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Development of low-cost impedimetric biosensors for the quantitative detection of cortisol and the human growth hormone

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ABSTRACT

Cortisol and human growth hormone can be used as reliable biomarkers for the response of the hypothalamus-pituitary-adrenal axis. The hypothalamus-pituitary-adrenal axis is complex and dependent on many factors. However, generally, increased cortisol production is the hypothalamus-pituitary-adrenal axis's physiological response to an external stressor. Growth hormone is also regulated through the hypothalamus-pituitary-adrenal axis and secreted from the anterior pituitary gland. Hence point-of-care sensing devices for the quantification of cortisol and growth hormone would be versatile for the diagnosis of hypothalamus-pituitary-adrenal axis activity. The practical uses for a stress indicator would be diverse, inclusive of the medical and pharmaceutical sectors as well as sports and performance. Therefore, this work discusses the development of two electrochemical biosensors, designed to accurately detect and quantify cortisol and growth hormone concentration levels with potential practical applications. The device relies on impedance values which are produced when cortisol and growth hormone are immobilized onto electrode surfaces through antibody-antigen bonding. Calibration data was achieved for both Gold (Au, 111) slide electrodes and inexpensive screen-printed gold electrode-based sensor biochips with a strong correlation between impedance and concentration of cortisol, with detection range 30–300 ng/mL for Au (111) platform and 60 – 360 ng/mL for screen-printed gold electrode platform, while recombinant human growth hormone detection range was observed 5–30 ng/mL using both Au (111) platform, and screen-printed gold electrode platform.

1. Introduction

Since the turn of the millennium there has been a great interest in the development and use of point-of-care (POC) devices [1,2]. POC devices are diagnostic devices used in close proximity to a patient, or by the individual themselves, in order to test for specific changes into their body [3–5]. Previous well-known POC devices include blood-glucose monitoring kits (used widely by individuals with diabetes) and home pregnancy tests [6–9]. Moreover, there is an increasing need for rapid response diagnostics which would ease the strain on medical professionals and allow for faster treatment of illness and ailment [10–14]. Further, analysis of biomarkers and pathogens in small-volume samples is increasingly important for the diagnosis of disease in real-time [15]. However, there is a gap in the market for an inexpensive POC biosensor for the accurate and quantitative detection of biomarkers especially cortisol and human growth hormone (hGH). Further, important aspects to consider in the design and fabrication of such devices include the cost, ease of use and time taken for results for the biomarker of interest [1, 16–20].

Therefore, the components discussed in this work are low-cost impedimetric POC biosensors designed to detect and quantify cortisol and hGH. Both biosensors would ideally run side-by-side, either in tandem or simultaneously, producing a response for the amount of both cortisol and hGH in blood samples, which would prove useful in monitoring the sports performance [21,22], physical health [23,24], pain management [25,26] and overall wellbeing of individuals [27,28].

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Moreover, developed POC biosensors is expected to diagnose possible tumors in the pituitary gland without exploratory surgery when used in conjunction with each other [29].

Cortisol is a steroid hormone from the glucocorticoid family [30] which is secreted from the adrenal gland in the hypothalamus-pituitary-adrenal axis (HPAA) [31,32]. It is responsible for maintaining glucose levels, blood pressure [33] and regulating metabolism [23,34]. The glucocorticoid is also commonly known as the "stress biomarker" [35] due to the increased release into the blood after a stressful or traumatic event, peaking after 15–30 min and decreasing back to regular pre-stress levels around 60–90 min later [36].

Although a surplus of cortisol is secreted when the body is under stress, shortly after a stressful situation has occurred the body resumes the natural circadian rhythm [32]. A continuous abnormal over-production of cortisol is usually due to a tumor either in the pituitary gland near the brain or in one of the adrenal glands above the kidneys [23]. Prolonged elevated levels of cortisol may lead to a serious condition called Cushing's syndrome. Symptoms of this illness include (but are not limited to) obesity [32], cardiovascular complications [37], osteoporosis [38], fat tissue deposits around the abdomen [39] and purple striae [40]. Alternatively, an insufficient production of cortisol may be indicative of damaged adrenal glands and may lead to Addison's disease [41]. Symptoms may include severe hypotension or hypovolemia [42], unintentional weight loss, persistent vomiting and fatigue [43].

While the hGH, also known as somatotropin [44], is a peptide hormone [45] which is synthesized and secreted from cells in the anterior pituitary gland called somatotrophs [46]. Its secretion is stimulated by growth hormone-releasing hormone (GHRH) [47] and is inhibited by somatostatin [48]. Somatotropin acts as an antagonist to insulin [49] and its main role is to stimulate the growth of all tissues and aid the breakdown of fat stores to provide energy for growth [50].

