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AN IMMUNOSENSOR UTILIZING REDUCED GRAPHENE OXIDE AS A POTENTIAL SENSING MATERIAL FOR CORTISOL DETECTION: OPTIMIZED SCREEN-PRINTED GOLD ELECTRODE DESIGN FOR STRESS HORMONE MONITORING

Ahmad Farid Mohd Azmi[a], Vayithiswary Kannan[b], Muhammad Hafiz Ahmad[b], Jahwarhar Izuan Abd Rashid[a, b, *]

[a] Department of Chemistry & Biology, Centre for Defence Foundation Studies, National Defence University of Malaysia, Sungai Besi Camp, 57000 Kuala Lumpur, Malaysia
[b] Centre for Tropicalization, Defence Research Institute, National Defence University of Malaysia, Sungai Besi Camp, 57000 Kuala Lumpur, Malaysia

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ABSTRACT

Monitoring cortisol levels enables us to assess the physiological stress on our body and mind. An electrochemical immunosensor was fabricated by modifying a screen-printed gold electrode (SPGE) with reduced graphene oxide (rGO) and immobilizing cortisol monoclonal antibodies via EDC/NHS coupling chemistry. The surface morphology and electrochemical behavior of the fabricated rGO-modified SPGE were characterized using Field Emission Scanning Electron Microscopy (FESEM) and cyclic voltammetry (CV), respectively. The utilization of rGO as a sensing material significantly improved the electrochemical current signal, both in the presence and absence of cortisol. We optimized four key parameters to enhance the sensor's performance: rGO concentration, EDC: NHS solution ratio, C-Mab concentration, and cortisol incubation time. Through optimization, we reduced the rGO concentration from 2 mg/mL to 0.5 mg/mL, adjusted the EDC:NHS ratio from 0.6:1 to 0.2:1, decreased C-Mab concentration from 0.7 mg/mL to 0.5 mg/mL, and shortened the cortisol incubation time from 15 minutes to 3 minutes. These improvements led to a significant enhancement in sensor performance, with the reduction of peak current increasing from 39.03% to 66%, representing a 69.1% overall performance improvement. Under these optimized conditions, the developed sensor exhibited good selectivity among various hormones and demonstrated reasonable within 0.001–10 µg/mL, encompassing typical human levels (0.1–0.3 µg/mL under normal conditions and up to 1–2 µg/mL under stress with a detection limit of 1.9677 µg/mL. The sensor shows promise as a potential platform for future stress-related cortisol monitoring in various body fluids.

1.0 INTRODUCTION

Stress is known as the condition of the body responding to the pressure arising from a factor, be it emotional or physical. In general, stress is perceived as being distressed and associated with negative connotations such as job-related stress, financial problems, family issues, injuries and many more [1-3]. These are the common stressors which might put an individual at risk of enduring chronic stress if left prolonged [4] This is because stress is also known as the major contributor to humans' psychosocial and physical pathological conditions [5-6]. Cortisol, a steroid hormone produced by the adrenal glands, is a primary stress biomarker due to its role in the hypothalamic-pituitary-adrenal (HPA) axis activation [7]. This hormone is also known as the primary stress biomarker as it enhances the activity of other stress biomarkers owing to its fat-soluble properties which enables them to enter into corresponding cells [8-9]. Other stress biomarkers include pro-inflammatory cytokines, salivary α-amylase, catecholamines

*Corresponding Author | Abdul Rashid, J. I. | jahwarhar@upnm.edu.my

(norepinephrine and epinephrine), dehydroepiandrosterone (DHEA), C-reactive protein, and brain-derived neurotrophic factor (BDNF)[10-12]. Since Cortisol is present in various human body fluids, such as serum, urine, saliva, sweat,[13-15] and even in hair and fingernails,[10, 16-17] making it a feasible stress biomarker for researchers. Traditional methods for the determination of cortisol, such as high-performance liquid chromatography (HPLC) [18], gas or liquid chromatography-tandem mass spectrometry (GC–MS/LC-MS),[19-20] electrochemiluminescence [21-22] fluorometry [23-24] as well as conventional enzyme-linked immunosorbent assays (ELISA) [24-25]. With the use of complex instrumentations and costly reagents, these tests require trained personnel and time-consuming procedures which outweighs their practicability for measuring cortisol constantly. Thus, there is a demand for a cost-effective, user-friendly, and capable of providing accurate results for determining cortisol level.

