolyte and the functional group present on the extended gate substrate causes a surface potential change. This change of potential causes a change in the flow of current to the gate of the FET. In turn, the gate current modulates the Drain-Source currents.

The Modulation of drain current due to the presence of biomolecules on the surface of the extended gate makes it possible to use the FET as a biosensor. This configuration also allows for the stabilization of FET by isolating it from changes happening due to light and temperature variations as the gate material is not directly exposed to the environment. In this research, we will be using EGFET configuration along with molecularly imprinted polymers for the sensing of Cortisol.

This configuration will result in a system that provides a direct output as a change in drain current due to variation in cortisol concentration. An SPCE was used to fabricate MIP specific to cortisol. We report the fabrication and characterization of MIP in detail in previous chapters.

6.4 Materials and methods

The SPCE was a three-electrode system with an Ag/AgCL reference and two Carbon electrodes. It was purchased from Zensor was used for the construction of MIP biosensor. The thiol-based crosslinker, DTSP, and NaBH4 were purchased from Thermo Fisher Scientific. Copper (II) Chloride (CuCl2), KCl and NaOH, used for deposition of copper nanoparticles were obtained from Sigma Aldrich. The buffer solution used for characterization was prepared from NaH2PO4 and Na2HPO4. These chemicals were also purchased from Sigma Aldrich. All the chemicals were of analytical grade and were used without further purification. The commercial prepackaged FET IC (CD4007ube) was purchased from Texas Instruments.

6.5 Fabrication of EGFET sensor

Formation of MIP on the SPCE – the fabrication and characterization of the MIP modified SPCE surface has been detailed in the previous chapter. The MIP electrode was connected to the gate of the FET sensor as shown in Figure 33 below. The gate of the FET was connected to the working electrode of the SPCE. The gate voltage source was connected to the Reference electrode of the SPCE. The source and drain were grounded and connected to the power source respectively. The output and transfer characteristics of the FET (CD4007UB) were measured using the Keithley 4200 source meter. The output and transfer characteristics were measured by connecting the gate directly to the Vg power supply and varying the gate voltage. After establishing the baseline measurements of the transistor, measurements were made with 50 µL of PBS with different pH values. Subsequent measurements were made in PBS solution (7.6pH) after incubating different cortisol concentrations on the MIP modified, extended gate.