Regularly, somatotrophs secret around 0.5 mg per day [51], however during puberty an increased amount of hGH is created resulting in a "growth spurt "[52]. An overproduction is also stimulated in response to a decreased diet and subjection to physiological stress [53]. As well as this, excess hGH, can often be caused by a benign tumor (or adenoma) in the pituitary gland [54]. Alternatively, a decrease in hGH secretion may be the response to food ingestion or can be indicative of hypothalamus or pituitary gland damage [55]. This results in short stature and dwarfism [56], which can lead to cardiovascular complications [57]. The benefits associated with surplus amounts of hGH in the body, such as added muscle growth [58], has led to the use and abuse of synthetic hGH by athletes [59]. For these reasons, alongside clinical interest for patient wellbeing and sports performance, it is important to be able to monitor the levels of hGH in real samples [60].

Popular methods of detecting hGH in blood samples include enzyme-linked immunosorbent assays (ELISAs) [61,62] and liquid chromatography-mass spectrometry (LC-MS) [63,64]. These laboratory-based methods are disadvantageous as they require trained technicians, specialist equipment and are time consuming [19,65]. However, there is a current market in both healthcare and athletics for POC devices for on-site detection with a real-time response for quantitative detection of hGH [21]. Further, internal tumors in the hypothalamus-pituitary-adrenal axis (HPPA) often go undiagnosed as it is difficult to identify which component, the hypothalamus, adrenal gland or pituitary gland, which is compromised. A POC device for the detection of cortisol and hGH could specifically identify which gland between the adrenal and pituitary gland (respectively). As well as this, the regulation of growth hormone is of paramount importance in international sporting events such as the Olympics. Previous synthetic hGH abuse has been known to boost an athletes' performance. Additionally, on-site cortisol sensing has many applications ranging from sport performance to patient's physiological well-being in a medical environment.

Unfortunately, detecting cortisol and hGH using ELISA kits is highly expensive, time consuming, labor intensive and requires chemicals and regents. Further, the current state of the art (ELISA) detection range and costs of the assays for cortisol and hGH varies from kit manufacturer company to company. However, the detection range and costs of ELISA for the cortisol and hGH from Abcam, UK are as follows: cortisol detection range from 10 to 500 ng/mL and this costs £550.00 [66], while hGH detection range falls between 9.4 and 600 pg/mL and this costs £580.00 [67]. Interestingly, by integrating electrochemical impedance spectroscopy (EIS) and screen-printed gold electrodes (SPGEs) platforms, for the first time Gwenin research group has been successful in creating the electrochemical biochips that can accurately detect both antigens (cortisol and hGH) in tandem on inexpensive SPGE platforms. Such created biochips are inexpensive, rapid, easy to use and require less chemicals and reagents [68,69]. Furthermore, there is a high demand POC diagnostic devices that do not require long incubation periods, bulky equipment and trained personnel to obtain dependable results [70,71]. Moreover, the comparison of the state of art (ELISA) and new POC technology has been summarized in Table 1.

For this reason, the field of electrochemical biosensors has become a popular area of research [72,73]. Electrochemical biosensors detect substances such as pathogens and biomarkers by recording the change current observed as these substances interact/bind with an electrode-immobilized biorecognition molecule, such as an antibody [74,75], capture probes [76,77] or enzymes [78,79]. More specifically, EIS based biosensors are favored due to direct detection at the surface of electrodes with high sensitivity eliminating the need for large, labelled detection molecules [80–82]. Therefore, an impedance-based POC biosensor for the quantification of cortisol and hGH could produce information about an individual's long-term physical and mental health conditions, and in turn could reduce the number of misdiagnosis instances. Moreover, biosensors capable of detecting cortisol and hGH would allow for diagnosis of tumors in the HPAA without the need for invasive surgery.

Therefore, the key objective of this work includes the development of two label-free and low-cost POC impedimetric biosensors, designed to accurately detect and quantify cortisol and hGH concentration. To perform layer-by-layer characterization of biosensors development using both cyclic voltammetry (CV) and EIS. Moreover, calibration data, ideally comparable to the ranges found in blood samples of a healthy human (both adult and child) will be evaluated employing both Gold (Au, 111) slide electrodes (AuSEs) as well as an inexpensive screen-printed gold electrodes (SPGEs)-based biosensors by leveraging electrochemical impedance spectroscopy (EIS). To perform the comparative analysis of the AuSEs and SPGEs-based biosensors in terms of cost.

2. Materials and methods

All antibodies and chemicals used were obtained from Stratech, Sigma Aldrich and Fisher Scientific. For all electrochemical observations, a Metrohm autolab PGSTAT302N potentiostat was used. AuSEs were purchased from Arrandee metal GmbH, whereas SPGEs were made by DropSens.

2.1. Enzyme-linked immunosorbent assays for antibodies and antigens compatibility studies

Polystyrene microtiter sterile 96-well plates were used for ELISA experiments. Two different assay configurations were established to detect both cortisol and hGH as described.

Monoclonal anti-cortisol antibody XM210 (100 μL) was added to each well (25 μg/mL) diluted in coating buffer (50 mmol/L Na_2CO_3 and 50 mmol/L $NaHCO_3$, pH 9.6). This was sealed and incubated at 4 °C overnight to develop an even monolayer. The plate was washed once using 200 μL of wash buffer (100 mmol/L tris-base with 0.9 % NaCl, pH 7.4). Next, all empty sites were blocked using 300 μL blocking buffer (0.25 % acetylated BSA (ac-BSA) in wash buffer) and incubated for one

Table 1
Comparison of the state of art (ELISA) with new POC technology.