With the rapid development of cutting-edge technology, researchers are now focused on developing biosensors as an alternative method in detecting this biomarker for its various advantages such as portability, flexible customization in detecting various types of target analytes and possess great commerciality. Sensing techniques such as Surface Plasmon Resonance (SPR) and Localized Surface Plasmon Resonance (LSPR) were utilised to detect cortisol by measuring changes in the refractive index on the sensing surface [26-28]. Using microfluidic assay, managed to quantify cortisol through competitive binding of cortisol onto aptamer-functionalized gold nanoparticles [29]. Lateral flow immunoassay has also been reported for a sensitive detection of cortisol employing colorimetric analysis on a paper based sensor and a thin strip of nitrocellulose membrane conjugated with sensing molecules [30-31]. Electrochemical sensing technique is also one of the most widely employed detection method for cortisol for its feasibility to be incorporated as point of care testing using miniaturized microfabrication of sensing materials coupled with sensitive electrochemical signal analysis [32-33]. Highly sensitive sensing materials such as carbon fiber, Nickel Oxide thin film on Indium Tin Oxide glass (NiO/ITO matrix) and enzyme tagging microchip array were specifically modified to detect cortisol from human samples [34-36].

The incorporation of nanomaterial as a sensing material in a biosensor has attracted many interests among research in improving the sensitive detection of a target analyte. In electrochemical biosensors, conducting polymers and their composites are widely being used as suitable matrices for biomolecule binding and an enhanced detection stability, speed, and sensitivity [35, 37]. Amongst a vast range of nanomaterial, Graphene and its derivatives are of the most utilized nanomaterial in biosensor research. It is a highly anisotropic material with carbon atoms linked by sp^2 bonds in a honeycomb lattice network and known to have high specific surface area that favours surface functionalization (in introducing carboxyl, hydroxyl, sulfonate, acid chloride and amine) in accommodating active probes immobilization and targets of interest [38-39]. In addition, its tremendous unique electronic, optical and mechanical properties offers excessive potential for a broad range of applications includes biological applications and energy storage (e.g. fuel cells, batteries and supercapacitors) [40]. Graphene is highly pure and its derivatives are chemically inert and cost-effective with greater homogeneous surfaces than carbon nanotubes (CNTs). Graphene exhibits superior electro-catalysis over CNT [41-42]. With the ability to enhance electrical activity through signal amplification and favours surface modification for the immobilization of various types of sensing molecules through functional group interactions, graphene and its derivatives pose great potential to be integrated in the development of a novel bio-interface for bio-sensing.

Recently, a number of studies have been reported in the utilization of different types of sensing matrix in the detection of cortisol such as polyaniline protected gold nanoparticles on gold electrode (BSA/C-Mab/PPAuNP/Au), Graphene-Nanoplatelet-Amphiphilic-diblock-co-Polymer Composite on square gold electrode, Room temperature ionic liquid (RTIL): BMIM[BF_4] modulated gold microelectrode-based sensor, Bifunctional protein interlayer-modified reduced GO (anti-cortisol antibody/d-BSA/rGO) electrodes but very few studies were documented on screen printed gold electrode and the combined utilization of GO and its derivatives [43-46]. In our previous research, we successfully utilized reduced GO as a matrix to immobilize the phosphodiesterase mutant YT enzyme for electrochemical detection of OP compounds [22]. This could be beneficial for studying cortisol levels. The objective of this study is to develop and optimize an electrochemical immunosensor based on rGO-modified SPGE for cortisol detection, evaluate its performance, and assess sensitivity and selectivity within physiological concentration ranges.

2.0 METHODS AND MATERIAL

Analytical grades of GO suspension (code: 777676) in water suspension (4 mg/ml), potassium hexacyanoferrate (II) trihydrate ($K_4Fe(CN)_6 \cdot 3H_2O$), phosphate buffered saline (PBS) solution,

*Corresponding Author | Abdul Rashid, J. I. | jahwarhar@upnm.edu.my

N-hydroxysuccinimide (NHS), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) were purchased from Sigma-Aldrich (St. Louis, MO). Cortisol monoclonal antibody (C-Mab) (XM210) was purchased from Abcam (USA). Hydrocortisone (Cortisol) was purchased from Tocris (Bristol, UK). Bovine serum albumin (BSA) was purchased from Sigma-Aldrich (St. Louis, MO). The screen-printed gold electrode (220-AT) and its connector were purchased from Metrohm DropSens. The SPGE features a ceramic body with a silver reference electrode and gold working (4 mm diameter) and counter electrode. Electrochemical analysis was conducted using a potentiostat (PGSTAT204 Autolab, Metrohm, Netherlands), controlled by the electrochemistry software NOVA version 2.1.