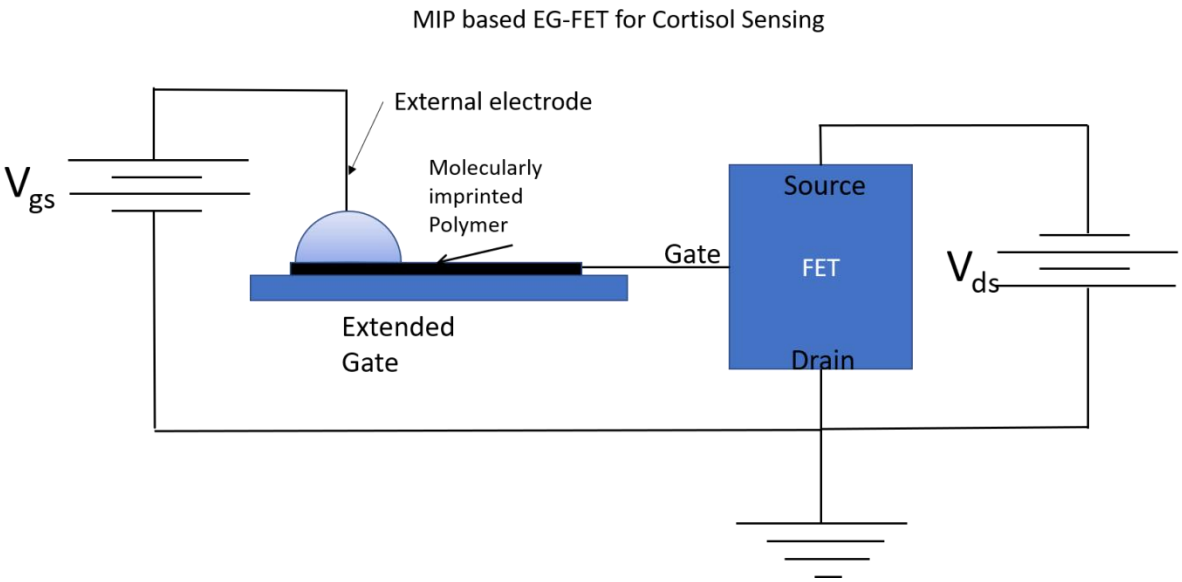

Figure 33: Scheme of the EGFET - MIP based sensing system

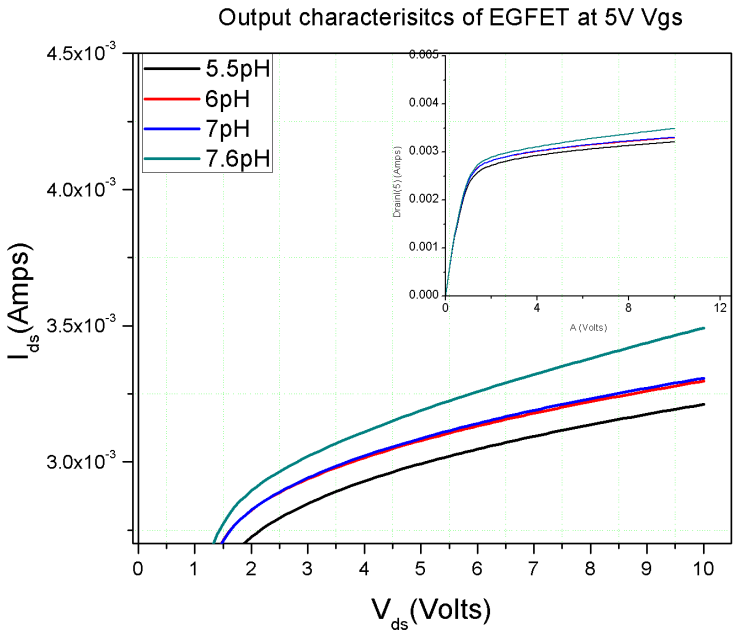

Figure 34: current response of the EGFET after the addition of PBS at different pH Values.

6.6 Results and Discussions

The system was first tested without the extended gate to compare the output and transfer characteristics as described in the Datasheet. The MIP modified,

extended gate was then connected to the FET and output characteristics were repeated with different pH solutions of PBS.

6.6.1 Study of pH response of the sensor

To evaluate the MIP EGFET sensor, it was tested with a series of PBS solution with different pH values. The gate voltage was kept constant at 5V for this study. The response of the system indicates that the current drops as the pH value change from more neutral to a hydrogen-rich environment at pH 5.5, indicating the formation of an ionic double layer. With lower pH values, the no of H+ ions in the solution is higher. This causes a stronger charge interaction at the surface of the MIP. This interaction results in a lower gate voltage. Thus, a lower drain current. The highest response was obtained when PBS solution of pH 7.6 was used. This value is closer to the pH of the biological system. Subsequent experiments were performed

