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Screen Printed Carbon Electrode Based Microfluidic Biosensor for Sweat Cortisol Detection

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SCREEN PRINTED CARBON ELECTRODE BASED MICROFLUIDIC BIOSENSOR FOR SWEAT CORTISOL DETECTION

BY

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This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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ABSTRACT

SCREEN PRINTED CARBON ELECTRODE BASED MICROFLUIDIC BIOSENSOR FOR SWEAT CORTISOL DETECTION

SHAILU SHREE POUDYAL

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A simple, cost-effective, microfluidic, field-deployable biosensor with screen printed carbon electrode (SPCE) was developed for detection of sweat cortisol with point of care applications. Cortisol detection in artificial sweat is an important screening tool for diagnosis and monitoring of various health conditions like Addison's disease, stress disorder, and Cushing's syndrome. A self-assembled monolayer of graphene oxide (GO) is functionalized on SPCE electrode, onto which cortisol antibodies are immobilized for cortisol detection. Microfluidic system ensured precise and controlled flow of reagents and antibodies. Electrochemical measurement is done using cyclic voltammetry, as a function of cortisol concentrations. Cyclic voltammetry measurement gives current magnitude with applied voltage as a function of time. Scanning Electron Microscopy (SEM) imaging shows the change in surface morphology with the addition of antibody, compared to bare electrode functionalized with GO. The images confirm the antibody binding to self-assembled GO nanosurface on the working electrode. Raman imaging also supports the advantages of surface functionalization with antibodies. It shows presence of GO and antibodies on the biosensor surface suggesting GO self-assembly and antibodies immobilization. Atomic Force Microscopy (AFM) imaging shows surface topography of the developed sensor upon immobilization of self-assembled GO. The evenly distributed

GO provided more surface area for antibodies immobilization. Cortisol was detected in the linear range of 0.1 ng/ml to 150 ng/ml, where current magnitude decreased with increasing cortisol concentration due to reduction in number of free electrons. The developed microfluidic biosensor for cortisol detection formed the base for sweat cortisol sensor with POC applications, and can also be used in personalized health diagnosis or monitoring.

CHAPTER 1. INTRODUCTION

1.1. Background

1.1.1. Biosensor and Need for Cortisol Sensor

Sensor is a device that assists in measuring or detecting the changes in an environment and sends information to the processor for further processing. Biosensor is an analytical device that uses biological molecules like enzymes or antibodies to detect the presence of some chemicals or analytes. The development of biosensors for highly selective and sensitive detection of biomolecules or pathogens has become very important for medical diagnostics and treatment, environmental pollution monitoring, food safety and others [1, 2]. Advantages of biosensors include: ease of use, cost-effectiveness, sensitivity, high accuracy. Research on miniaturized biosensors is widely increasing due to its demand in diagnostic applications. Interest in developing small sized devices for biomedical applications is increasing rapidly, and the existing techniques are inadequate to meet the requirement of selective analyte response, fast response time and point of care applications.

Self-assembled monolayers (SAMs) have various attractive features for biosensor applications. SAMs act as a novel substrate for immobilized biomolecules like enzymes and antibodies by mimicking the cellular microenvironment of lipid bilayer structures [3, 4]. They provide special benefits for biosensor applications which involve current and potential measurement. These monolayered structures remain chemically stable even after reaction with immobilization molecules for biosensor applications, increasing their potential as biosensor or immunosensor. Nanomaterials like graphene oxide (GO) have the ability of forming self-assembled structure [5]. Nanomaterials like GO help improve the sensitivity of biomolecule detection due to their high surface area to volume ratio and size

similar to biomolecules. GO is a potential nanomaterial for applications like biosensor, drug delivery, and nanocomposites. Its ease of surface modification, size control, and unique optical properties have made significant improvement for its use in biomolecule detection or as biosensor [6]. These properties when incorporated to make a biosensor for detection of cortisol can make a huge impact for whole mankind and medical industry. Cortisol is a steroid hormone that plays an important role in balancing blood pressure and glucose levels, and controls carbohydrate metabolism [7, 8]. As cortisol is released in response to stress and low-blood glucose concentration, cortisol level measurement could be used as a powerful tool in medical field for diagnosis, real time and point of care applications. Studies have shown a direct positive relationship between the decline in work performance and salivary cortisol levels after anxiety and stress induction.

1.1.2. Cortisol and Its Role in Human Health

Individuals are suffering from psychological stress and the number is increasing each day, due to globalization, life style, and competition [8]. Due to increased stress level, life-threatening conditions like heart-attack, brain-problems, depression have become major issues in developed countries. Stress creates problem not only to the brain, but also to the entire system. Psychological stress has been increasingly alarming due to people's everyday lifestyle with competition in every field like education, business and even social life. Due to prolonged stress exposure, signaling pathways from brain are triggered, leading to release of cortisol from adrenal cortex. The increased stress level causes stress cycle to find its way into the nervous system and affect the whole-body chemistry.

Cortisol is a glucocorticoid hormone synthesized in the adrenal cortex of the kidney that plays an important role in balancing blood pressure and glucose levels, controls

carbohydrate metabolism, cognitive function and electrolyte balance [9-11]. But the cortisol hormone is not released with consistency. Due to various stress condition, there is deviation of cortisol concentration from the standard levels in sweat, saliva and serum [12-14]. This deviation thus influence the endocrine, circulatory, and immune system to achieve homeostasis in human body [15]. The pituitary gland in the brain regulates the amount of cortisol produced by adrenal glands. The pituitary gland secretes the hormone adrenocorticotropin (ACTH) to stimulate adrenal glands to release more cortisol, when the level of cortisol is low. But when the cortisol levels are higher, pituitary gland decreases its output of ACTH [16]. The main component of body's adaptive system responsible for maintaining regulated physiological process is hypothalamic–pituitary–adrenal (HPA) axis, which changes with changing environmental factors. Cortisol is the end- product of the HPA axis.

Cortisol is the key biomarker for determining individual's stress level. It takes charge of regulating variety of physiological functions in human body. Cortisol hormone regulated different body functions like glucose metabolism, immune function and inflammatory response [17]. The measurement of cortisol level in saliva can also help determine aggressive nature of teenagers [16]. This hormone is also responsible for balancing blood pressure and glucose level. It also affects the immune system, and endocrine and circulatory systems to maintain homeostasis. Studies have shown that cortisol follows circadian rhythm through 24hours cycle with variation in cortisol levels. Other factors affecting cortisol level are eating habits and physical activities. Variation in cortisol level is mainly dominated by psychological and emotional condition, and is thus commonly known as **"stress hormone"**. There is growing interest in measurement of

cortisol to develop a system where cortisol variation can be used as an indicator for medically and physiologically relevant events like stress, post-traumatic stress disorder [12]. It also plays important role in sports medicine. Determination of natural glucocorticoid and other substances is important for athletes before and after stress for doping control purposes. There is increased need for regular testing for illicit drug content in clinical or forensic samples [18]. Levels of cortisol in the body change throughout the day [19]. They are highest in the morning, dropping rapidly until mid-day, and gradually declining throughout the rest of the day and lowest at night [20].

Abnormal increase in cortisol concentration leads to various disorders like inflammation inhibition, immune-system depression, increased amino and fatty acid levels in blood. Studies have shown that increased cortisol level helps developing Cushing's syndrome with symptoms of obesity, fatigue and fragile bone, while its decreased level contribute to Addison's diseases with symptoms like weight loss, fatigue, darkening of skin folds and scars. Abnormal cortisol levels can also influence health conditions like type II diabetes [21, 22] constant stress, obesity, metabolic syndrome and others. Increased level of cortisol can also influence the severity of epileptic seizures due to increases in the adrenocorticotropic hormone as well as cortisol levels. Symptoms of cortisol deficiency include: fatigue, nausea, vomiting, low blood pressure, orthostatic hypotension, low blood sugar, shock and coma [23].

Increase in cortisol levels in plasma, serum, urine have been observed in different space missions. This can lead to elevated stress level, as well as motion sickness during launch or re-entry to the space. So, it is very important to detect cortisol levels before,

during and after space flights to study and ensure human health in space, and more importantly during long-term missions [18].

Different cortisol sample sources are urine, blood, sweat, saliva, interstitial fluid (ISF). Urine sample collection is done over a period of 24-hours, which is time consuming and inconvenient for patients. Cortisol level in ISF is much higher than in saliva, but low harvesting rate limits its applicability for point of care. Sweat cortisol is the best source for biosensor applications and for continuous monitoring. Compared to other cortisol sources, sweat can be used for regular cortisol level detection without patient discomfort.

1.1.3. Cortisol Sensing at Point of Care

The state of art techniques used for detecting cortisol are limited due to requirements like portability, cost, analysis time, need of skilled personnel for system operation, long time for analysis. There is a need for development of point of care microfluidic system for timely diagnosis and treatment. Microfluidic systems have the advantages of small sample volume and reagent, precise control of fluidic flow, cost-effective, and applicable for point of care treatment [24]. The error that can be created due to human handling can be prevented with the use of microfluidics, minimizing occurrence of false positive results.

Here, we propose a portable, handheld, rapid, cost-effective, flexible, field-deployable carbon-based paper sensor electrode with POC for sweat cortisol detection. The developed biosensor used a novel nanomaterial GO which has the ability to form SAM to improve the sensitivity of the sensor. The biosensor has been developed for field or on-site cortisol detection. Different antibodies were functionalized on GO biosensor surface for artificial sweat cortisol detection.

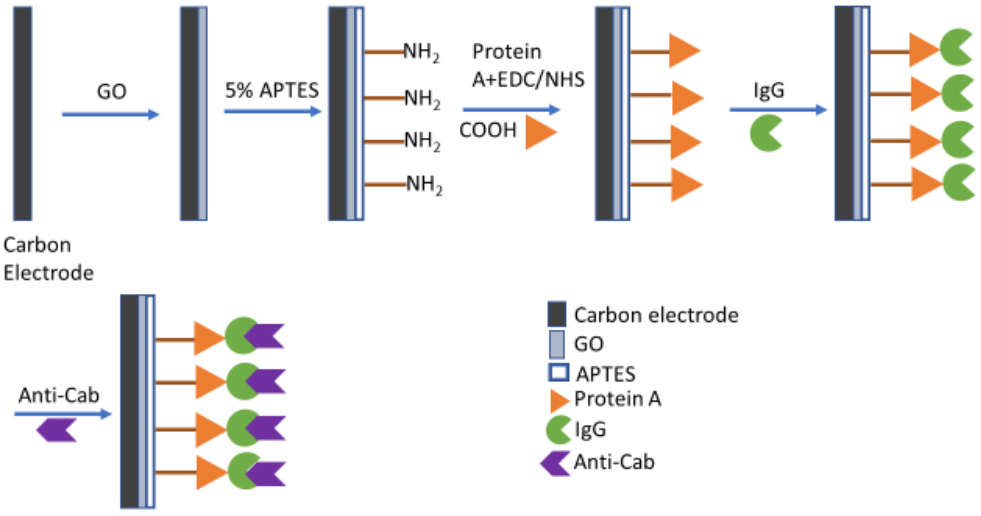

Figure 1. 1 Overview of Developed Cortisol Sensor

1.2. Previous Works

1.2.1. Currently Used Approaches for Cortisol Measurement and their Drawbacks

As the cortisol is released in response to stress and low-blood glucose concentration, cortisol level measurement could be used as a powerful tool in medical field for diagnosis and real-time application. Cortisol affects not only physical performance, but also, cognitive functions by altering information processing [25, 26]. Studies have shown a direct positive relationship between the reduction in work performance and salivary cortisol levels, after stress and anxiety induction. Disposable, microfluidic, low-cost cortisol sensors can overcome the limitations of existing measurement techniques like cortisol detection from hair or saliva. Cortisol measurement from hair or saliva is not feasible for real-time measurement, and use of wearable system can generate a detection

tool with ease for patients. The current cortisol detection methodologies like ELISA, QCM, SPR, and chromatograph are limited to laboratories including complex instrumentation, longer processing time and need of a professional, making the job complex [15, 27, 28]. The other shortcoming of existing techniques is that it only provides information about the cortisol sample taken in the laboratory, and lack information about the variation in cortisol level observed with environmental changes.

Chromatography technique is based on the mass transfer induced adsorption and detects cortisol from hair, saliva, blood and others. Liquid chromatography and mass spectroscopy were developed for identification and quantification of cortisol, but the results were influenced by the sex [29]. The continuous monitoring of cortisol at different point of time with variation in cortisol level, can help clinicians provide better diagnosis and treatment for cortisol related health issues. With the current technologies, for the continuous monitoring of cortisol for 24-hours, patients must be admitted in the hospital and should go through stress like pain and discomfort. Development of a portable, disposable and cost-effective system can become a boon for point of care application and for medical industry. Here, to overcome the limitations of state-of-art techniques, we propose to develop a highly sensitive disposable, microfluidic sweat cortisol sensor from sweat, which has application for point-of-care detection and making it easier to get useful information for timely diagnosis and treatment.

1.2.1.1. Chromatographic Techniques for Cortisol Detection

One of the oldest approaches for detection of cortisol are chromatographic techniques. In this technique, cortisol is separated from hair, saliva, urine, serum samples by the process of mass transfer induced adsorption. Raul et al. developed a system using

LC-MS for detection of cortisol from hair samples [29]. The results obtained were not influenced by hair color, but influenced by sex. Chen et al. developed a nanofiber packed solid-phase-extraction (SPE) tip based device to achieve high throughput sample extraction and detect cortisol from saliva [30]. The SPE system was able to overcome low extraction recovery and bad cleanup effect of conventional methods, and thus improved the sensitivity and selectivity. But the sample preparation procedure through this technique is time-consuming and can also cause manual handling error. Gao et al. developed the high-performance liquid chromatography (HPLC)- fluorescein labeled (FLU) method for detection of human hair cortisol [31]. The technique detected cortisol over a physiological range. But the system lacked specificity required for measuring low concentrations and also the effect of interference from other substances from the sample limits their point of care applications. HPLC also requires many preprocessing procedures.