The 0.5 mg/mL GO suspension was drop-casted onto the SPGE surface and incubated for 18 hours at room temperature. In order to remove oxide layer on the surface of GO, the deposited GO was electro-reduced in 0.1 M potassium chloride (KCl) solution using cyclic voltammetry (CV) scans from -0.2 V to 1.0 V for 11 cycles at a constant rate of 50 mV/s until a stable CV response was observed at both oxidation and reduction potential. The electrode was rinsed with distilled water and dried at room temperature, resulting in the formation of rGO-modified SPCE. The electrode surface was then treated with an EDC-NHS solution at a ratio of 0.6:1 for one hour at room temperature to functionalize the modified electrode surface with Cortisol monoclonal antibody. The excess EDC-NHS solution was washed using PBS solution to remove the excess and left air-dried at room temperature. Cortisol monoclonal antibody (C-Mab) (0.5 µg/mL) of 10 µL was drop-casted onto the rGO-SPGE for 30 minutes and washed using PBS solution. BSA solution was used to block any unbound surface of SPGE and to prevent non-specific binding of analytes. The modified electrodes were stored at 4 ^{o}C for further usage. To determine the level of Cortisol hormone, a volume of 10 µL of 0.5 µg/mL solution was incubated on modified SPGE for 3 minutes and then washed with PBS solution. The electrochemical activity of the SPGE was measured using CV in a range from -0.4 A to 0.6 A at a scan rate of 50 mV/s, employing 5 mM potassium ferricyanide in 0.1 M KCl as the electrolyte.

Surface morphological studies of modified electrode were performed using Field Emission Scanning Electron Microscope (FESEM) (GeminiSEM 500). Electrochemical measurement was analysed using Autolab PGSTAT 204 (Metrohm, Switzerland). Electrochemical characterization of the modified electrode was conducted in the presence of $[Fe(CN)_6]^{3-/4-}$ ions in terms of scan rate study across ranging applied voltage (50 – 600 mV/s). The optimization of the fabrication modification of the modified electrode, such as varying rGO concentrations, EDC-NHS solution ratios, C-Mab Antibodies concentrations, and incubation time, was studied. In the optimization work for this cortisol biosensor, the measurement is based on the percentage reduction of peak current (%). This approach allows for a standardized comparison of the sensor's response across different experimental conditions and cortisol concentrations. The percentage reduction is calculated using the following formula of Percentage Reduction of Peak Current (%)

$$= \frac{\text{Peak current before immobilization of cortisol} - \text{Peak current before immobilization}}{\text{Peak current before immobilization of cortisol}} \text{X } 100 \qquad (1)$$

The optimized conditions were further employed for analytical performance including determination of different concentrations and interference study at various hormones such as cortisol, estradiol, estriol, and progesterone. The data for each parameter were derived from three replicates.

3.0 RESULTS AND DISCUSSION

Figure 1 illustrates the fabrication procedure of cortisol immunosensor using electrochemical approach. Firstly, the GO was immobilized onto the SPGE surface by drop casting. GO was electrochemically reduced into rGO to enhance the electrochemical properties of GO-SPGE by removing the oxide layer on the GO films. The successful formation of rGO films on the SPGE surface was evident in the increased electrochemical current responses and enhanced surface area for immobilizing cortisol monoclonal antibodies on the electrode. The advantages of rGO surface, which contains various functional groups such as hydroxyl, carboxyl, and epoxide groups, were utilized to be functionalized with C-Mab through an amide linkage. This involved the activation of carboxyl groups using an EDC–NHS solution and their subsequent reaction with the amine groups of the C-Mab [47]. To minimize and block nonspecific binding on the surface of the C-Mab/rGO-modified SPGE, BSA protein was utilized. The immunoreaction between C-Mab and cortisol on the rGO-SPGE surface was monitored using the CV approach in a $[Fe(CN)_6]^{3-/4-}$ solution. The interaction between C-Mab and cortisol hindered electron transfer in the $[Fe(CN)_6]^{3-/4-}$ solution, resulting in a reduced current signal. Therefore, the difference in current signal with and without the presence of cortisol became the main analytical parameter in this study.