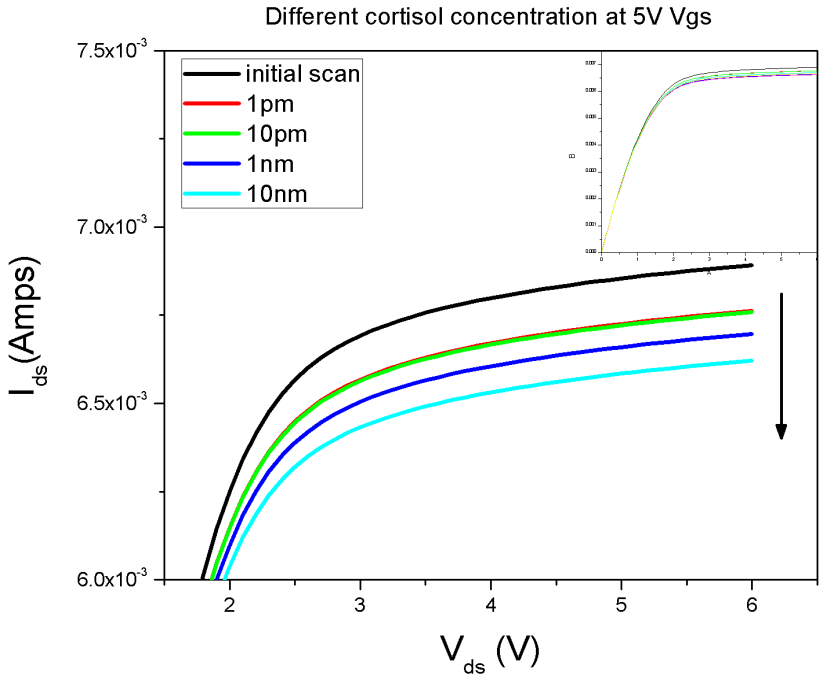

Figure 35: Output characteristics of the EGFET after adding different cortisol concentrations at a constant gate voltage of 5V

using the same.

6.6.2 Drain current response of the sensor with respect to different cortisol concentrations

Cortisol stock solution was prepared by dissolving Lyophilized Cortisol powder in Ethanol and PBS solution of pH~7.4. The stock solution was then diluted to obtain different cortisol concentrations. The MIP modified electrode was incubated with 10 µL of cortisol for 30 minutes. It was then washed away with DI water and dried with nitrogen. 50 µL of PBS was then dispensed on the electrode, and the I-V measurements were made then. Figure 35 And Figure 36 shows the output characteristics of different cortisol concentrations immobilized on the MIP modified, extended gate. The Drain-source voltage was swept while the Gate voltage was kept

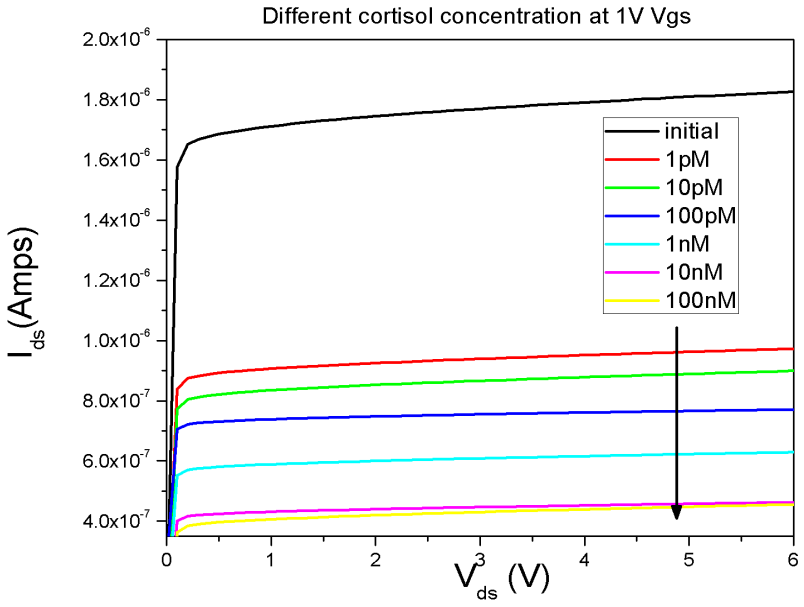

Figure 36: Different cortisol concentration at a lower gate voltage(1V). The reduction in drain current with increasing cortisol can be hypothesized due to cortisol molecules occupying empty sites in the MIP matrix and reducing electron transport in the polymer matrix. The reduced gate current, causes a reduction in the accumulated charges at the actual gate. Thus, causing a decrease in source drain current due to the reduced gate voltage.

at 5 Volt and 1 Volt respectively.

Figure 35 And Figure 36, shows a decrease in drain current can be observed as cortisol concentration increases. The cortisol molecules occupy the empty sites in the polymer matrix. The occupation of conductive sites by cortisol causes a change in the interaction of charge carrier with the polymer surface. The change in surface charges restricts the flow of current across the MIP thin film. The reduced current, in effect, decreases the electron density in the gate region of the n-type MOSFET working in enhancement mode. This change in Drain current due to increased cortisol concentration was used for sensing purpose [144].