1.2.1.2. Immunoassays for Cortisol Detection

The ability of an antibody to recognize and bind selectively to a specific antigen forms the basis of immunoassays. Immunoassay is also known as the gold standard for the detection and measurement of concentration of the analyte due to high degree of selectivity and specificity of antigen-antibody complex. Different research groups have applied radioimmunoassays (RIA) for the detection of cortisol [32-34]. This system uses radioisotopes as labels for cortisol detection. But due to harmful effects of handling radioisotopes, the use of RIA has been limited. Appel et al. used fluorescein isothiocyanate (FITC) as a fluorescent tag with mixture of sulfuric acid and acetic acid as the labels in cortisol immuoassays [35]. The concentration of fluorescence-labeled analyte is proportional to intensity of fluorescence. The drawback of the system is that it requires

costly fluorescent microscope and also the fluorescein label degrades with time. Shi et al. developed a chemiluminescence (CL) system for cortisol detection which was based on the reaction between silver complex and luminol [36]. The detection method was applied with flow injection analyses (FIA) to find free cortisol in human serum. The developed system was highly sensitive and convenient for cortisol detection, but it used blood serum as a source for cortisol. It used an invasive approach and has limited applicability.

Manenschijn et al. used salivary ELISA kit for detection of cortisol from hair samples [37]. The system was able to find positive correlation between hair cortisol and waist circumference and waist to hip ratio, but no correlations were found between hair cortisol levels and blood pressure or age. The ELISA method has limited applicability due to need for large sample reagents, complexity due to multiple assay steps and longer incubation times. Immunosensor based on quartz crystal microbalance (QCM) was applied for detection of cortisol by Atashbar et al. In this method, piezoelectric crystal with protein A was used for cortisol detection. The developed sensor was simple, with short analysis time, cost-effectiveness and selectivity, but the system needed improvement in terms of its limit of detection [38].

1.2.1.3. Surface Plasmon Resonance (SPR) for Cortisol Detection

When the conducting substrate is irradiated with light, valence electrons start oscillating which forms the basis for SPR based detection system. It is highly sensitive to the molecules adsorption onto the substrate, where adsorption of molecules onto the substrate is observed when resonance curve shifts to higher wavelength. It is used for quantitative measurement of captured analyte on antibodies coated substrate [15]. Mitchell et al. developed a SPR based immunosensor using cortisol-linker conjugate for cortisol

detection in saliva to provide high assay sensitivity. It used a coating antigen in a microfluidic SPR immunoassay using secondary antibody for enhancement of signal. The automatic immunosensor showed high sensitivity in short time period. But the immunosensor system still lacks POC applications and treatment approach for patients [39].

Richard C. Stevens et al. developed a biosensor for detection of cortisol in saliva using portable SPR system. A six-channel portable biosensor system was developed which used cortisol specific monoclonal antibodies [40]. A portable biosensor for cortisol detection with flow cell was designed which reduced non-specific binding by delivering only small molecular weight analytes to sensor surface. But the system generated salivary cortisol curve by subtracting the reference channel data from cortisol channel data. During this procedure, data generated can also overlap with the reference sample, affecting the complete reliability of developed system.

Marco Frasconi et al. developed an immunosensor for cortisol detection using SPR based system. The immunosensor was developed for real-time detection of cortisol and cortisone levels in urine and saliva samples. The developed system was simple, rapid, sensitive and reproducible due to use of polycarboxylate-hydrogel based coatings for antibody immobilization [18]. The results obtained from the developed system correlated well with reference LC-tandem MS method for cortisol and cortisone detection from urine and saliva samples. The SPR based system used urine and saliva samples for detection of cortisol. Collecting urine or saliva samples for continuous monitoring of cortisol is not convenient for patients and also cannot be applied for continuous monitoring.

1.2.1.4. ELISA/ GC-MS/ LC-MS

E. Pujos et al. analyzed corticosteroid using three different techniques: ELISA (Enzyme-linked Immunosorbent Assay), gas chromatography-mass spectroscopy (GC-MS) and liquid chromatography-mass spectroscopy (LC-MS). Urine sample was used for analysis of corticosteroids in different human samples. The possibilities and sensitivity of each approach were analyzed [41]. The experimental procedure followed for analysis showed that ELISA cannot be used for the assay when screening for the presence of steroids. Problems were observed while interpreting the results, due to differences between compound cross-reactivity, which can lead to the situation of false-positive results. The GC-MS and LC-MS methods used are time consuming approaches with expertise required for instrumentation handling. Urine sample used for analysis is not feasible for continuous monitoring and point of care applications.

Liquid-chromatography and mass spectroscopy are also used for measuring salivary cortisol but requires a slow cortisol extraction step [42]. The LC-MS technique also suffers from long detection time, long sample preparation time and high detection limit. Detection limit of cortisol obtained from both bioluminescent immunoassays and electrochemical immunoassays are in the range of pg ml^{-1}, but limit of detection for LC-MS is limited to $ngml^{-1}$ range.

Different commercial kits using ELISA technique are available to measure and monitor cortisol in saliva. These are the reliable techniques which provide accurate and reproducible results but takes several hours for analysis. Other immunoassays like time-resolved fluoroimmunoassay, [43] rapid quantitative immunodetection using a lateral flow

assay,[44] and luminescence immunoassays [45, 46] are also used for measuring salivary cortisol level. Theses immunoassays are also time-consuming or lack quantifiable results.

Mara Mirasoli et al. developed a solid-phase competitive immunoassay for detection of cortisol in saliva using mutant of the photoprotein aequorin as a label. Bioluminescent protein, aequorin was used as a highly sensitive label for cortisol measurement in saliva [46]. Cortisol levels in saliva samples collected from 15 healthy subjects were detected and analyzed with sensitivity, accuracy and precision. The use of immunoassay system is a time-consuming procedure with possibility of getting false positive results.

1.2.1.5. Electrochemical System with Metal Electrodes

Label-free electrochemical immunosensing method has become the most promising alternative to optical detection. These are gaining in popularity due to their low cost, high sensitivity and very low limit of detection [15]. Sun et al. developed a micro-fabricated Au electrode based immune-electrochemical system using alkaline-phosphatase (AP) enzyme for salivary cortisol determination [47]. In the reaction between AP enzyme attached to cortisol and antibodies in p-nitrophenyl phosphate (pNPP) solution, p-nitrophenol (pNP) was generated which was detected by CV measurement. The system showed lower limit of cortisol detection, but lacked continuous monitoring of salivary cortisol. SAM of Dithiobis (succinimidyl propionate) modified with interdigited microelectrodes were used by Arya et al. for cortisol detection using electrochemical impedance (EIS) method. They also developed the same system for detection of cortisol in ISF (Interstitial Fluid) in vitro [48, 49]. But the cortisol detection from ISF is not feasible for continuous monitoring of cortisol which varies at different times of the day.

Xiaoqiang Liu et al. developed an electrochemical immunosensor with gold nanoparticles for detection of cortisol from serum [50]. The electrochemical immunosensor consisted of Au electrode modified by Au nanoparticle/Protein G–DTBP-scaffold to enhance the detection of the model analyte cortisol. Au nanoparticles deposited on Au electrodes provided larger surface area for biomolecules interaction, and improved the sensitivity, dynamic range and detection limit of the analysis. But the developed sensor detected cortisol from serum sample, which is an invasive method for sample collection. This system lacks continuous monitoring of patient cortisol and also the device is not cost-effective.

Electrochemical immunosensor was developed by Phani KiranVabbina et al. for the detection of cortisol using zinc oxide nanorods and nanoflakes synthesized on Au coated substrates [51]. 1D nanorod provided high surface area, while 2D nanoflakes provided high surface charge density and also promoted higher Anti-Cab loading and thus better sensing performance. The prepared sensors provided good sensitivity for detection of saliva cortisol. Both the nanorods and nanoflakes were synthesized by sonochemical method on Au-film by e-beam evaporator. The process used for sensor fabrication is complex and not cost-effective. The sensor cannot be applied for point of care evaluation and applications.

Chaker Tlili et al. developed chemiresistor immunosensor for stress biomarker cortisol in saliva based on single walled carbon nanotubes [52]. The nanotube based immunosensor provided rapid, label-free measurement of salivary cortisol. Easy to use, field deployable immunosensor was developed for detection of stress biomarker cortisol in saliva. But the developed immunosensor failed the matrix selectivity test. The resistance

of immunosensor remained same, neither increased or decreased when exposed to ten-fold diluted artificial saliva, the matrix in which cortisol should be determined for clinical diagnostic applications.

Arun Kumar et al. developed an ultrasensitive cortisol detection system using functionalized gold nanowires [53]. The enzyme fragment complementation (EFC) technique used make it possible to detect cortisol from a system with very low concentration by enzymatically amplifying the signal using functionalized gold nanowires. EFC technique was used to improve the sensitivity and minimize the signal to noise ratio of the developed sensor. The functionalized nanowires provided better electron transfer due to increased surface to volume ratio and high electrical conductivity, and thus improved the sensitivity and detection limit of the biosensor. The electrode system used for the detection of cortisol used gold nanowires as WE and platinum deposited on silicon chip as WE, which make the system cost-ineffective and non-flexible.

1.2.1.6. Disposable Sensor with Magnetic Particles

Maria Moreno Guzman et al. developed an electrochemical immunosensor for detection of cortisol using screen-printed carbon electrodes functionalized with magnetic particles [54]. Magnetic beads functionalized Protein A with alkaline phosphatase labeled cortisol and anti-cortisol were functionalized onto the working electrode of SPCE. A neodymium magnet was placed on the bottom of working electrode to localize magnetic beads onto the working electrode. The developed system provided high analytical performance in terms of sensitivity, selectivity and wide range of quantifiable analyte concentrations. But when the magnet was placed below the working electrode, there can be variation in bead layer thickness or the spreading area of the working electrode surface

can be variable between different measurements. This limits the sensor's application for use in point of care or for proper diagnosis.

Moreno-Guzman et al. fabricated nanomaterials based system for cortisol detection with enhanced sensitivity and selectivity. They developed an immunoassay with anti-cortisol antibody immobilized onto protein A modified magnetic particles [54]. The system was developed for detection of cortisol in human sera samples.

1.2.2. Electrochemical Approach for Cortisol Detection

Measurement of changes in electrical properties of a conductive material forms the basis of the electrochemical immunosensing, which is due to the adsorption of an analyte on antibodies functionalized surface. Electrochemical cortisol immunosensor have been growing rapidly due to their fast speed, low cost and excellent performance [50]. The high specificity is observed in these immunosensors due to the strong affinity between an antigen and its specific antibody immobilized on the sensor. With the change in the concentration of the electro active redox species, electrical change is observed at the electrode. The electrochemical system using nanomaterials on metal electrodes have been gaining popularity for detection of cortisol. These sensors also provide with high sensitivity and selectivity for timely diagnosis and treatment.

However, cortisol hormone is not released with consistency. There is substantial deviation of cortisol concentration from the standard levels in sweat, saliva and serum, which is related to various stress conditions. This deviation influences the endocrine, circulatory, and immune system to achieve homeostasis in human body.

Different researchers have been using different sources of cortisol: hair, salivary and sweat because of their ease of use and non-invasive nature. But hair and salivary cortisol have some limitations as mentioned earlier and are not suitable for their application in continuous monitoring, despite of their several advantages. Hence, sweat cortisol could be the promising future sensor research target for continuous monitoring of cortisol with comfort ensured to patients. There are many challenges in terms of sensitivity and selectivity using sweat cortisol, but different functionalization procedure using nanomaterials can help overcome these problems and thus a health monitoring system with point of care service can be developed.

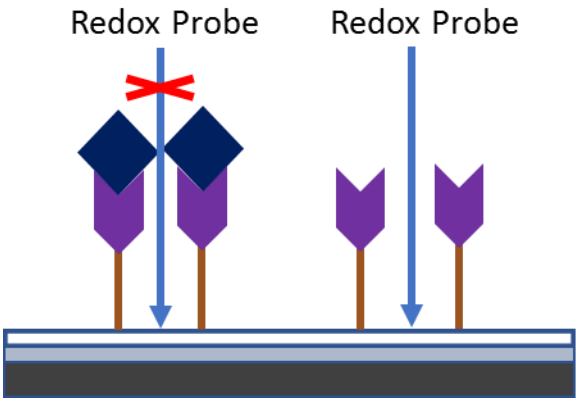

Figure 1. 2 Schematic Diagram for Electrochemical Immunosensing

Here, we propose to develop a novel electrochemical biosensor system for cortisol detection from sweat using a microfluidic based disposable and flexible paper sensor with point of care application, that can be used for continuous health monitoring and provide patients with timely diagnosis and treatment. The detection of cortisol from sweat is a non-invasive approach that performs a specific monitoring of target analyte in physiological conditions. Portable miniaturized analytical devices for cortisol detection and its

continuous monitoring at POC could be useful to personalize health diagnostics for appropriate effectual and exact treatment [55, 56]. The sensor system is screen printed carbon electrode functionalized with nanomaterial GO, incorporating protein A along with anti-Cab and cortisol. The three electrodes system consisting of working electrode (WE), reference electrode (RE) and counter electrode (CE), is highly sensitive and provide low limit of detection. The simple structure with low fabrication cost has increased the applicability of three electrode system.

Paper based three electrode systems are becoming popular over conventional Si-SiO_2 based electrode system. Some of the advantages of paper sensor include: cheap flexible disposable system with point of care applications. Paper sensors are more popular compared to other electrode based system as the paper is ubiquitous, inexpensive, easy to transport and store [57]. Paper also has the ability of capillary action, eliminating the need for an external source of power. Paper can filter particulates from a sample. It can also be used for storing blood, saliva samples. Another advantage of paper sensor is the possibility channel patterning is by photolithography.