Modality	Detection Range	Equipment	Accessibility	Usability	Costs of Assay
State of the art	Dynamic linear range	Laboratory-based	Centralised	Trained technician	~ US$ 800.00
POC technology	Comparable or wider linear range	Portable	On-site	User-friendly	~ US$ 4.00

hour before washing once. The cortisol-HRP (horseradish peroxidase) conjugate was added (100 μL) to each well at various concentrations between 0 and 360 ng/mL, with one well-containing wash solution only for normalization, and incubated at 37 °C for 1 h. There were four repeats for each tested concentration. The plate was washed once using wash buffer (200 μL). The substrate, 3,3′,5,5′-tetramethylbenzidine (TMB), was added (100 μL) to each well and incubated for 15 min at 37 °C. After color development, STOP solution containing 1 M HCL (100 μL) was added to terminate the reaction and absorbance was measured at 450 nm, enabling the quantitative detection of cortisol.

Recombinant human growth hormone rhGH (100 μL) in coating buffer (50 mmol/L Na_2CO_3 and 50 mmol/L $NaHCO_3$, pH 9.6) was used to coat wells of a polystyrene microtiter sterile 96-well plate (100 μL) in the concentration range of 0–100 ng/mL. The plate was incubated overnight at 4 °C and then washed twice using phosphate-buffered saline (PBS) solution (200 μL). Next, all wells were blocked using 0.25 % ac-BSA (300 μL) in PBS for 1 h at 37 °C, before washing twice with PBS. Monoclonal growth hormone antibody conjugated the horseradish peroxidase (Gh29-HRP) was added to each well (100 μL, 50 $ngmL^{-1}$) and plates were incubated for 1.5 h at 25 °C then washed with PBS. Color development using TMB substrate (100 μL) was undergone for 15 min at 25 °C and terminated using 1 M HCl (100 μL). Absorbance was observed at 450 nm.

2.2. Development of impedimetric biosensors using auses

AuSEs were annealed under flame 3 times to achieve a consistent (111) terraces [83]. To coat antibody onto the surface, slides were submerged in 10 mL of coating buffer (50 mmol/L Na_2CO_3 and 50 mmol/L $NaHCO_3$, pH 9.6) containing monoclonal XM210 (100 ng/mL) for 48 h at 4 °C. After this period all slides were washed 3 times using 200 μL wash buffer (100 mmol/L tris-base with 0.9 % NaCl, pH 7.4). Next, empty sites on slides were blocked using 0.25 % ac-BSA in wash buffer (10 mL) for one hour at 37 °C. After blocking, slides were again washed 3 times. For calibration data slides were then submerged in a solution containing cortisol in wash buffer for 1 h at 37 °C. Concentrations of cortisol used were as follows: 0, 30, 60, 90, 120, 150, 180, 210, 240 and 300 ng/mL. Slides were washed a final 3 times and impedance tests were completed.

Further, AuSEs were annealed under flame 3 times to achieve a consistent (111) orientation [83]. To coat antibody onto the surface, 200 μL of anti-growth hormone antibody Gh29 (10.6 μg/mL) in coating buffer (50 mmol/L Na_2CO_3 and 50 mmol/L $NaHCO_3$, pH 9.6) was pipetted onto AuSEs for a period of 24 h at 4 °C. After this period all slides were washed 3 times using 200 μL wash buffer (1X PBS). Next empty sites on slides were blocked using 0.25 % ac-BSA in wash buffer (200 μL) for one hour at 37 °C. After blocking, slides were again washed 3 times. For calibration, data slides were then covered in a solution containing recombinant growth hormone rhGH in PBS for 1 h at 37 °C. Concentrations of rhGH used were as follows: 0, 5, 10, 15, 20, 25, 30, and 35 ng/mL. Slides were washed a final 3 times and impedance tests were completed.

2.3. Development of impedimetric biosensors using SPGEs

The same format, direct assay, was adapted to SPGEs. Purchased from Dropsens Metrohm, SPGEs consist of an Ag reference electrode, a Pt counter (or auxiliary) electrode and an Au working electrode. To achieve consistency between SPGEs surfaces, the electrodes were pre-treated at 200 °C for 24 h. Further, for cortisol detection: XM210 antibody was coated onto the working area on SPGEs (10 μg/mL, 50 μL) at 4 °C overnight. SPGEs surface was washed using PBS to remove any excess antibody. Blocking agent ac-BSA (0.25 % in PBS) was added to the electrode surface for 1 h at 37 °C and then washed with PBS. Cortisol concentrations used were 0, 60, 120, 180, 240, 300 and 360 ng/mL in 50 μL volumes. Each step of impedimetric biosensor development for the detection and quantification of cortisol using SPGEs is illustrated in Scheme 1A. While the working area on SPGEs was coated in Gh29 (9 μg/mL, 50 μL) at 4 °C overnight. Any excess antibody is washed from the surface using PBS. Electrodes were then blocked using ac-BSA blocking buffer (0.25 % in PBS) for 1 h. A final washing step took place before the addition of rhGH (50 μL) and the concentrations used are 5, 10, 15, 20, 25 and 30 ng/mL. Each step of impedimetric biosensor development for the detection and quantification of hGH using SPGEs is illustrated in Scheme 1B.