Figure 37 shows the concentration curve for cortisol as plotted from the Vd vs. Id data obtained in Figure 36. Drain current values at 2 Volts were chosen and plotted against their respective concentration on a logarithmic scale.

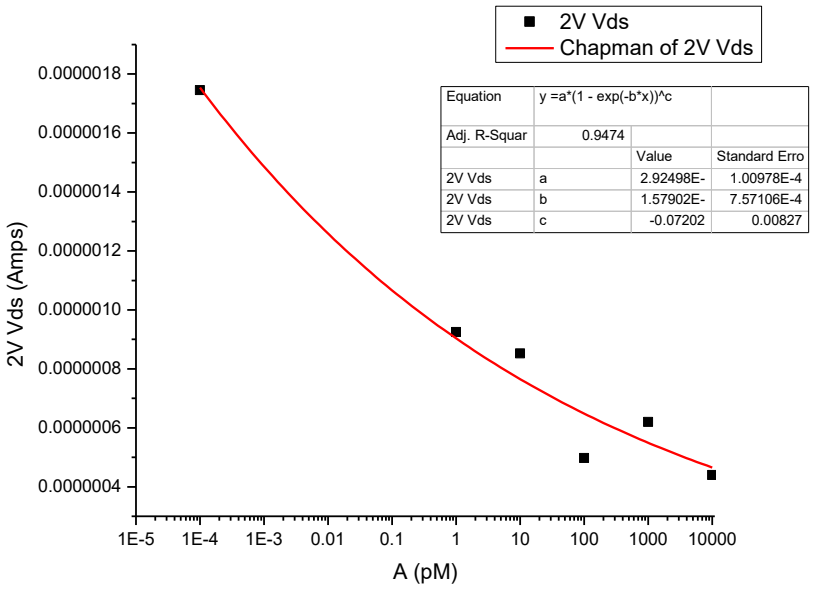

Figure 37: The concentration curve of the EGFET device from 1 pM to 100 nM cortisol concentration vs. the current measured at 2 Volts Vd and 1Volt Vgs

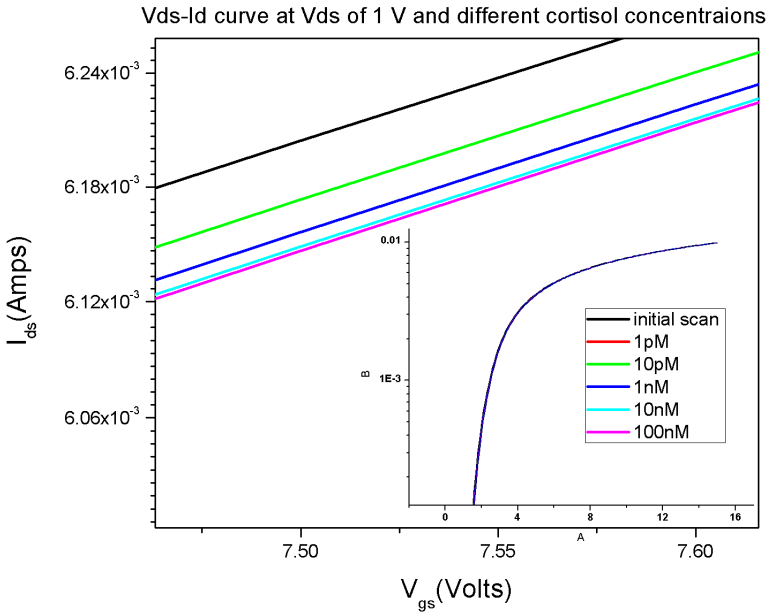

Figure 38: Vgs-Id curve of the EGFET-MIP device at different cortisol concentrations. The drain voltage was kept as 1V

6.7 Conclusions

MIP based EGFET sensors were fabricated in this chapter. The MIP cortisol sensor fabricated on SPCE in the previous chapter was used for this research. The working electrode was connected to the gate of the FET and the Ag/AgCl reference electrode was supplied with a gate voltage using an external power supply. The EGFET could detect the changes in PBS solutions of Different Concentrations. It was also able to detect the various concentration of Cortisol in PBS solution. The Limit of detection of this sensor was found to be 1 pM. The fabricated sensor paves the way for label-free, fast, low cost and direct sensing of cortisol. This work has potential applications in POC sensing of cortisol.