*Corresponding Author | Abdul Rashid, J. I. | jahwarhar@upnm.edu.my

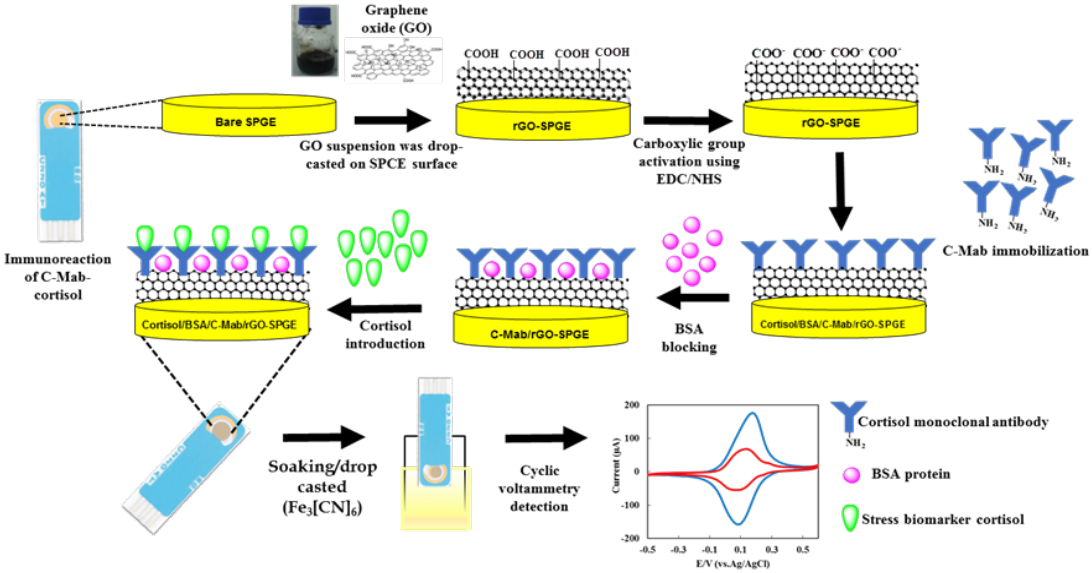


Figure 1. Schematic overview of the development of immunosensor based on rGO-modified SPGE for the electrochemical detection of cortisol

The surface morphology of the rGO-modified SPGE significantly impacts the performance of our immunosensor, as depicted in Figure 2. It features a wrinkled-like shape with numerous creases and a highly porous, rough structure that provides an extensive surface area for C-Mab immobilization. This promotes greater interaction with Cortisol hormone to enhance current signal. This suggesting its potential to improve biomolecule loading on the rGO surface. This finding also indicates successful deposition of rGO on the bare SPGE surface, demonstrating effective chemical modification in this work [48].