1.2.2.1. Paper Based Electrochemical System with Nanomaterial

The paper based electrode system is flexible, disposable system consisting of three electrodes. The WE is coated with carbon, while RE and CE are coated with sliver-silver/chloride (Ag/Ag-Cl). The carbon coated WE helps in easy functionalization. The paper sensor has following dimensions:

- WE: 3mm (carbon coated)
- RE & CE: Ag/Ag-Cl

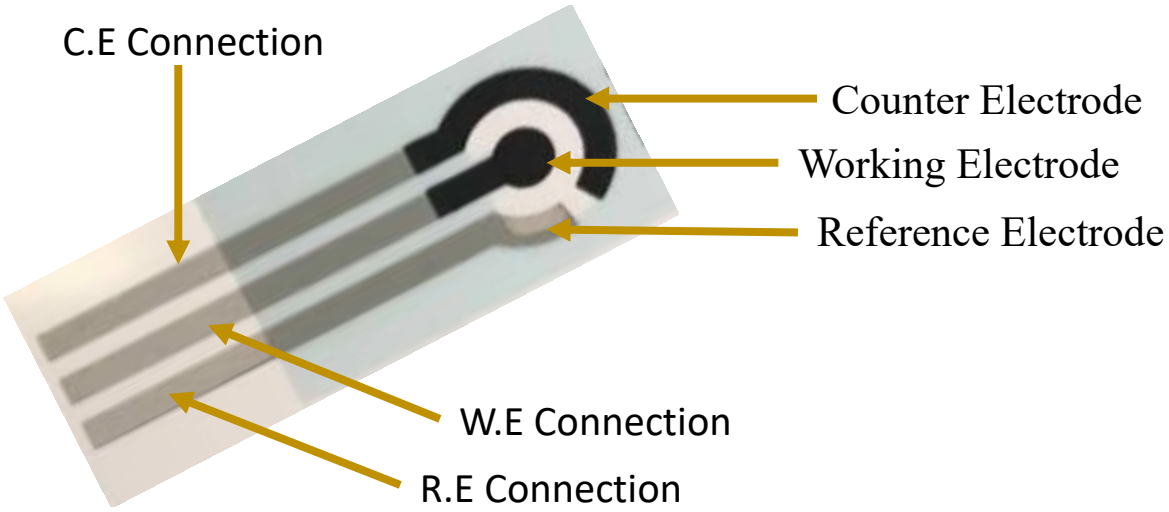

Figure 1. 3 Picture of a Paper Sensor with Different Electrodes and Connections

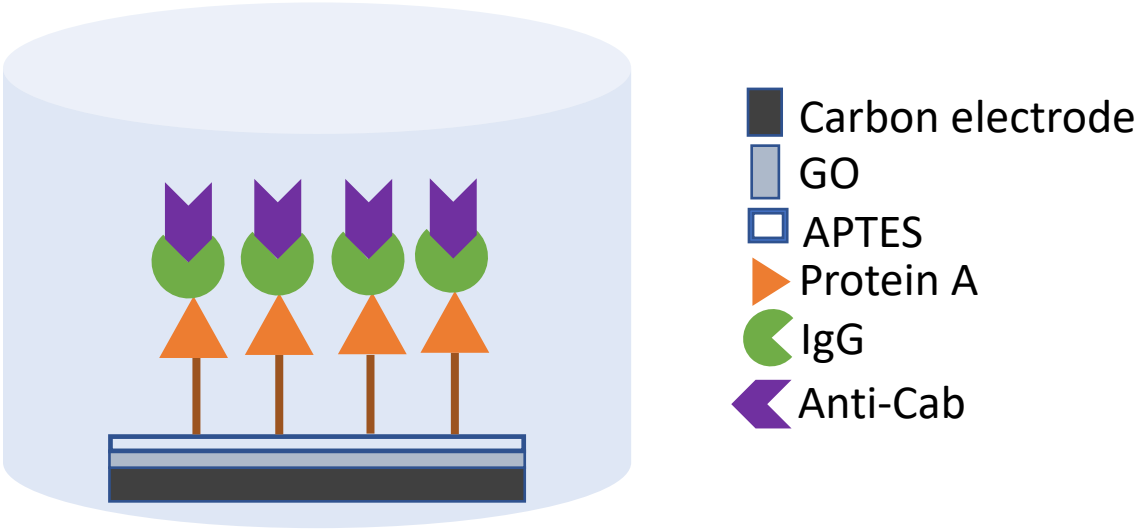

Figure 1. 4 Functionalization on the Carbon Working Electrode with Microfluidic Block

There are various advantages of nanomaterials for biosensor applications. Nanomaterials have been widely used for biosensor application due to their improved sensitivity for biomolecule detection by offering high surface area to volume ratio and also have size similar to biomolecules like enzymes or antibodies [6]. The use of nanomaterials for application in biosensor is gaining popularity due to its various advantages. Some of the advantages of nanomaterials are self-assembly property, nanorough surface or increased surface area for more interaction with antibodies and target antigens. Increased surface area provides specific interaction between the nanomaterial and antibodies [58]. Graphene oxide is the novel nanomaterial with several advantages. GO is one of the promising nanomaterial with variety of applications in biosensing, drug delivery and nanocomposites. Its ease of surface functionalization and unique optical properties make it an attractive material for biomolecule detection [6].

1.3. Motivation

There is a need for cost-effective, sensitive, flexible, microfluidic, field deployable sweat cortisol sensor with point of care applications.

1.4. Objectives

The objective of this work is to develop a biosensor system with high performance and sensitivity for cortisol detection. The tasks performed to achieve the objectives are: (a) prepare GO solution to form the novel nanomaterial platform, (b) functionalize different antibodies and cortisol on paper sensor, (c) perform cyclic voltammetry, CV measurement for detection of cortisol in sweat, (d) SEM imaging of GO on the gold/carbon electrode surface, (e) Raman imaging to show the surface structure after GO and antibodies

functionalization, (f) AFM imaging to show surface topography of only GO film on sensor surface.

CHAPTER 2. THEORY

2.1. Different Sources of Cortisol Sample

2.1.1. Hair Cortisol

Human hair grows with the predictable growth rate of about 1 cm/month which makes hair cortisol useful for cortisol research. Naturally growing hair can be a source of regular and consistent supply to research laboratory. Being the non-invasive source of cortisol, it has become popular for use in cortisol sensor. There are different factors affecting hair cortisol concentration (HCC) which limits its validity and reliability. Study by Nicolass H. Fourie et al. showed that hair cortisol concentration is affected by age and sex [59]. The variation in cortisol concentration limits the applicability of HCC as reliable source for medical diagnostics.

2.1.1.1. Limitation of HCC

HCC is variable for patients with bipolar disorder and also for anxiety patients [60]. Researches have shown that hair cortisol concentration has no proper correlation with human body stress level. Since, several factors affect HCC, it cannot be used as a tool to measure rapid changes in stress. Results obtained from HCC research must be validated with well-established methods like ELISA, radio immunoassay, chemiluminescence, GC/MS or LC/MS [61, 62]. All these problems hinder the implementation of HCC as a tool for point of care cortisol monitoring.

2.1.2. Salivary Cortisol

Saliva cortisol is used as a tool for monitoring and measuring cortisol concentration. It has been widely used because of its non-invasive nature and ease of use. Sample collection can be done in a short period of time, which enables quick sample collection and

decreases patient's stress as well [63]. Another benefit of salivary cortisol is that, it is usually available in biologically free state, compared to other cortisol like blood cortisol bound to other proteins [64]. The biologically free state cortisol makes it more attractive for cortisol sensor researches. Both traditional immunoassay methods and recent nanomaterials based methods use salivary cortisol as a tool for monitoring cortisol level.

2.1.2.1. Limitation of Salivary Cortisol

Despite many advantages of salivary cortisol, its application is limited for continuous real-time monitoring of cortisol. Saliva sample cannot be collected continuously from the patients for longer period of time. Hence, its application is limited for reflecting real-time stress level of patients. But it can be still used as a significant tool as a reference for the cortisol research.

2.1.3. Sweat Cortisol

Sweat is the body fluid that provides significant information about health for diagnostic purposes. Different proteins and metabolites contained in sweat help identify disease or infection and also provide information about general health status [65]. Researches are being done for real-time health monitoring using sweat to detect glucose, lactate or other electrolytes. But no biosensor system has been successfully developed to detect cortisol from real sweat. Development of a sensor system using sweat cortisol is a challenging process, as it requires extremely high sensitivity compared to other forms of cortisol. According to M. Jia et al. in the experiment quantified by HPLC-SRM, eccrine sweat cortisol concentration ranged from 0.24 ng/ml to 2.80 ng/ml, when the sample was collected from 1pm to 4pm [42]. This range is more than 10 times smaller than salivary cortisol concentration making it more difficult to develop point-of-care cortisol sensor.

Different approaches have been applied to enhance the sensitivity and selectivity of cortisol in sweat. Nanomaterials like Zinc oxide has been used by R. D. Munje et al. to immobilize number of linker molecules for specific detection of cortisol [66]. However, the existing techniques have still not achieved enough sensitivity and specificity to detect sweat cortisol.

Sweat is the next level tool that can be applied to develop point of care cortisol sensor system which allows continuous monitoring of sweat compared to saliva or hair, to assist health treatment and diagnosis. To obtain a cortisol biosensor system with enhanced sensitivity and selectivity, various approaches can be applied. These include:

- Novel nanomaterial

  Kim et al. used nanomaterial reduced graphene oxide (rGO) to detect salivary cortisol [67]. Cross-linking chemicals like EDC and anti-cortisol were functionalized to detect salivary cortisol. The results obtained were highly promising and showed high sensitivity with a limit of the tens of picomolar range and outstanding selectivity with little reaction to aldosterone and progesterone which are cortisol analogs. GO can be one alternate novel nanomaterial that can be applied to develop cortisol sensor with higher sensitivity. Other nanomaterials like metal nanoparticles, quantum dots, magnetic nanoparticles can also be used. One major advantage of nanomaterial is its higher surface to volume ratio which enhances the sensitivity.

- Microfluidics

  Biosensor incorporating microfluidics have various advantages like automation, efficiency, reduction of cost. PDMS chamber when used as a

microfluidic platform allow precise and controlled flow. Less volume of reagents is required when using microfluidic chamber. It also provides better analysis when evaluating through electrochemical system like cyclic voltammetry. Miniaturized sensing system like microfluidics help decrease the probability of human errors and sample volume required [68, 69]. The results can be used for the timely disease diagnostics and be used as health informatics. Various advantages of microfluidic systems include: small sample volumes, small reagent volume, precise control of fluid flow, controlled environment for biomolecule reaction, point of care application with reduced form factor. Microfluidic systems also minimize human errors like handling and operation and thus reduce the probability of false positive results.

2.2. Biosensor

An analytical device that converts a biological response into an electrical signal is known as biosensor or biological sensor. Biosensor can also be defined as the chemical sensing device in which biological response is coupled with transducer for developing some complex biochemical parameter. Biosensor also includes sensor devices which are used for determining the concentration of substances and other biological parameters, which are not directly related to biological systems [70]. It is basically comprised of two main components: biological system acting as a sensor and an electronic component for signal detection and transmission. Biosensor is composed of transducer and biological elements like an antibody, enzyme, or a nucleic acid. When the analyte interacts with the bioelement, biological response is converted into electrical signal by the transducer. The interaction of sensor with analyte generates signal in the form of electrical, optical or

thermal. The signal is then converted by a suitable transducer into a measurable electrical parameter like current or voltage. The application of biosensor is widely increasing for measurement of wide spectrum of analytes like organic compounds, gases, hormones or ions, bacteria or viruses.

The paper based sensor that was used for the experiment was composed of cortisol as an analyte and an electrochemical transducer. The electrodes are commonly made of gold, silver, platinum, or carbon-based materials. In the experimental scenario, silver/silver-chloride was the electrode for signal transduction. The amperometric transducer detect changes in current which occurs due to oxidation or reduction. The current will show the reaction that occurred between the analyte and the bioelement [71].

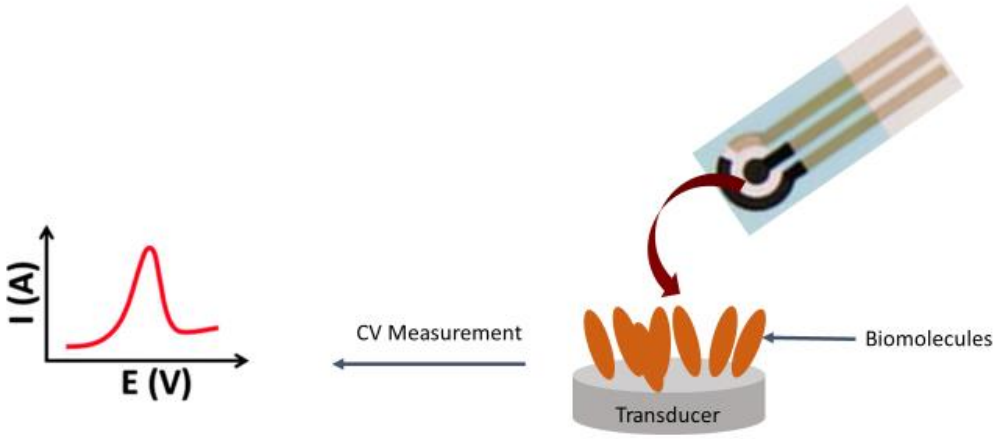

Figure 2. 1 Electrochemical Measurement of Biomolecules

Electrochemical transducer provides the changes in the form of electrical signal which is proportional to the analyte concentration. Electrochemical reaction takes place at the electrode-electrolyte interfaces and help electricity to flow between the electrode and solid or liquid electrolyte.

2.3. Cyclic Voltammetry

Cyclic voltammetry is a powerful, simple and rapid method that characterizes the electrochemical behavior of analytes that can be oxidized or reduced electrochemically. The electrochemical measurement technique that measures the current developed at the working electrode in an electrochemical cell when voltage is applied is called cyclic voltammetry[72, 73]. It is used for studying the electrochemical properties of an analyte in solution. It is used for monitoring redox behavior of chemical species within a wide potential range. It monitors the current at the working electrode as a triangular excitation potential is applied to the electrode. The voltammogram obtained can be used for analyzing fundamental information regarding the redox reaction.

Cyclic voltammograms are also regarded as the electrochemical equivalent to the spectra in optical spectroscopy. The working electrode is controlled compared to reference electrode, Ag/AgCl electrode. The excitation signal is the controlling potential applied across the WE and the auxiliary electrodes. The excitation signal is linearly varied with time, it is first scanned positively. The potential is then applied in reverse, which cause negative scan back to the original potential to complete the cycle. According to the requirement, single or multiple cycles can be used on the same surface. A CV plot is the plot of response current at the working electrode against the applied electrical potential.