2.4. Cyclic voltammetry

All electrochemical characterization steps were conducted using a Metrohm autolab PGSTAT30 potentiostat. AuSEs as working electrode were analyzed in conjunction with a platinum electrode as a counter electrode (CE) and a silver/silver chloride (Ag/AgCl) electrode for the reference electrode (RE). Further, SPGEs were analyzed using CV after they were coated as mentioned in Section 2.2.

Briefly, the electrode was removed from the layer-containing solution, washed thoroughly using sterile wash solution (100 mmol/L tris-base with 0.9 % NaCl, pH 7.4) and then dried under nitrogen before being sealed in the electrochemical cell. CV assays were completed in the scanning potential range of −0.5 to + 0.9 V at a scan rate of 50 mV/s. Assays contained a redox species of $[Fe(CN)_6][4]^-$ (5 mM) [and $[Fe(CN)_6][3]^-$ (5 mM) prepared in 1 M phosphate-buffered saline solution (KCl).

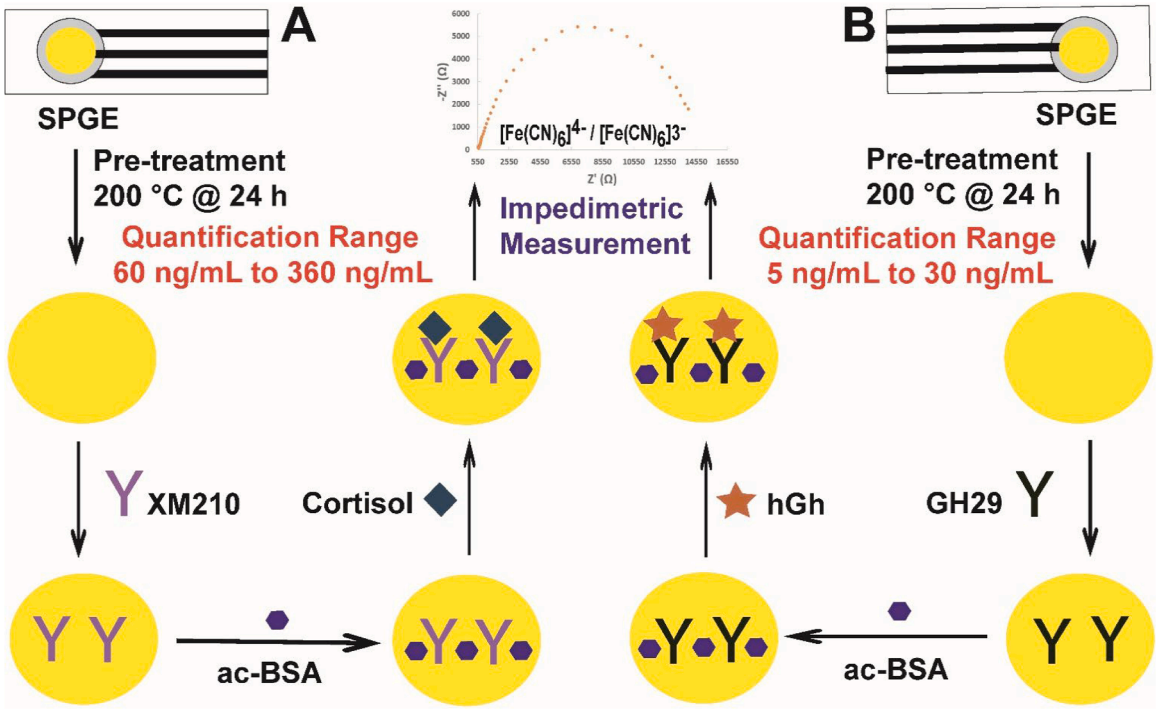
2.5. Electrochemical impedance spectroscopy

Electrodes were analyzed using EIS after they were coated as mentioned in Section 2.2. Briefly, the electrode was removed from the layer-containing solution, washed thoroughly using sterile wash solution (100 mmol/L tris-base with 0.9 % NaCl, pH 7.4) and then dried under nitrogen before being sealed in the electrochemical cell. Impedance measurements were performed using a ferri/ferrocyanide (5 mM) redox probe in KCl (100 mM) electrolyte. A potential of 300 mV was applied with a perturbation amplitude of 10 mV/s for a frequency range of 10 kHz to 0.1 Hz.

3. Results and discussion

3.1. Enzyme-linked immunosorbent assays for antibodies and antigens compatibility studies

ELISA is a well-established, laboratory-based method which is widely utilized for the identification and quantification of biomarkers [84–86]. It can often be used to determine the concentration of a specific biomarker in a complex mixture due to the antibody-antigen binding interaction [87,88]. For the development of a biosensors capable of detecting the stress biomarker cortisol and physical health biomarker hGH it was important to conduct preliminary ELISA tests which could determine the process of an antibodies/antigens binding events.