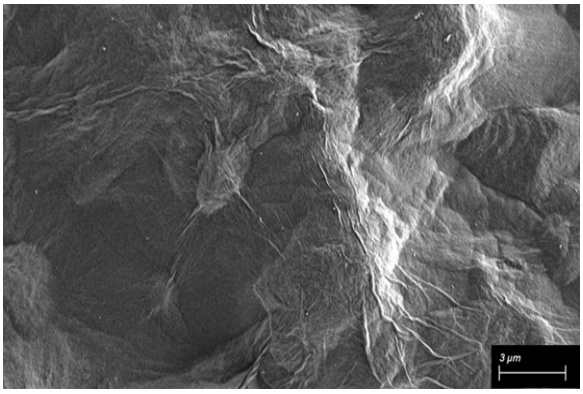
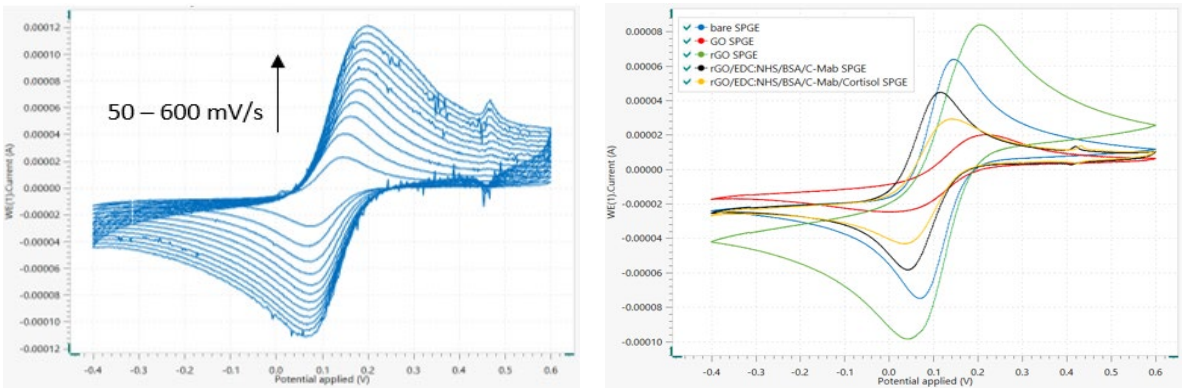


Figure 2. FESEM image of rGO on SPGE at 5000X magnification

Figure 3(a) demonstrates the effect of scan rates on rGO-modified screen-printed gold electrodes (SPGE) at varying scan rates of 50-600 mV/s. The cyclic voltammetry (CV) curves displayed a linear increase in anodic and cathodic peak currents with rising scan rates, indicating a diffusion-controlled process governing the electrochemical reaction on the surface of the rGO-modified SPGE. This process facilitates the efficient electron transfer of $[Fe(CN)_6]^{3-/4-}$ species [41, 49]. In Figure 3(b), the CV characterization of various SPGE modifications reveals a decrease in electrochemical current following the deposition of GOsheets onto the SPGE surface. This reduction is attributed to the oxide layers of GO, which hinder the electron transfer of $[Fe(CN)_6]^{3-/4-}$ species. Subsequent electrochemical reduction treatment of GO, which removes its oxide layers, significantly enhances the electrochemical peak current by 63.88 μA. The largest CV curve in Figure 3(b) clearly showed that the role of rGO in improving electron transfer kinetics across the active sensing surface [50]. The distinct CV curves between the bare SPGE (blue line) and the rGO-modified SPGE (green line) demonstrate the successful utilization of rGO as a sensing material on the bare SPGE. The enhanced performance of the rGO-modified SPGE was further observed in the development of an immunosensor for cortisol hormone determination. The enhanced performance of rGO-modifed SPGE was further applied to development of immunosensor for the determination of Cortisol hormone. Chemical modification for C-Mab immobilization resulted in a reduction of the electrochemical

*Corresponding Author | Abdul Rashid, J. I. | jahwarhar@upnm.edu.my

peak current due to the deposition of C-Mab and BSA on the rGO surface, blocking the active area of the electrode for $[Fe(CN)_6]^{3-/4-}$ ions and resulted in a decreased electrochemical peak current [51]. This observation is in agreement for the modification patterns of electrochemical immunosensors that are mostly being reported [32, 37]. The further decrease in peak current recorded after the addition of cortisol confirms that the immobilized capture molecule (C-Mab) is specific to cortisol and the interaction of cortisol-C-Mab has occurred. Consequently, monitoring the percentage reduction of CV electrochemical peak current in the presence and absence of cortisol becomes a key analytical aspect for future studies.