During the forward scan, potential is positive to oxidize $Fe(CN)_6^{-4}$, resulting in anodic current due to the electrode process,

$$Fe(CN)_6^{-4} \rightarrow Fe(CN)_6^{-3} + e$$

During the procedure, electrode acts as an oxidant and the oxidation current increases to a peak. When the concentration of $Fe(CN)_6^{-4}$ decreases at the electrode surface, current start decaying. When the scan direction begins in negative, reverse scan potential is still positive enough to oxidize $Fe(CN)_6^{-4}$. This allows anodic current to continue even though the potential is scanning in reverse direction. $Fe(CN)_6^{-3}$ will be reduced by the electrode process when the electrode is sufficiently a strong reductant, that was formed adjacent to the electrode surface. This will lead to cathodic current formation which will form a peak and then decay as $Fe(CN)_6^{-3}$ and is used up in the solution adjacent to the electrode.

$$Fe(CN)_6^{-3} + e \rightarrow Fe(CN)_6^{-4}$$

In the positive scan, as per the anodic process, $Fe(CN)_6^{-3}$ is generated electrochemically from $Fe(CN)_6^{-4}$, and while in the reverse scan, as per the cathodic process, $Fe(CN)_6^{-3}$ is reduced back to $Fe(CN)_6^{-4}$.

Its versatility and ease of measurement have made it a popular tool in the fields of electrochemistry, organic chemistry and biochemistry. CV measurement is done for the electrochemical study of biological material, compound or an electrode surface. It has the capability of rapidly observing the redox behavior over a wide range of potential.

In cyclic voltammetry, the potential of working electrode is swept at specific sweep rate (volts/second), and resulting current is measured vs time. At specific switching potential, the sweep is usually reversed, and thus is called cyclic voltammetry [74].

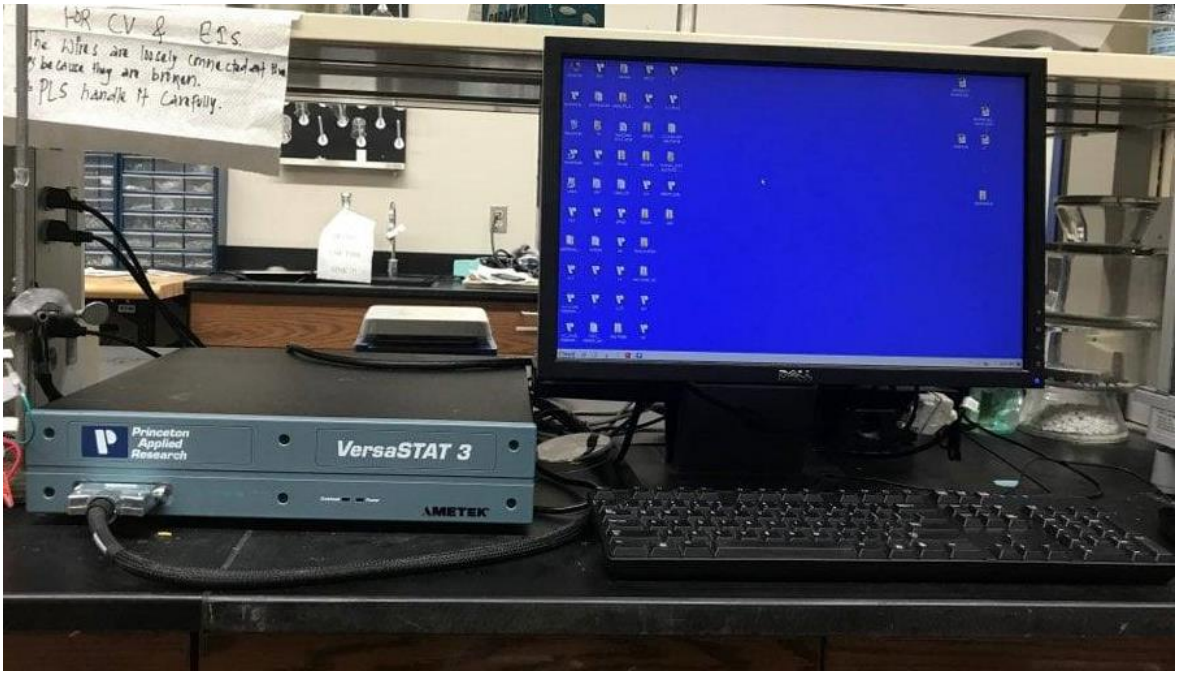

Figure 2. 2 Cyclic Voltammetry Measurement System

All the electrochemical reactions are governed by Nernst equation. It deals with the relationship of the potential of an electrode and concentration of two species O and R involved in redox reaction at that electrode.

$$O + ne^{-1} \leftrightarrow R$$

$$E = E^{0'} \frac{RT}{nF} \ln\left(\frac{C_0}{C_R}\right)$$

In the above equation, $E^{0'}$ is the redox potential containing O and R, concentration of oxidized half couple is C_0 and concentration of reduced half is C_R. In case of the redox moieties, fe3+/2+, Fe3+ is represented as O and Fe2+ is represented as R. Either of them

can be the analyte. In an amperometry experiment, potential is applied to an electrode to make a specific value of the ratio Co/Cr at the electrode surface. The concentrations near the electrode surface vary with bulk concentration. If both the concentrations are same, then no driving force is observed for transport of analyte to or from the electrode surface. If that is the condition, then oxidation/reduction current of analyte will be zero. But generally, the potential applied at the electrode surface will not be same as that in the bulk. The driving force acting for transport of analyte to or from the electrode surface is diffusion. If the concentration of Co at the electrode surface is less than Co in the bulk, then to maintain the equilibrium, O will be transported from the bulk to the electrode surface. The magnitude of current in the amperometry experiment is determined by the transport of analyte to the electrode surface, coupled with the oxidation or reduction of analyte when it arrives at the surface.

2.4. Raman Spectroscopy

Raman spectroscopy is a type of scattering spectroscopy that observes vibrational, rotational, and other low frequency modes in atomic and molecular systems [75]. It uses a laser in visible, near infrared, or near ultraviolet range to interact with molecular vibrations, phonons, or other excitations in the system, resulting in the energy of the laser photons being shifted. Infrared spectroscopy and Raman spectroscopy are the most common vibrational spectroscopies for assessing molecular motion and fingerprinting species; however, some vibrational modes are IR active, some are Raman active, and some are both IR and Raman active. In order to become Raman active, the polarizability of the sample needs to change. Raman spectroscopy uses a laser to measure molecular vibrations or phonons. It does this by observing the scattering of the laser due to polarizability changes

in the molecules. Therefore, Raman spectroscopy can be used to sense the differences in crystallization in samples of the same material. Raman spectra of same material give peaks at different wavenumber. In electrical engineering, this is a useful tool for measuring the degree of crystallization of silicon (amorphous versus crystalline) samples [76].

In Raman spectroscopy, various wavenumbers of the laser are sent across the sample and the intensity of the scattered beam is recorded and plotted for each wavenumber. In this vibrational technique, different bonds present in the molecule give rise to its own series of Raman bands that act as a fingerprint unique or specific to that particular molecular structure [77]. Crystalline silicon, for example, is expected to have a peak intensity or Raman band at a Raman shift wavenumber of 520 cm^{-1}. This spectroscopy is a useful tool providing both quantitative and qualitative information about a material. It gives information about the peak position including chemical species and symmetry, shift in position including stress and temperature effects, width including structural disorder, and intensity which is proportional to concentration.

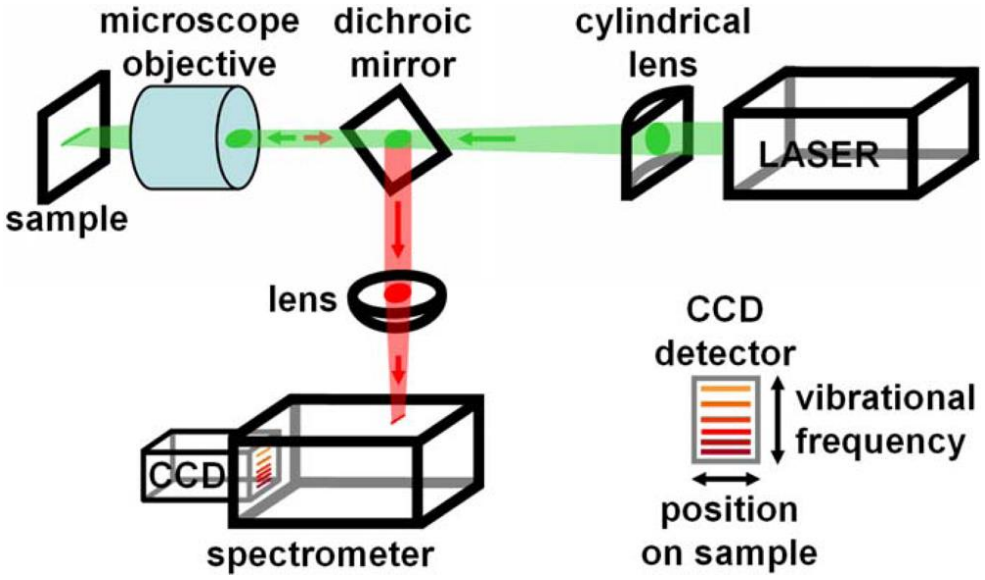

Figure 2. 3 Schematic Structure of Raman spectrometer [78]

2.5. Atomic Force Microscopy

Atomic force microscope (AFM) is a type of scanning probe microscopes (SPM). SPMs have the potential of measuring local, electrical, and magnetic properties like surface potential, height, friction by using suitable probe. The probe raster scans over a small area of the sample with the help of a probe mounted in a flexible vibrating cantilever and measures the local property simultaneously to generate an image. The force between the probe and sample is measured by AFM [79, 80]. The interaction force in non-contact mode between this probe and the sample surface is monitored and the force distribution along the surface of sample is mapped using a photodiode. This photodetector tracks the laser beam that is reflected back from the flat top of the cantilever, the bending in the cantilever causes the change in direction of reflected beam.

In non-contact mode, a precise feedback loop that corrects the oscillations amplitude deviations as the surface of the sample changes is used to restrict tip from

crashing into the sample and construct the image of surface topology. AFM examines the interaction force between the probe and the sample, and the atomic forces involved. These atomic forces involved are mainly Van der Waals, Electrostatic or Columbic and capillary or adhesive forces. Van der Waals force is described as the force between dipoles of individual particles whereas Electrostatic force is the force between ionic bonding. Similarly, Capillary force is observed due to the cohesive and adhesive pressure that causes the liquid to defy the field of gravity.

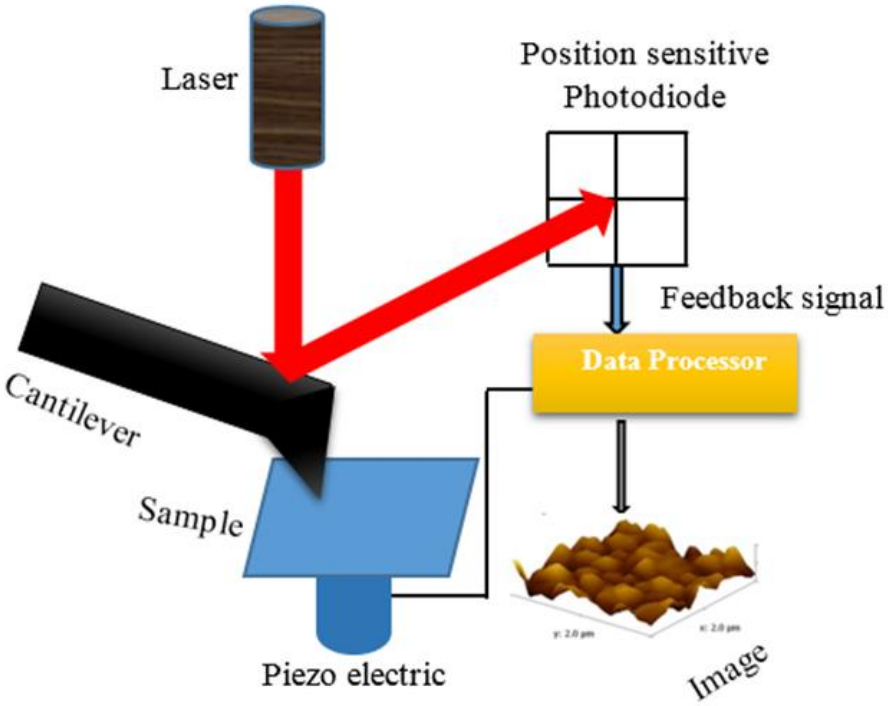

Figure 2. 4 Schematic Structure of AFM Imaging

Furthermore, Cantilever is one of the key components in AFM that holds the scanning tip and reflects back the incident laser light used for detection. The three different modes of AFM are: contact mode, non-contact mode and tapping mode. When the distance between the tip and sample is bigger, there is no interaction. When the tip is closer to the

sample, imaging in this regime is called non-contact mode. When the tip is much closer, the repulsive van der Waals force is predominant and imaging in this regime is in intermittent contact regime. When the distance between tip and sample is few angstroms, the forces balance and net force drops to zero. The atoms come in the contact regime when total force becomes positive or repulsive. In tapping mode and non-contact mode, cantilever is close to its resonance frequency. The change in oscillation amplitude and resonance frequency is due to tip-sample interaction. These changes in amplitude and frequency are used as feedback signal to control the imaging.

2.6. Scanning Electron Microscopy

SEM is an electron microscope where a specimen surface is scanned by a beam of electrons in a vacuum chamber, and the signals are produced at that time from the sample to form an image. Electron beam is thermionically generated from an electron gun containing tungsten filament cathode. Tungsten filament is typically used in thermionic guns due to its highest melting point and lowest vapor pressure of all metals [81]. Its low cost also makes it more popular with ability to be heated electrically for electron emissions. SEM is the most versatile of nanotechnology measurement devices. It provides a much higher resolution than an optical microscope, and has several advantages over AFM or a profilometer. SEM can measure nanoscale topography of a surface, determine the composition and surface properties like stiffness and phase, as well as provide information about the ratio of atoms on the surface and further into the material [82].

Main components of the microscope are column for electron beam generation, specimen chamber, vacuum pump, monitor and control panel. The column is used to generate electron beam. High vacuum is generated inside the whole microscope. The

specimen stage can be moved in left or right directions as required. To generate electron beam, thin tungsten wire is used, also called as thermionic cathode. When heated by the electric current, tungsten emits electron into the vacuum. A metallic disk with a central hole or anode is placed below the cathode. The anode is connected to the positive pole of the high voltage source and thermionic cathode with the negative port. The strong electric field between cathode-anode accelerate electrons downward. These accelerating electrons are called primary electrons. They form a broad diverging beam which hits the specimen and stage. An electromagnetic lens focuses the beam finely on specimen surface.