Scheme 1. Step of impedimetric biosensor development for the detection and quantification of **(A)** cortisol and **(B)** hGH using SPGE.

Therefore, a direct ELISA was designed for the detection of cortisol, whereby monoclonal antibody XM210 was the capture antibody, acetylated bovine serum albumin (ac-BSA) as the blocking agent and HRP was the identifying tag. The results obtained for this ELISA has been depicted in Fig. 1A. Results from ELISA indicate that there is an antibody-antigen binding occurring in a directly proportional manner. This is clear from the strong correlation ($R^2 = 0.992$) observed between absorbance and concentration of cortisol-HRP between the range of 25–300 ng/mL. ELISA is a laboratory-based method which requires bulky equipment [89], long incubation periods [35] and trained personnel [65] to complete and therefore is not a suitable method for POC. XM210 is an immunoglobulin G 2A cortisol-specific monoclonal antibody which has never been previously incorporated into a biosensor. The binding information proven through ELISA shows that this antibody would act as a successful capture for cortisol when included in a biosensor for POC detection.

Further, HRP conjugated monoclonal antibody GH29-HRP was used for light intensity-dependent photometric detection of immobilized rhGH. Calibration results are illustrated in Fig. 1B. This calibration set shows a strong relationship ($R^2 = 0.996$) between the concentration of rhGH and the results absorbance, with reliable results and a small deviation between the repeat readings at each concentration. It was found that the direct assay format was the most successful assay type when compared with previous sandwich ELISA results. To reduce background, signal the target antigen (rhGH) was directly coated to the surface of wells, eliminating the need for a capture antibody. Blocking agent, ac-BSA, was used and monoclonal antibody Gh29 conjugated to HRP was added with TMB substrate for quantitative colorimetric detection. A linear direct correlation is observed between the concentration range of 0–100 ng/mL. This range is inclusive of the values for healthy children,

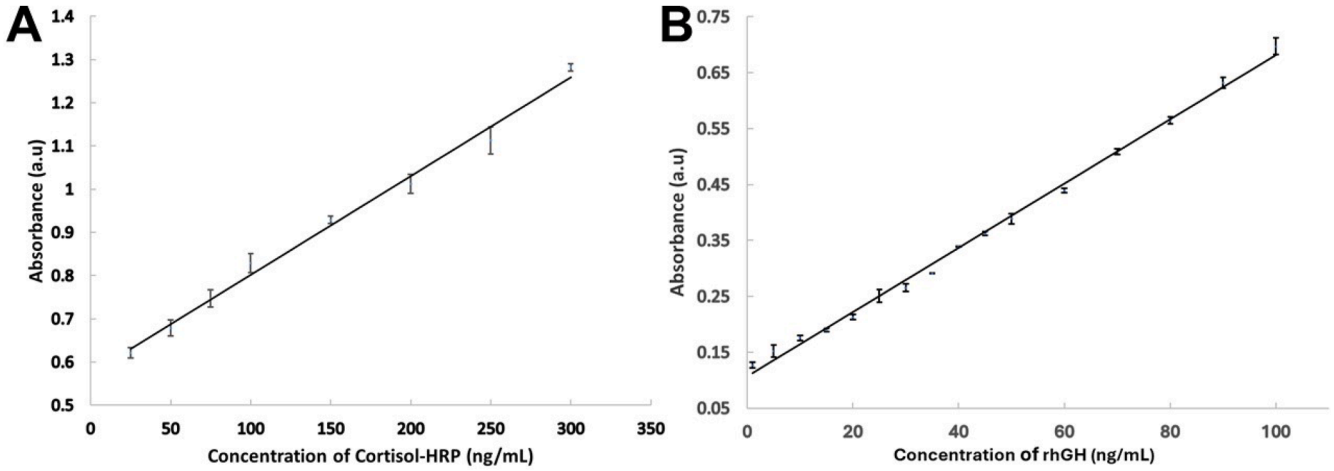


Fig. 1. (A) A graph displaying data collected from a sandwich assay ELISA for cortisol through concentration range 0 – 300 ng/mL whereby XM210 is the capture antibody, ac-BSA is the blocking agent and HRP is the detection molecule. **(B)** Calibration data from direct assay ELISA for the quantification of recombinant growth hormone (rhGH) for concentrations 0–100 ng/mL. The blocking agent is ac-BSA and the detection antibody is anti-growth hormone conjugated to HRP enzyme (Gh29-HRP). Error bars are representative of ± one standard deviation.

adult males and adult females (including pregnant women and newborn) [62]. As the concentration ranges for each individual varies depending on age, body mass index and gender [90], the final biosensor must consider this in the development of the device/user interface. To note the regular levels of hGH can be affected by various factors including age, weight and physical wellbeing of an individual [90].