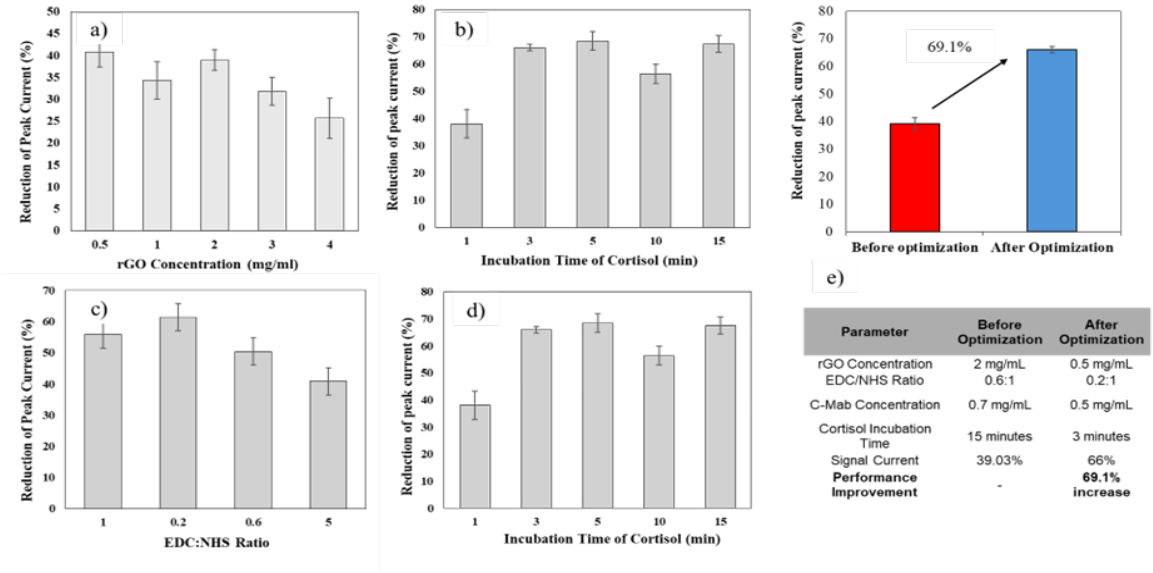
(a) (b)

Figure 3. (a) Scan rate study of rGO-SPGE at 50–600 mV/s and (b) Electrochemical characterization of different modifications of SPGE using [Fe(CN)6]3-/4- as the redox probe

In optimizing the performance of our developed immunosensor for Cortisol hormone determination using fabricated SPGE, four different parameters were investigated: concentration of rGO, ratio of EDC-NHS solution, concentration of CC-Mab, and incubation time of cortisol. Figure 4(a) shows the rGO concentration optimization. The highest average peak current difference of 40.8% (± 3.4%) was observed at 0.5 mg/mL, gradually decreasing with increasing concentration. The highest percentage of reduction in peak current was assumed that at the concentration of 0.5 mg/mL is optimal for providing larger surface area for the immobilization of C-Mab and its interaction with Cortisol hormone. Besides rGO can act as a good sensing matrix for the immobilization of biomolecules, its advantages in electrical properties also contribute to the enhancement of current signal in this study [52]. However, increasing the rGO concentration above 0.5 mg/mL resulted in decreasing the peak current reduction percentage which was due to a few possible factors. One of the probable factors suggested the incorporation of high concentration of GO on the SPGE could lead to increased thickness of the layer, resulting in presence of functional groups on the rGO surface that hinders the electron flow process thus decreased its active surface area for C-Mab attachment and consequently reduced the sensitivity for cortisol detection [33, 51]. Figure 4(b) displays the EDC-NHS ratio optimization. The 0.2:1 ratio of EDC-NHS offered the optimum peak current reduction of 61.4% (± 3.4%). This ratio was chosen as optimal due to its highest performance among the tested ratios. The 0.2:1 ratio effectively activates the carboxyl group (COOH) into (COO-) for the attachment of C-Mab through amide bond interaction in which the formation of amine reactive intermediate promotes the attachment of NH_2 ending of the C-Mab onto the rGO surface [47, 53].

In Figure 4(c), concentration of immobilized C-Mab at 0.5 mg/mL is the optimum concentration to reach the highest peak current reduction of about 65%. The reduction of peak current was then decreased with further addition of C-Mab. This phenomenon signified a lowering in sensitivity for cortisol detection through the low electrochemical current response recorded at high concentration of C-Mab [34]. However, the current reduction percentage was seen to decrease at C-Mab concentration beyond 0.5 mg/mL, due to the occurrence of steric hindrance as the concentration of C-Mab increases [34, 51]. Figure 4(d), the incubation time of 3 minutes shows a peak current reduction of about 66% (± 3.4%). As a function of incubation time of cortisol, which is very close to the maximum reduction observed at 15 minutes (approximately 68%). The 3-minute incubation time was chosen as optimal due to its time efficiency while maintaining high sensitivity. This shorter incubation time offers advantages in terms of rapid analysis, higher throughput, and potential preservation of sample integrity, making the sensor more practical for real-world applications.