At the point of incidence, primary electrons knock out electrons of the specimen. These knocked out electrons are called secondary electrons. Secondary electron detector with positively biased grids collect more secondary electrons and then form an image on the computer screen. The basic principal of SEM is to use secondary electrons to form an image [83]. Many recorded secondary electrons lead to bight image point and few electrons lead to grey image point and if no electrons, then form a completely black image point. Finally, a rectangular area on the specimen is scanned and a complete image is formed.

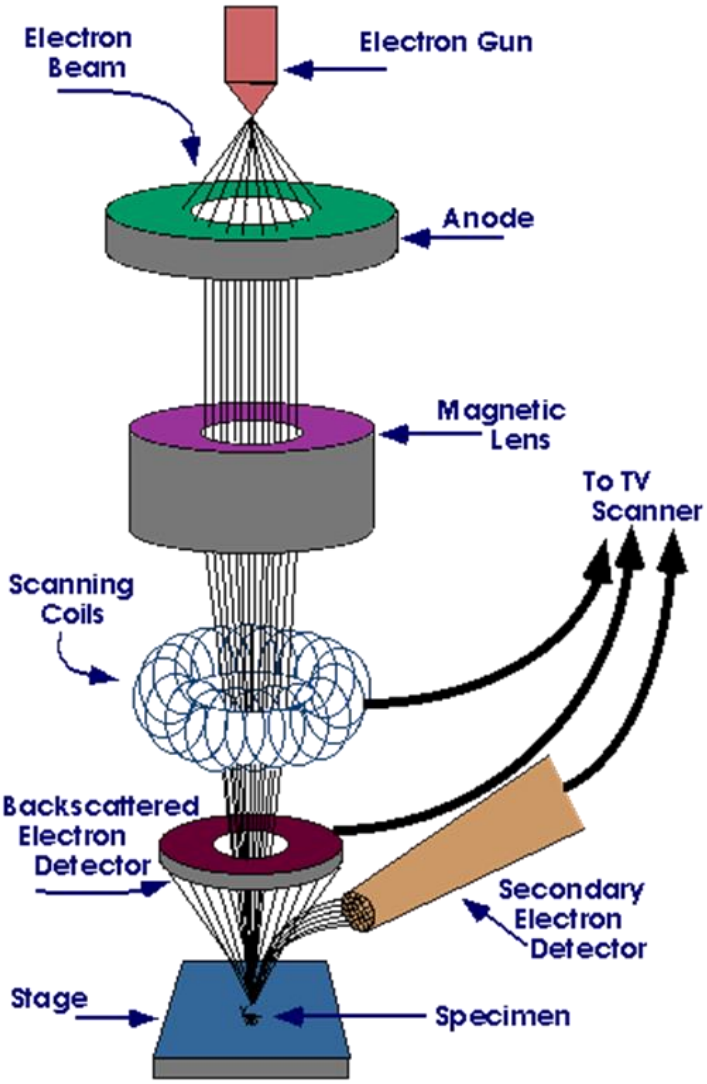

Figure 2. 5 Schematic Structure of SEM

In SEM, electrons are admitted from a filament and then drawn to an anode. From there they are focused into a beam by two condenser lenses, and a scanning coil deflects the beam onto the sample either linearly or in the raster fashion. After interaction with specimen, primary electrons decelerate and loose energy and electrons are generated from the surface of specimen known as secondary electrons. But when primary electrons enter underneath the sample surface, they come out with decelerated energy known as

backscattered electrons. Primarily, secondary electrons are used for imaging. Inside the microscope, there are detectors for the three-primary electron-beam sample interactions: backscattered electrons, secondary electrons, and characteristic x-rays. Primary electrons when hit the loosely bound outer electrons of specimen, generated electrons from the specimen are called secondary electrons. If the primary electrons penetrate the surface of specimen and come out with decelerated energy, it is called backscattered electrons. These all can be used to create an SEM image for analysis.

Secondary and backscattered electrons are most commonly used for imaging purpose. But secondary electrons are most valuable for showing the sample morphology and topography, while backscattered are responsible for showing contrasts in composition in multiphase samples or for rapid phase discrimination [84]. X-rays of fixed wavelength are generated then the excited electrons return to their lower energy states. Thus, for each element, characteristic x-rays are produced that is excited by the electron beam [85].

The signals generated from the electron-sample interactions provide information about the sample like external morphology or surface texture, chemical composition, crystalline structure and orientation of materials that make up the sample. Data are usually collected over a selected area of the sample surface, and a 2D image is generated which shows spatial variations in these properties.

CHAPTER 3. EXPERIMENTAL PROCEDURE

3.1. Materials

The paper based carbon electrode was generous gift from GSI technologies. Graphite powder was purchased from Alfa Aesar, and DMF (Dimethylformamide) and TBA hydroxide (Tetrabutylammonium) were purchased from Sigma-Aldrich for preparation of graphene oxide powder. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and sulfo-NHS (N-hydroxysulfosuccinimide) were purchased from Thermo Fisher Scientific for the preparation of crosslinking reagent together with MES buffer (2-(N-morpholino) ethanesulfonic acid). Other antibodies like Anti-rabbit IgG, Protein A, Anti-cortisol were purchased from Sigma-Aldrich. Cortisol solution was purchased from Cerilliant. To prepare all the reagents distilled water was used. For the preparation of microfluidic chamber, PDMS (polydimethylsiloxane), silicone elastomer base and silicone curing agent were purchased from Dow Corning. The redox moieties potassium ferricyanide was purchased from Sigma-Aldrich.

3.2. Sensor Fabrication Procedure

The paper sensor provided by GSI technologies have carbon working electrode with Ag/AgCl (Silver/Silver-Chloride) reference electrode and counter electrode. The working electrode was covered with microfluidic block to ensure precise flow of chemicals and reagents. The working electrode was first functionalized with nanomaterial GO to provide larger surface area for antibodies interaction. On the working electrode 10 µl of GO solution was dropped through the microfluidic chamber. It was then followed by incubation for an hour in room temperature. The electrode was then washed vigorously with 10 µl of PBS for three times. The 5% APTES solution in acetone was dropped in the working

electrode (10 µl). It was followed by incubation for an hour in room temperature and then washed with same amount of PBS for three times. The interaction between GO and APTES formed a thin layer.

After the activation of the protein A with EDC/sulfo-NHS, 10 µl solution was dropped on the working electrode. The Fc binding site of protein A bonded with IgG. Both the antibodies were incubated for an hour in room temperature followed by washing step with PBS. Anti-Cab antibody was then dropped on the working electrode (10 µl) and incubated for an hour in room temperature, followed by washing step with PBS three times. Finally, different cortisol concentrations were prepared and dropped on the working electrode (10 µl). It was incubated at room temperature for 30 minutes. After 30 minutes, electrodes were again washed with PBS thrice.

All the functionalization procedures were completed and the microfluidic block over the working electrode was removed. Sensors were now ready for CV measurement. The microfluidic block with 6mm hole was placed over the sensor covering all three electrodes. Redox moieties 20 µl was dropped over all the electrodes. The three legs of the sensor were connected to the respective electrodes of the CV measurement system.

3.2.1. Preparation of Microfluidic Chamber

PDMS chamber was prepared by mixing PDMS base and curing agent in the ratio of 10:1. After mixing thoroughly, it was poured on a petri-dish and kept in the desiccator. When all the bubbles were removed, it was cured in hot plate at 65ºC for about 15-20 minutes. A sticky PDMS mold was ready which was cut into small pieces as required to cover the working electrode and also to cover all the three electrodes. To cover only the

3mm working electrode, a 3mm punch was used for making a hole. This microfluidic block was placed over the working electrode to ensure proper distribution and functionalization of reagents. A 6mm punch was used to make a bigger microfluidic block to cover all the three electrodes: WE, RE, and CE. This was prepared to drop redox moieties precisely to get proper CV measurement from the biosensor.

3.2.2. Preparation of Graphene Oxide Solution

The commercially available GO was used as received. The protocol for preparing GO solution was followed as mentioned in the product. The 10 mg of GO powder was weighed and was added to the mixture of 10 ml dimethylformamide (DMF) solution and 300 μl tetrabutylammonium (TBA) hydroxide (40%). The solution was then sonicated using the tip sonicator for 30 minutes. The sonication process was done in ice bucket to maintain the temperature. The solution was then incubated at room temperature for 2 days. Only supernatant was removed from the incubated tube and it underwent bath sonication for an hour. To obtain proper centrifugation, samples were distributed in centrifuge tube (about 1 ml each). Samples were then centrifuged at 12,000 rpm for 3 minutes and again supernatant was collected. Finally, the GO suspension was obtained and stored at 4°C until further use.

3.2.3. Preparation of APTES Solution

The (3-Aminopropyl) triethoxysilane (APTES) solution was used as obtained without further modification. The 5% APTES solution was prepared in acetone as required. The 5% APTES solution was chosen for amine group activation.

3.2.4. Preparation of EDC/sulfo-NHS

EDC (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide) and sulfo-NHS (N-hydroxysulfosuccinimide) were used as obtained. The cross-linking solution of EDC (4mg/ml) and sulfo-NHS (11mg/ml) were prepared in 0.1 M MES (2-(N-morpholino) ethane sulfonic acid) buffer. This crosslinking solution was used for the activation of carboxyl groups.

3.2.5. Activation of Protein-A by EDC/sulfo-NHS

Protein A stock solution (1mg/ml) was prepared in 0.1 M MES buffer. The working solution was prepared by taking 100 µl of Protein A stock solution and diluted with 890 µl of MES buffer. To this protein A solution, 10 µl of EDC/sulfo-NHS solution prepared earlier was added. The solution of protein A with EDC/sulfo-NHS was incubated in room temperature for 15 minutes to activate the carboxyl group.

3.2.6. Anti-Rabbit IgG Preparation

The stock solution for anti-rabbit IgG was 1.99 mg/ml, from which working solution of 160 µg/ml was prepared. The anti-rabbit IgG antibody was used as obtained. The working solution was prepared by taking 80.402 µl of stocking solution and adding to 919.598 µl of 1% filtered BSA solution.

3.2.7. Anti-Cab Antibody and Buffer Preparation

The buffer required for anti-Cab antibody preparation is 0.05 M Tris-HCl. 0.05 M buffer solution was prepared in DI water. The stock solution was reconstituted again 10-fold with the buffer to prepare the working solution.

3.2.8. Preparation of Cortisol in Tween-20

Different cortisol concentrations were prepared in Tween-20 and PBS solution. Different cortisol concentrations were prepared: 0 ng/ml without any cortisol to 150 ng/ml were prepared.

3.2.9. Preparation of Redox Moieties

Redox moieties used were 5 mM (Fe[CN]6 3-) containing 0.1M KCl was used. After completion of functionalization step in working electrode, redox moieties were added to all the electrodes. This solution was used as an electrolyte for the experiment.

3.3. SEM Imaging

The SEM imaging was done with Hitachi S-4300N system. SEM was used to observe the surface at various stages of electrode fabrication. The surface of carbon electrode was functionalized with GO solution in room temperature for an hour. After washing with PBS and drying, sample was ready for imaging. The CRC-sputtering system was used to coat gold on the surface of electrode functionalized with GO to observe under SEM. Another sample was prepared by functionalizing the working electrode with GO, APTES, protein A, IgG and anti-cab to observe the change in surface morphology with addition of antibodies. 10 KV voltage was applied for the imaging.

Figure 3. 1 Experimental Setup of Hitachi S-4300N SEM

3.4. Atomic Force Microscopy

Topography images were taken using Agilent 5500 SPM in tapping mode. Silicon tip (Budget Sensors, Multi75 Eg) of spring constant ~1-4 N/m and tip normal radius of ~1Ao coated with Cr/Pt having a resonance frequency ~75 KHz was used. An off-resonance of 100-200 Hz was applied to the resonance peak of cantilever to achieve the required resolution. The operating regime of the tip-sample interaction can be changed from attractive to repulsive either by applying small positive or negative off-resonance. The obtained images were processed with Gwyddion software.

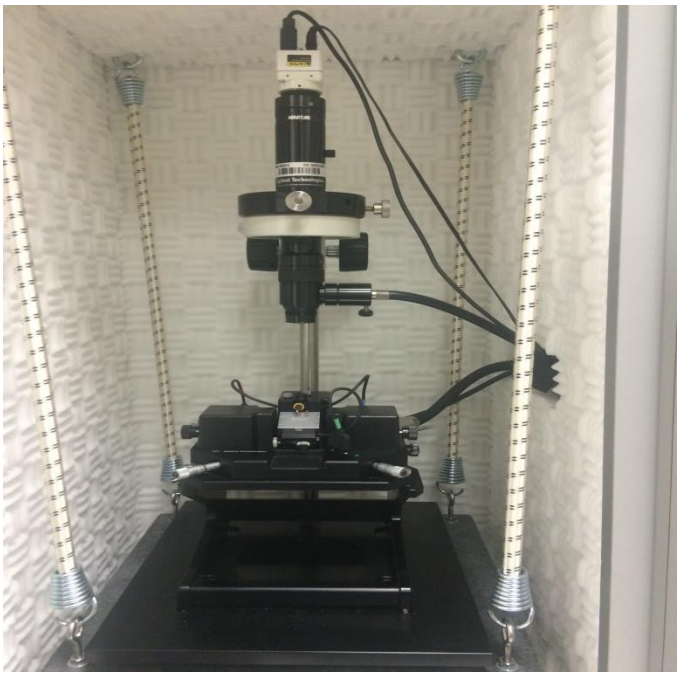

Figure 3. 2 Experimental Setup of Atomic Force Microscope

3.5. Raman Spectroscopy

Raman spectroscopy was measured using HORIBA Jobin Yvon Raman Lab RAM HR800 System using 532nm green laser. Using the computer system, the laser was focused on a single part of the sample through the microscope. Grating and filter were adjusted. 100x lens was used to focus the sample. Raman spectroscopy system requires four components: light source, sample, wavelength selector, detector. A dispersive Raman spectrometer uses monochromator as wavelength selector. Lasers are used as light source and are known as excitation light or incident beam. Incident beam path light is the optical path from light source and sample.