3.2. Specificity studies of antibodies using ELISA

In the development of POC devices for real samples it is important to consider the type of analyte used [91,92]. Real samples are often complex mixtures and in real samples, such as blood, urine and saliva, there may be other non-specific antigens which could interfere with the outcome of POC biosensor if capture antibodies are not specific to the antigen of choice [3,30]. The molecules most likely to interfere with a bioassay would be similar in structure, size and charge.

Further, the molecules that are similar to cortisol are also cholesterol derivatives progestagens, glucocorticoids, mineralocorticoids, androgens, and estrogens [93]. These steroid hormones were chosen to "compete" with HRP conjugated cortisol (C-HRP) *via* ELISA in order to analyse the level of interference that these non-specific antigens would have due to the level of specificity of capture antibody XM210. Concentrations of these molecules were comparable to the maximum amount of each substance in healthy human blood samples. Results from this experiment were analysed and displayed in the bar chart in Fig. 2A.

While a similar ELISA was completed in order to determine if various non-specific antigens from both a synthetic mixture and real blood samples would interfere with the binding interaction between Gh29 and rhGH. The synthetic hormone mixture consisted of insulin, somatostatin, ghrelin, growth hormone-releasing hormone. Concentrations of these synthetic hormones were also comparable to the maximum amount of each substance found in healthy human blood samples. The results obtained for the evaluation of antibody (Gh29) specificity using ELISA have been depicted in Fig. 2B.

ELISA-based specificity study of both XM210 and Gh29 antibodies showed the suitability for the development of impedimetric biosensors for the quantitative detection of cortisol and hGH in complex mixtures containing non-specific antigens including blood.

3.3. Layer-by-layer study using cyclic voltammetry

Electrochemical techniques such as cyclic voltammetry and impedance may be used to show electrical changes at the surface of an electrode which may indicate a layer formation [73,94–96].

Therefore, firstly CV was performed on Au (111) electrodes to show the layer formation of a monoclonal cortisol-specific antibody (XM210), blocking agent (ac-BSA) and the antigen of this assay (cortisol) Fig. 3A. As shown in Fig. 3A, CV of the direct cortisol assay on Au (111) electrodes was achieved, indicated by the decrease in the magnitude of current output as each layer/component of the assay is coated onto the working electrode surface. A reversible reaction is observed, indicating the reduction and oxidation of the electrolyte (potassium ferri/ferrocyanide) due to electron transfer at the surface of the electrode. While the electron transfer between electrolyte/electrode is impeded as layer of biochemical matter bound to the surface of the working electrode. If there is antibody bound to the surface of the Au (111) electrode the charge transfer is inhibited slightly; this is indicated by the reduction in current peak height for oxidation (E_{pa}) and reduction (E_{pc}) seen in Fig. 3A. When both antibody and blocking agent are added to the Au (111) surface a greater blocking occurs, resulting in a greater current peak height reduction. The greatest reduction in peak height is observed when all three assay layers (XM210, ac-BSA and cortisol) are present on the electrode surface. There is also a sideways shift in E_{pa} and E_{pc} which signifies the presence of a fully formed ac-BSA layer in these results [68,73].

Further, electrodes were developed incorporating a direct assay for hGH detection, similar to previous ELISA (discussed in Section 3.1). CV was performed to determine the relationship between redox probe (potassium ferri/ferrocyanide) and the surface of electrodes. Results are representative of surface coverage of a) primary/capture antibody – Gh29 b) Gh29 with an ac-BSA blocking agent and c) Gh29 with ac-BSA and target rhGH onto Au (111) slides. A blank AuSE was also studied for comparison. Results are shown in Fig. 3B A decrease in the magnitude of current output (regarding both forward oxidations, E_{pa}, and reverse reduction, E_{pc}, peaks) is observed with the addition of each component to the surface of the electrode. Electron transfer between electrolyte/electrode may be blocked if there is a layer of biochemical matter bound to the surface of the working electrode, this is indicated by the reduction in current peak height for oxidation (E_{pa}) and reduction (E_{pc}) seen in Fig. 3B As antibody (Gh29), blocking agent (ac-BSA) and target antigen (rhGH) are added sequentially, a resistance increase is observed, obstructing charge transfer between electrode and electrolyte [68,73].

Moreover, an increase in faradaic impedance, which is an accumulation of the resistance and capacitance acting simultaneously at the