Chapter 7: SUMMARY AND FUTURE WORK

7.1 Summary

The POC sensors are essential tools for providing healthcare and offer accurate, timely, affordable and manageable diagnostics of targeted diseases. This research aimed to explore different methodologies for sensing cortisol that would enable fast, selective, sensitive, label-free and temperature stable sensors. Thin Film-based electrochemical immunosensors were fabricated using self-assembled monolayer functionalized by the anti-cortisol antibody. These sensors were validated against Standard ELISA process using human saliva samples. The developed system was fast, reliable and utilized electrochemical approach towards sense. However, it suffered from some shortcomings. A redox label is required for making electrochemical measurements. The sensors were sensitive to temperature variations, and the system provided indirect output.

Further research in Nanomaterial modified electrode for label-free sensing was conducted. Copper/Copper oxide nanoparticles were fabricated on SPCE. These nano-modified electrodes were used to fabricate immunosensors specific to cortisol. The significant advantage of using this methodology was the avoidance of a Redox medium for electrochemical measurements. The fabricated immunosensors had a linear correlation from 100 µM to 100 pM (cortisol concentration) with a sensitivity of 4.21 µA/M and the standard deviation of 0.0094. The estimated Limit of detection was found to be 6.6 nM. However, the sensing system was still plagued with temperature instability. The detecting moieties (antibodies and enzymes) were sensitive to temperature changes and were denatured easily at higher than room temperatures. A biomimetic sensor based on molecular imprinted polymer

(polypyrrole) was explored at this for cortisol detection to mitigate this challenge. The MIP based sensors were cheap to fabricate, temperature stable, gave label-free detection and were reusable. The performance of the resulting sensors was comparable to those of commercially available technologies (ELISA). The MIP sensors were fabricated on SPCE and biosensing of cortisol was demonstrated using CV. Though CV is a viable technique for POC biosensing, it does not provide a direct readout. A much simpler system is reported where MIP is along with a FET in an extended gate configuration. This system was easy to construct, can be ported to miniaturized electronics and can provide a direct readout. In this last study, cortisol of different concentration was detected in a label-free setting to pave the way for truly miniaturized reusable, cheap, fast and accurate biosensing system for POC. The performance of all the sensors fabricated in this study is summarised in Table 6 below. The MIP-EGFET sensor meets all the criteria in the table that makes it the best candidate to explore POC/wearable sensors for detection of Cortisol.

Table 6: Comparison of Cortisol sensors fabricated during this research

	SAM based Electrochemical immunosensor	Cu/CuO Nanomodified Electrochemical immunosensor	MIP based Electrochemical Sensor	MIP-EGFET sensor
Sensing element	Cortisol specific Monoclonal Antibodies	Cortisol specific Monoclonal Antibodies	MIP	MIP
Mode of detection	CV	CV	CV	EGFET
Label free	No	Yes	Yes	Yes
Reusable	No	No	Yes	Yes
Direct readout	No	No	No	Yes
Selective to	Yes	Yes	Yes	Yes

Cortisol				
Limit of detection for cortisol	10 pg/mL	0.002 pg/mL	0.0004 pg/mL	0.0004 pg/mL

7.2 Future work

This research has validated techniques and methods which has potential application for POC sensing of cortisol (a vital Steroid Biomarker for detection of stress). This work focused on addressing critical issues that face sensing systems for POC application. There are still challenges that require addressing before a commercially viable system is available. The molecularly Imprinted Technique is a promising technique for biosensing application. However, there is much room for improvement regarding having an extremely selective polymer.

The selectivity of the MIP can be improved by computationally exploring the interaction between the polymer and the bioanalyte.

The Simulation studies of different binding sites at the polymer can be useful for better understanding of loading/elution mechanism of the target molecule. Computationally exploring the energy required for effective removal of the target molecule without affecting the molecular backbone etc.

At the device end, the EGFET configuration is promising. It can be incorporated with miniaturized electronics to provide a direct readout in handheld or wearable device form and integrated with MIP based sensing electrodes.

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