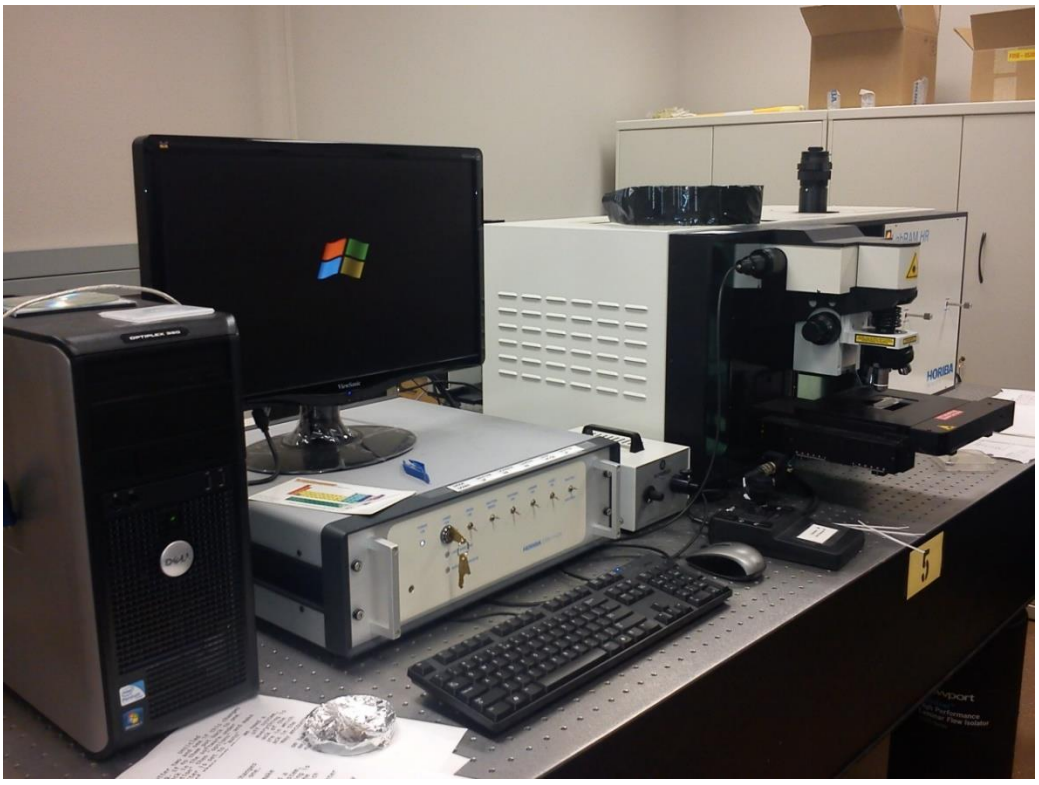

Figure 3. 3 Experimental Setup for Raman Spectrum Measurement

CHAPTER 4. RESULTS AND DISCUSSION

4.1. GO Solution Preparation

The commercially available GO used for the experiment had both larger and small nanoparticles. After the tip sonication process followed by incubation in room temperature for 2 days, all the large particles settled down on the tube. Only the supernatant was used for further processing. After bath sonication and centrifugation, all of the heavy particles settled down and only pure GO nanoparticle suspension was obtained as the supernatant. Finally, GO nanoparticles suspension for further experiments were obtained.

GO is the nanomaterial with self-assembly property. When immobilized on the working electrode of SPCE, it forms SAM. A weak physical bonding is present between the carbon electrode and GO due to pi-pi bonding between them. The SAM layer thus formed on the working electrode increase the surface roughness and also increase the surface area for antibody interaction. This helps in improving the sensitivity of the biosensor. The formation of SAM on the working electrode is supported by the SEM imaging. The change in surface roughness with functionalization of GO on the electrode surface is proved by AFM imaging which shows the surface roughness.

The positively charged GO-APTES layer form physical bonding with carbon electrode. The carboxyl or hydroxyl group on the surface of GO form covalent attachment with the amine group of APTES. The GO-APTES structure formed on the carbon surface form the base for further functionalization on working electrode.

4.2. SEM Imaging

The SEM images were obtained for self-assembled GO film on the working electrode and also for electrode functionalized with antibodies. From the images, we can see the change in the electrode surface with addition of antibody. The electrode surface becomes more smoother and uniform after the antibody was added. The change in the morphology also suggests the successful immobilization of antibody on the surface of carbon working electrode with GO.

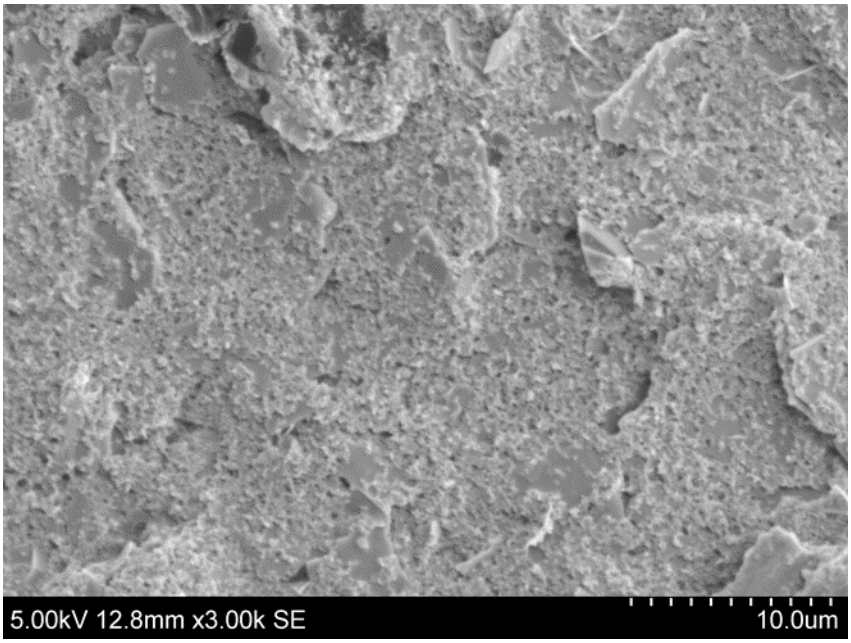

Figure 4. 1 SEM Image of only GO on the Working Electrode

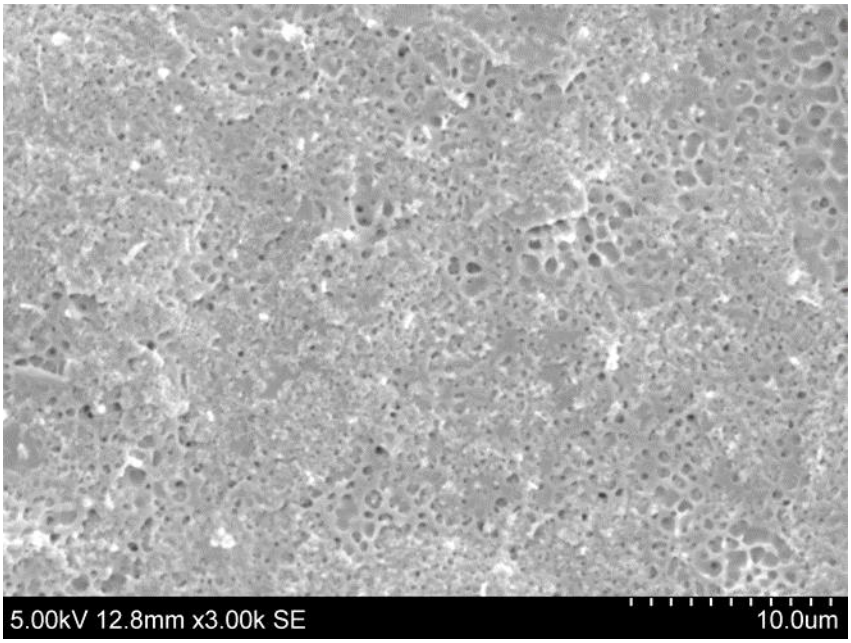

Figure 4. 2 SEM Image of Antibody Functionalized Working Electrode

4.3. AFM Imaging

AFM imaging was done for the GO layer on the working electrode of SPCE. AFM imaging shows the surface topography of GO film on the sensor surface. The imaging shows evenly distributed GO on the biosensor. This illustrates successful self-assembly of GO the developed biosensor. The obtained topography suggests that antibodies can be attached uniformly to the GO surface. The nanoparticles of GO provide more surface area for antibodies attachment, which ensures more cortisol attachment. The 3D imaging also illustrates the smooth even surface of GO on the sensor.

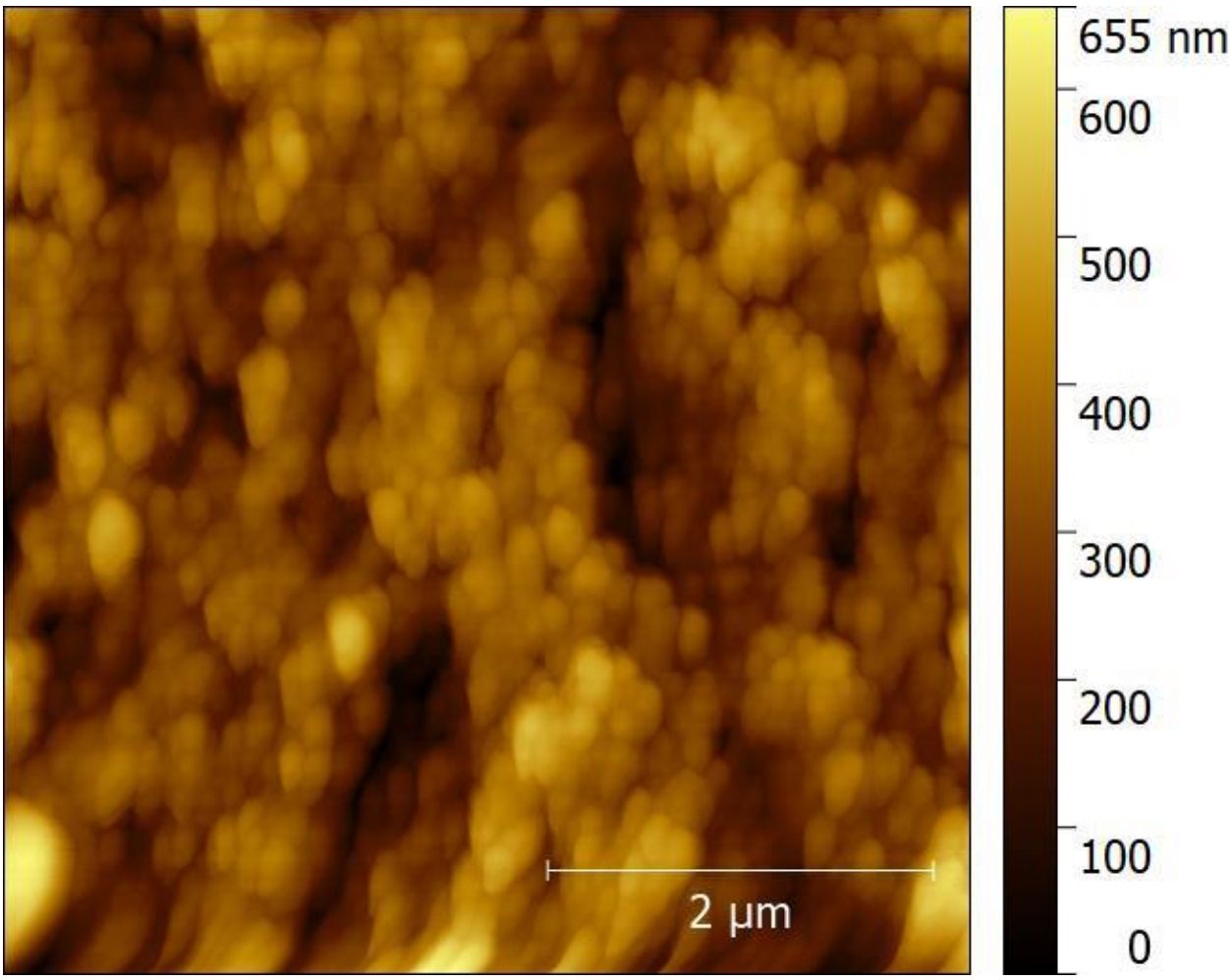

Figure 4. 3 AFM Imaging of only GO on the Sensor Surface

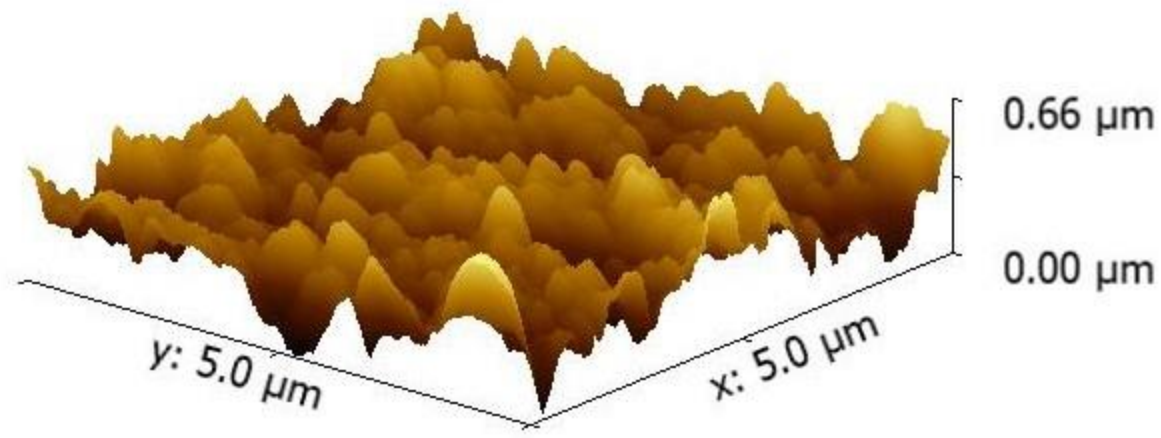

Figure 4. 4 3D View of GO layer on the Sensor Surface

4.4. Raman Spectrum

Raman spectroscopy measurement of different samples: GO only, antibodies functionalized with and without GO showed different peaks at different wavenumbers. The Raman peak for only GO sample shows two specific peaks at 1350 and 1600 cm^{-1}, which is specific to D and G bands of GO respectively. In the graph with GO functionalized antibodies, along with two specific peaks for GO, other non-specific peaks are also present indicating the presence of antibodies. In contrary, Raman spectrum for antibodies functionalized without GO shows no peaks for GO. Random peaks are obtained for different antibodies at different wavenumbers.