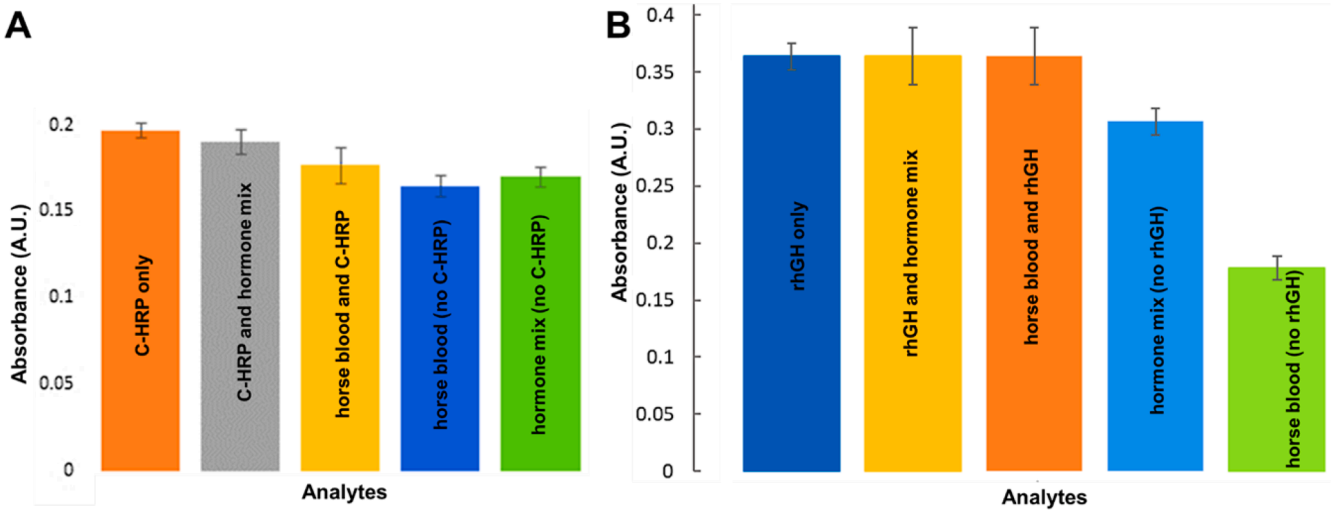


Fig. 2. **(A)** Results from ELISA, showing the level of specificity for antibody XM210 using specific target antigen (cortisol) conjugated to HRP (C-HRP) for reference when compared with a mixture of hormone molecules and a complex mixture from horse blood. **(B)** Results from ELISA, showing the level of specificity for antibody Gh29, using Gh29 conjugated to HRP (Gh29-HRP) to identify binding to both specific and non-specific antigens in a mixture of hormone molecules and a complex mixture from horse blood. Error bars are representative of ± 1 standard deviation.

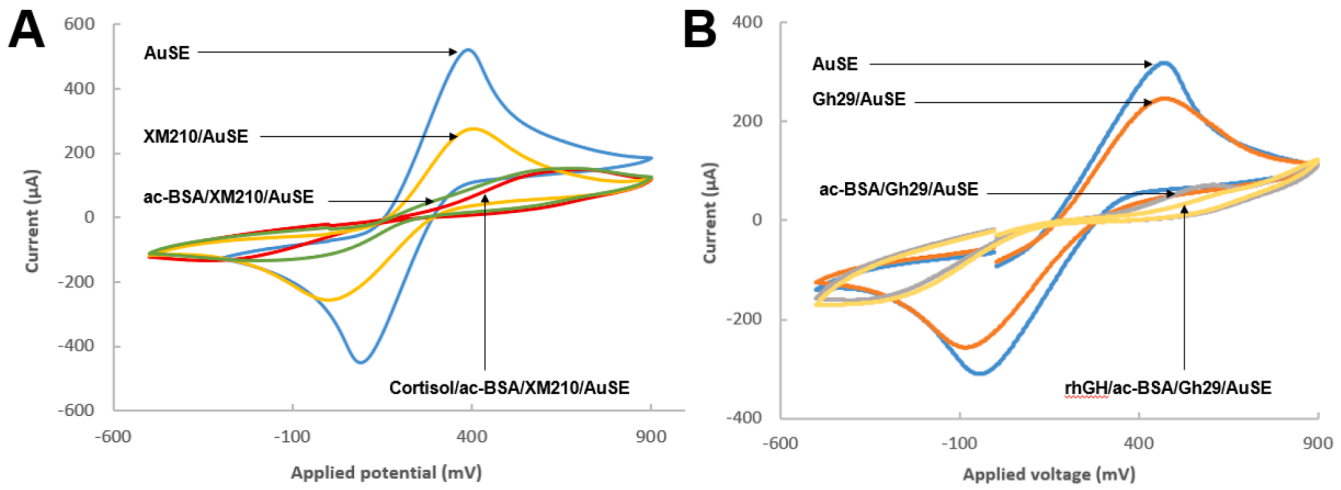


Fig. 3. **(A)** CV of bare AuSE (blue); XM210 coated AuSE (yellow); XM210 and ac-BSA coated AuSE (green); XM210 with ac-BSA and cortisol (in excess) coated AuSE (red) in 1 M PBS containing 5 mM $K_3[Fe(CN)6]/K_4[Fe(CN)6]$. **(B)** CV of bare AuSE (blue); Gh29 coated AuSE (red); Gh29 and ac-BSA coated AuSE (grey); Gh29 with ac-BSA and rhGH (in excess) coated AuSE (yellow) in 1 M PBS containing 5 mM $K_3[Fe(CN)6]/K_4[Fe(CN)6]$. All CV assays were completed in the scanning potential range of −500 mV to + 900 mV at a scan rate of 50 mV/s against Ag/AgCl reference electrode.

electrode surface, is shown by the integrated area increase between each step of the assay layer formation [97]. Considering the capacitive properties of the electrode, equation 1 is used to determine the ability that the electrode possesses to store charge.