*Corresponding Author | Abdul Rashid, J. I. | jahwarhar@upnm.edu.my

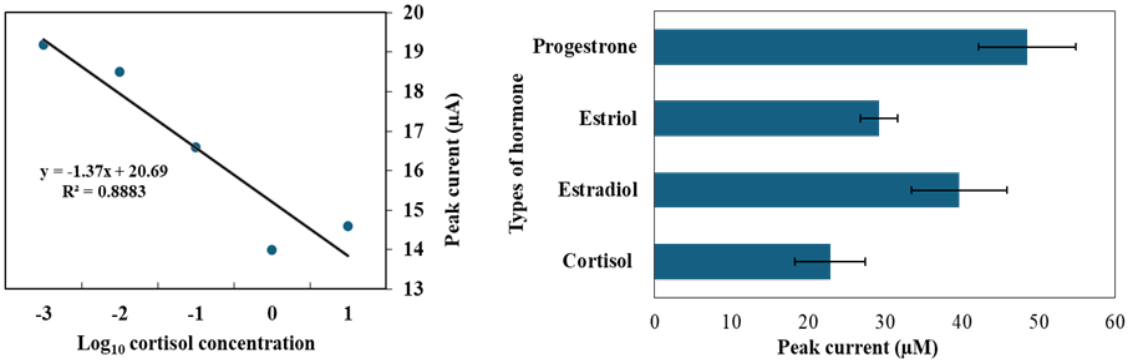


Parameter	Before Optimization	After Optimization
rGO Concentration	2 mg/mL	0.5 mg/mL
EDC/NHS Ratio	0.6:1	0.2:1
C-Mab Concentration	0.7 mg/mL	0.5 mg/mL
Cortisol Incubation Time	15 minutes	3 minutes
Signal Current	39.03%	66%
Performance Improvement	-	**69.1% increase**

Figure 4. Electrochemical detection of cortisol optimized by (a) rGO concentration, (b) EDC-NHS ratio, (c) C-Mab concentration and (d) Incubation time of cortisol e) Before and after optimization

Figure 4(e) below shows the comparison of reduction of peak current before and after optimization of parameters. The initial parameters used in this study were 2 mg/mL concentration of rGO, 0.6:1 EDC/NHS ratio, 0.7 mg/mL C-Mab concentration, and 15 minutes of cortisol incubation time. Through optimization work, we successfully improved the signal current of electrochemical detection of cortisol from 39.03% to 66%, which represents a significant increase of 69.1% in sensor performance. This substantial improvement demonstrates the effectiveness of our optimization process in enhancing the sensitivity and efficiency of the cortisol immunosensor.

Figure 5 displays the peak current of the modified cortisol biosensor using linear regression analysis in the different range concentration of 0.001 µg/mL to 10.0 µg/mL. The electrochemical current response was linearly reduced with increasing cortisol concentration. The linear regression function obtained was $i(\mu A) = 2 \times 10^{-5} - 1 \times 10^{-6}$ [cortisol] (µg/mL), with a correlation coefficient (R^2) of 0.8872. This signifies that the C-Mab modified rGO-SPGE is sensitive to capturing cortisol over a wide range of concentrations, from 0.001 µg/mL to 10.0 µg/mL, which corresponds to normal human cortisol levels. In addition, a high degree of reproducibility was achieved among the randomly modified SPGE electrodes. The relative standard deviation (%RSD) from the modified SPGE was less than 10% (6.65%), indicating a high level of reproducibility for the modified SPGE. Using the calculation LOD = 3.3 × (SD/Slope), the limit of detection was determined to be 1.9677 µg/mL. Table 1 summarizes the recent developments in cortisol biosensors. This study is noted for its simplicity in the construction of the sensing matrix and the electrochemical analysis method, demonstrating the ability to detect cortisol at levels as low as 1.9677 µg/mL.