Raman spectroscopy measurement for different conditions have shown different results. The graphs with antibodies functionalization have many peaks compared to graph without antibodies functionalization. Both graphs with antibodies functionalization show

several peaks corresponding to different antibodies. This illustrates successful binding of anti-cab antibody over the screen-printed carbon electrode.

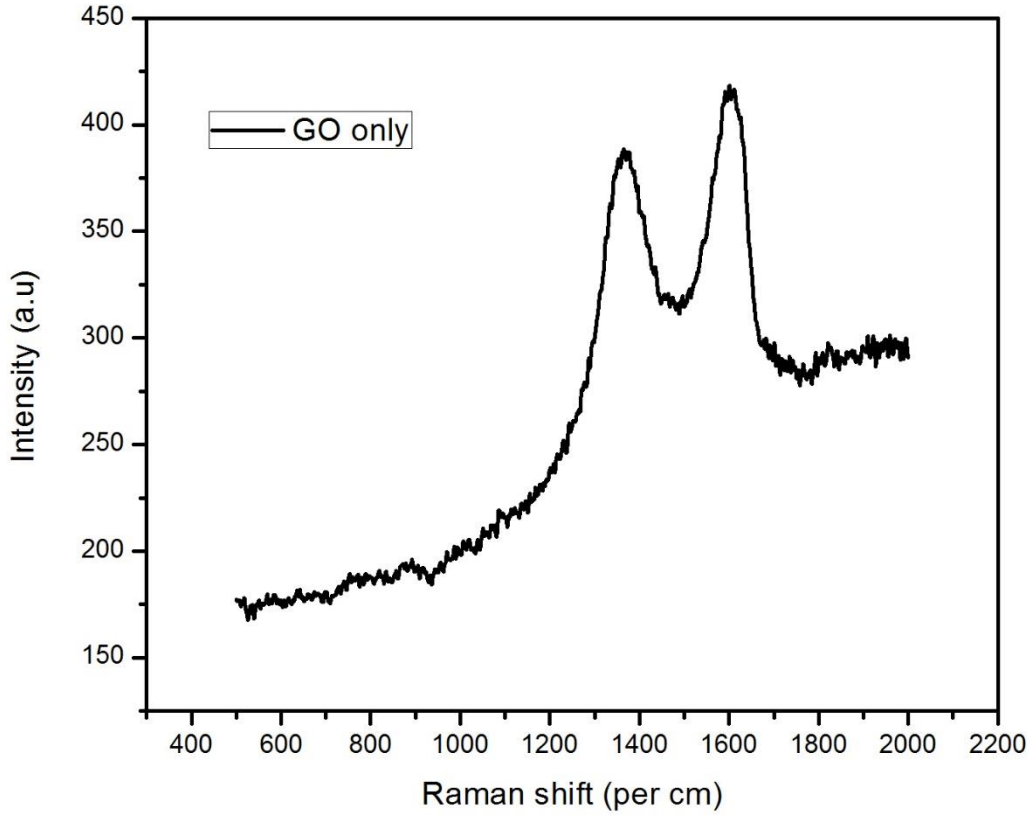

Figure 4. 5 Raman Peak of only GO Layer

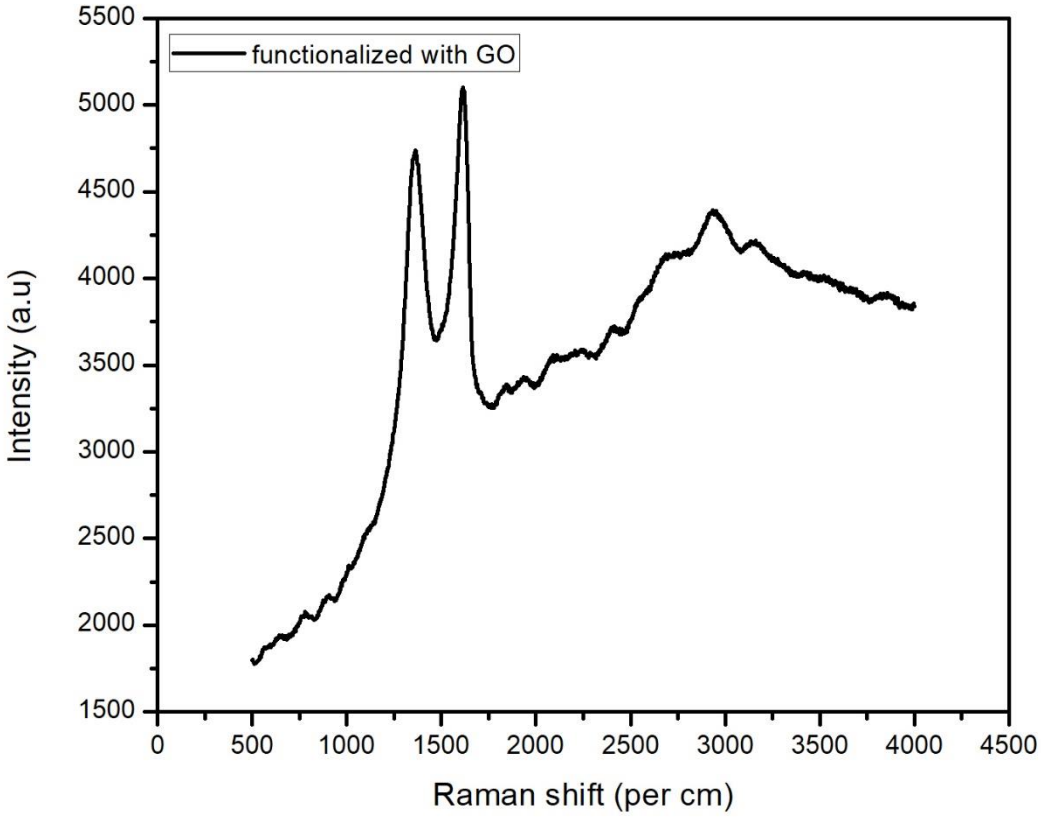

Figure 4. 6 Raman Peak of Sensor Functionalized with GO and Antibodies

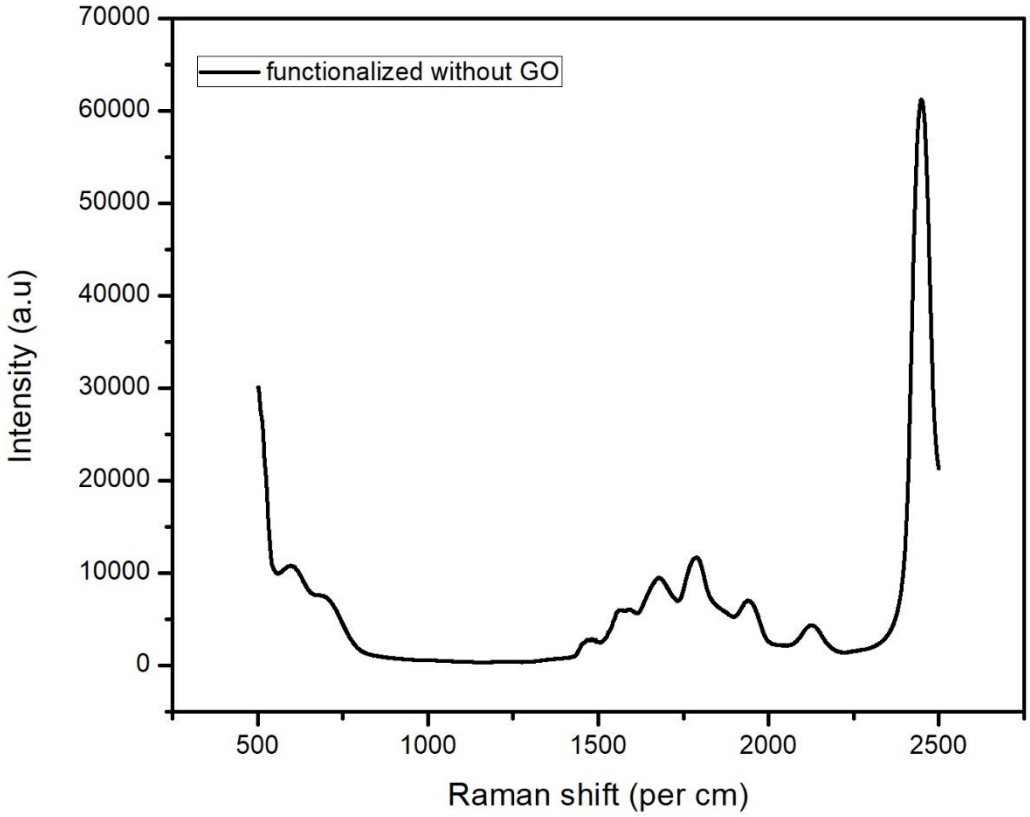

Figure 4. 7 Raman Peak of Sensor Functionalized only with Antibodies (without GO)

4.5. Protein A with EDC/sulfo-NHS and IgG Activation

EDC/sulfo-NHS help activate the carboxyl group of the antibody, Protein A. It helps in crosslinking of protein A with the GO-APTES surface. Protein A contains specific binding site for Fc region of antibodies, keeping the fragment antigen-binding (Fab) region free for antigen binding thus eliminating the need for further chemical modification of the antibody. Protein A has high affinity for Fc fragment of antibody IgG. Protein A was first functionalized on the electrode to prevent random immobilization of antibodies and also to maximize the ability of antibodies to bind to antigens.

4.6. Anti-Cab

Anti-cortisol antibody is immobilized on the surface of IgG. The two antibodies complex form the base for attachment of cortisol antigen prepared in artificial sweat. The complex structure of Anti-Cab/IgG/Protein A (EDC/sulfo-NHS)/GO carbon bioelectrode was used for cortisol detection.

4.7. Redox Moieties

The redox moieties work as the electrolyte for the experiment. With the addition of redox moieties, it hiders the transfer of electrons to the electrode.

4.8. Cyclic Voltammetry Measurements

Cyclic Voltammetry measurement was done to optimize the stepwise fabrication of electrochemical biosensor developed for cortisol detection. Figure shows the stepwise fabrication of the developed biosensor. CV measurement was done using the redox moieties K_4FeCN_6 in PBS at the potential range from -0.6 to 0.6 V. Well-defined oxidation and reduction peaks were observed in CV measurement due to redox moieties present in the electrolyte.

As seen in the figure 4.8, the magnitude of electrochemical current response decreases with modification in each step. This suggests that after the SAM formation of GO with APTES on the electrode, it prevents electron transport from redox moieties to the electrode surface due to electrostatic interaction between positively charged self-assembled GO-APTES and electrode. With further addition of carboxyl activated protein A, the amine group of APTES form bonding with carboxyl group of activated protein A with EDC/sulfo-NHS, this leads to the decrease in current magnitude. The magnitude of current was lower

after IgG addition due to its binding with protein A, which has high binding affinity for Fc fragment of antibody IgG. Finally, the current was lowest due to binding between IgG and anti-Cab, which further reduce the electron transport from electrolyte to the electrode surface. With increasing functionalization step, binding between the layers further increases. This reduces the number of free electrons in the electrolyte solution decreasing the current magnitude.

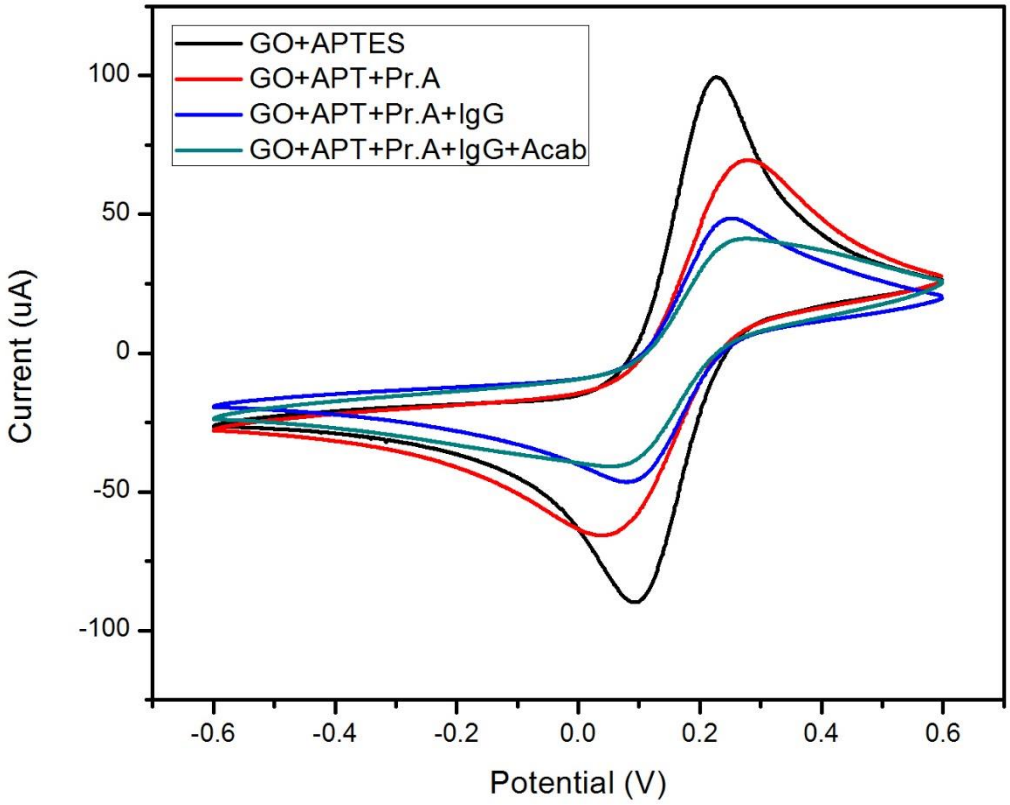

Figure 4. 8 CV Measurement for Each Functionalization Step

The electrochemical response of the developed biosensor was studied as a function of cortisol concentrations. Different cortisol concentrations from 0.1ng/ml to 150ng/ml

were tested under the experimental condition: K_4FeCN_6 as redox moieties in PBS with potential range from -0.6 to 0.6 V. The results obtained from CV measurement show that magnitude of current response decreases with increasing cortisol concentration. This decrease in current is observed due to formation of immunocomplex between anti-cab and cortisol which leads to electron charge transfer hindrance at the electrode electrolyte interface.