(a) (b)

Figure 5. a) Calibration curve of peak current vs Log_{10} cortisol concentration (0.001 µg/mL to 10.0 µg/mL); b) Selectivity study of electrochemical performance at difference types of hormones

*Corresponding Author | Abdul Rashid, J. I. | jahwarhar@upnm.edu.my

Table 1. Summary of recent cortisol biosensors

Modified electrode	Detection technique	Detection range & sensitivity	Ref
Polyaniline protected gold nanoparticles on gold electrode (BSA/C-Mab/PPAuNP/Au)	Cyclic Voltammetry	1 pM–100 nM & 1.63 μA M^{-1}	[43]
Graphene-Nanoplatelet-Amphiphilic-diblock-co-Polymer Composite-based paper electrode	Electrochemical Impedance Spectroscopy & Cyclic Voltammetry	3 pg/mL–10 μg/mL & 50 Ω $(pg\ mL^{-1})^{-1}$	[44]
Gold Interdigitated electrode	Chronoamperometry & Electrochemical Impedance Spectroscopy	1 pg/ml–150 ng/ml & NA	[54]
ionic liquid/BMIM[BF_4]-gold microelectrode-based sensor	Electrochemical Impedance Spectroscopy & Chronoamperometry	8–141 ng/mL & 5 ng/ml	[45]
Bifunctional protein interlayer-modified reduced GO electrodes	Electrochemical Impedance Spectroscopy	10 pM–100 nM & 10 pM	[46]
Molecularly imprinted polymer (MIP)-modified Screen-printed carbon electrode	Differential Pulse Voltammetry	0.1–100 nM & 0.036 nM	[55]
rGO/AuNPs-modified glassy carbon electrode	Differential Pulse Voltammetry	0.1–100 nM & NA	[56]
β-Cyclodextrin/rGO-modified molecularly imprinted polypyrrole (PPy)	Cyclic Voltammetry	5 pg/mL–5000 ng/mL & 19.3 pM	[57]
rGO-modified screen-printed gold electrode (rGO-SPGE)	Cyclic Voltammetry	1 ng/mL–10 μg/mL & 1.9677 μg/mL	This work

*1 nM ≈ 0.0000001 μg/mL

Various types of hormones were applied on the modified SPGE included cortisol, estradiol, estriol and progesterone in which they belong to the same group of steroid based hormones. A clear distinction of the electrochemical peak current was seen on the modified SPGE. As shown in Figure 5b, the lowest current activity (22.85 μA) was provided by cortisol, followed by estriol of 29.2 μA, estradiol of 39.65 μA and the highest current activity was progesterone at 48.5 μA. Low current of electrochemical peak of the rGO-SPGE added with cortisol might be attributed to the high deposition of cortisol hormones towards the C-Mab on the modified SPGE surface as the active sensing surface is highly specific to cortisol. From the finding, estriol has the closest current activity to cortisol among the other hormones applied. This scenario might be resulted by its molecular structure mimicking the structure of hydrocortisone (cortisol) where high deposition of estriol hormones managed to bind with the cortisol antibody [24]. Nevertheless, at the highest peak current reduction attained, the rGO-SPGE shown high specificity towards cortisol hormone.

4.0 CONCLUSIONS

This study found that the use of rGO as a sensing material significantly improved the performance of the immunosensor based on the electrochemical approach. This performance enhancement was evident in the detection of cortisol hormone in the range of 1.0 ng/mL to 10 μg/mL, which corresponds to human cortisol levels in both normal and stressed conditions. Future studies will focus on simpler sample preparation methods and explore new biorecognition elements such as aptamers to develop more practical and reliable methods. The results of this study clearly demonstrate that one way to improve sensor performance is by incorporating materials that enhance electrochemical properties, enabling the detection of low cortisol concentrations. This finding suggests that the fabricated C-Mab/rGO-SPGE biosensor offers extensive potential for future applications in assessing stress levels using cortisol as a biomarker.

*Corresponding Author | Abdul Rashid, J. I. | jahwarhar@upnm.edu.my

5.0 CONFLICT OF INTEREST

The authors declare no conflicts of interest.

6.0 AUTHORS CONTRIBUTION

Mohd Azmi, A. F. (Data Curation; Writing – Review & Editing; Supervision)
Kannan, V. (Data curation; Writing – Review & Editing; Visualisation)
Ahmad, M. H. (Investigation; Formal analysis; Writing – Original Draft)
Abdul Rashid, J. I. (Conceptualization; Methodology; Investigation; Project administration)

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*Corresponding Author | Abdul Rashid, J. I. | jahwarhar@upnm.edu.my