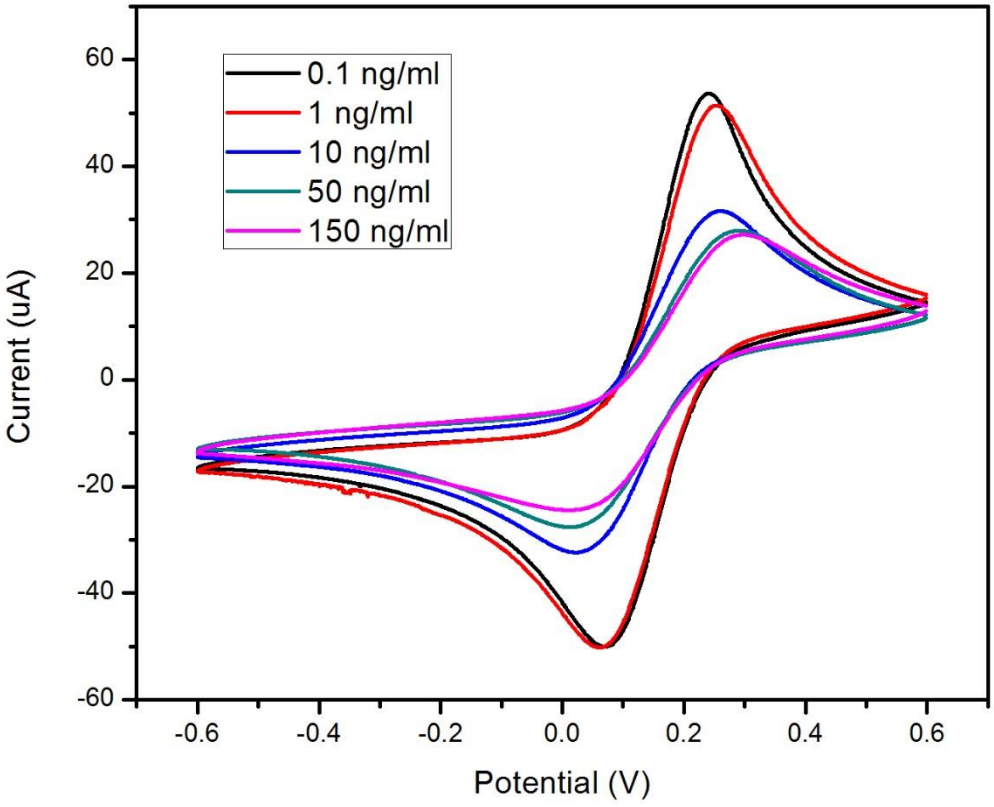

Figure 4. 9 CV Measurement for Different Cortisol Concentration

The comparison between SPCE with and without GO functionalization is shown in the figure below. The CV measurement of electrode without GO has higher current

magnitude compared to with functionalization. Comparison for these two conditions were done till anti-cab functionalization and also for cortisol functionalization. For both conditions, current magnitude was lower in case of GO functionalized carbon electrode. The less number of free electrons in case of GO functionalized electrode decreased the current magnitude, due to successful binding of antibodies on the electrode surface.

Figures 4.10 and 4.11 demonstrate the advantages of using nanomaterial GO as a biosensor for detection of cortisol. The proper binding between each step upon self-assembled GO, reduces the number of free electrons thus decreasing the current magnitude. Due to several advantages of nanomaterial GO, there will be more surface area for antibodies immobilization and thus for cortisol attachment. The nano-roughness created by self-assembled GO also enable more antibodies immobilization and help in successful antibodies binding to electrode surface.

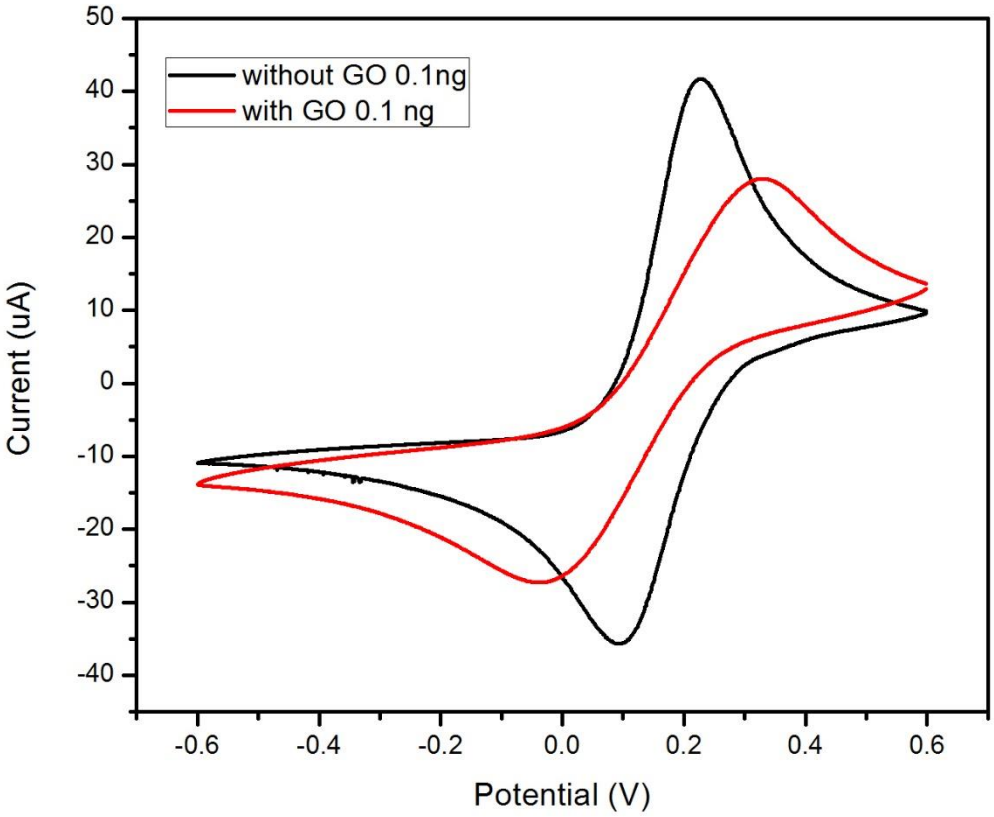

Figure 4. 10 CV Measurement with/without GO Functionalization (with cortisol)

The comparison between non-functionalized electrode surface and functionalized electrode surface, shows lower current magnitude for the device with GO. The lowest cortisol concentration, 0.1 ng/ml was used for comparing the results. When functionalized with self-assembled GO, it increases the surface roughness and provides more surface area for antibody immobilization. Electron transfer from the electrode surface is reduced upon functionalization with GO, lowering the current magnitude.

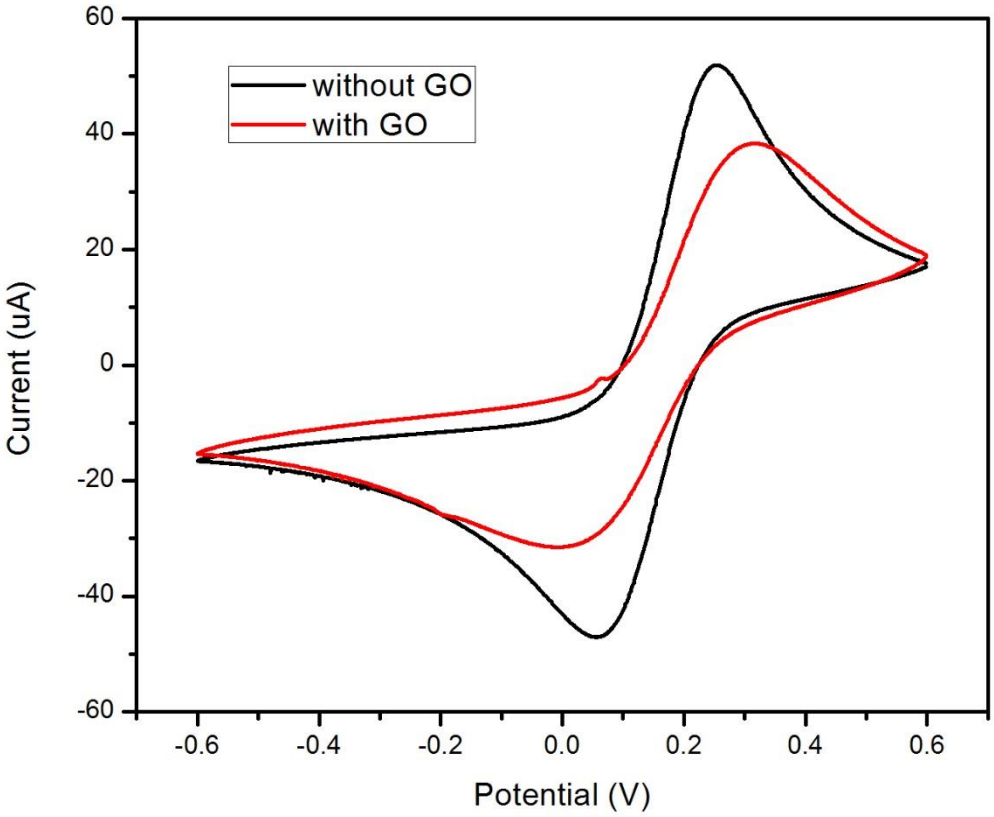

Figure 4. 11 CV Measurement with/without GO Functionalization (without cortisol)

CHAPTER 5. SUMMARY AND CONCLUSIONS

5.1. Summary

The standard of lifestyle in today's modern world is increasing day by day. The change in lifestyle has become necessity for some, while for others it has become a compulsion. Along with the advantages of globalization, it also brings disadvantages with it. The changes in lifestyle or competition due to it has become a serious issue. It can lead to serious life-threatening diseases like heart disease, mental problems, depression which have become major issue in developing countries. Due to these problems, rate of psychological stress is increasing alarmingly. Personalized measurement and monitoring system are gaining popularity for precise and accurate measurement of psychological stress. Psychological stress affects the nervous system and thus affect the physiological health. A variation is observed in cortisol level with change in stress level. Cortisol, a glucocorticoid hormone synthesized in the adrenal cortex. It is released in response to stress and popularly known as "stress hormone".

In addition to different physiological conditions, cortisol is also closely related to circadian clock in human body and significantly affect physical performances. Circadian clock and sleep physiology are responsible for regulating daily patterns in most behavioral and physiological system. Circadian disruption negatively influences metabolic, cardiovascular, endocrine, immune, bone, stress, neurological, and cognition health and function. In addition, disrupted circadian rhythm leads to sleep loss, which is a physiological stressor that leads to high cortisol levels. Increased cortisol levels can also lead to alertness, impeding relaxation, and sleep. High cortisol levels can be referred as both cause and tracker of circadian desynchronization.

Abnormalities in cortisol level leads to different disease conditions including Cushing's syndrome, Addison's disease, adrenal insufficiencies and other stress disorders. The conventional chromatography techniques including ELISA, SPR, RIA used for cortisol detection are complex laboratory based methods. The multistep purification process, processing time make these methods inconvenient for point of care detection. The simple, cost-effective electrochemical immunosensor based on nanomaterials have become popular for timely detection of stress hormone cortisol. The SPCE based electrochemical immunosensor incorporated with microfluidic system is a simple, cost-effective, field-deployable immunosensor with fast response time with advantages for point of care applications. GO is a novel nanomaterial for detection of cortisol with higher sensitivity due to their high surface area to volume ratio. One major advantage of nanomaterial is its higher surface to volume ratio which enhances its sensitivity for cortisol detection. Other advantages of GO are self-assembly property, nanorough surface or increased surface area for more interaction with antibodies and target antigens. The microfluidic system incorporated over the SPCE allows use of less sample and reagent volume, precise fluid flow, along with controlled environment for biomolecule reaction. Its advantages like automation, flow control, reduction in human error add to its point of care application.

The SPCE based electrochemical immunosensor is a simple, efficient and cost-effective approach applied for detection of cortisol. The paper based sensor was composed of cortisol as an analyte and an electrochemical transducer. In the experimental scenario, silver/silver-chloride was the electrode for signal transduction with carbon based working electrode. The amperometric transducer detect changes in current which occurs due to oxidation or reduction. The current will show the reaction that occurred between the

analyte and the bioelement. The CV method used for cortisol detection is an amperometric approach. It is a powerful, simple and rapid method that characterizes the electrochemical behavior of analytes that can be oxidized or reduced electrochemically. This approach measures the current developed at the working electrode in an electrochemical cell when voltage is applied. The change in the current with application of voltage is obtained in a short period of time, making detection and analysis faster. Accurate measurement is done within short time frame.

5.2. Conclusions

A simple, rapid and cost-effective microfluidic, field-deployable screen-printed carbon electrode based biosensor was developed for cortisol detection. The developed microfluidic system with self-assembled GO was functionalized on SPCE electrode. The use of SPCE and microfluidic system allowed development of simple and rapid sensor with less cost compared to state-of-art techniques. The developed system can be applied for point of care application with timely detection of cortisol level along with treatment of cortisol related diseases. Different antibodies were functionalized over the GO layer for detection of artificial sweat. Electrochemical measurement was done using cyclic voltammetry, as a function of cortisol concentrations. CV measurement was done for each antibody functionalization step, which showed decrease in current magnitude with each functionalization step due to reduction in number of free electrons. CV measurement also proved the advantage of using self-assembled GO compared to without GO functionalization, in both cases: with or without cortisol sensing. Similarly, CV measurement for different cortisol concentration also showed decrease in current magnitude with increasing cortisol concentration due to immunocomplex formation

between anti-cab and cortisol which leads to electron charge transfer hindrance at the electrode electrolyte interface.

Other measurement system like Raman, SEM, AFM showed change in the device structure with or without GO functionalization and with/without antibodies functionalization. SEM images with and without antibodies functionalization showed the surface morphology. SEM imaging shows the change in surface morphology with the addition of antibody, compared to bare electrode functionalized with GO, confirming the antibody binding to self-assembled GO nanosurface on the working electrode. Raman imaging also supports the advantages of surface functionalization with antibodies, also with/without GO. A simple, rapid, low-cost, field-deployable microfluidic SPCE cortisol sensor was developed with point of care applications.

5.3. Future Works

The developed sensor measured artificial sweat cortisol effectively with functionalization of novel nanomaterial GO and different specific antibodies. The working electrode can be functionalized with other nanomaterials to detect cortisol or also can be applied for other biomolecules detection. Other cross-linkers can be used to immobilize the antibodies which can yield more sensitivity. The developed microfluidic SPCE biosensor system can be applied for detection of different biomolecules.

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