Equation 1 – Capacitance, a characteristic of electric double layers, is equal to the change in surface charge (σ) as a fraction of the electric surface potential (ψ).

$$C = \frac{d\,\sigma}{d\,\psi}$$

Due to the nature of the electrical double layer, it would be expected that as more biological matter binds to the surface of the electrode it would become more difficult for exchange of electrons between the working electrode surface and the redox-active electrolyte, as shown in Fig. 3.

3.4. *Layer-by-layer study using impedance spectroscopy*

Impedance is a versatile method that can identify small changes at the surface of electrodes [98,99]. Therefore, firstly, impedance measurements were conducted on 1) blank AuSE, 2) XM210 coated AuSE, 3) XM210 and ac-BSA coated AuSEs and 4) XM210, ac-BSA and cortisol coated AuSEs. While a similar trend as CV was observed when layer formation was assessed using EIS. Results are overlaid in Fig. 4.

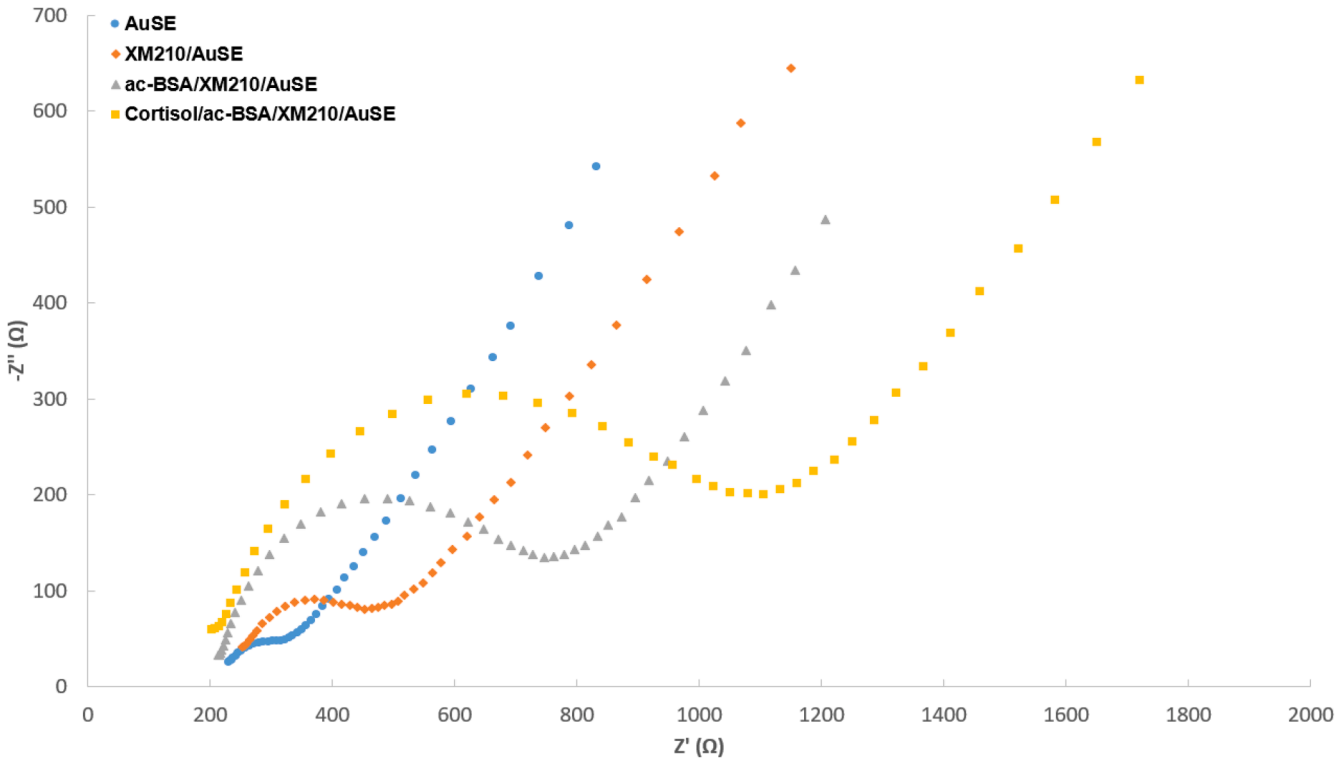


Fig. 4. Nyquist plots of bare AuSE (blue); XM210 coated AuSE (orange); XM210 and ac-BSA coated AuSE (grey); XM210, ac-BSA and cortisol, in excess 360 ng/mL (greater than 1:1 ratio for antibody sites) coated AuSE (yellow) in 100 mM KCl containing 5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$.

Generally, an increased impedance loop is indicative of a greater resistive force at the surface of a working electrode [96,99]. This may be due to a biochemical species, such as an antibody or antigen, binding to the electrode surface. The charge transfer resistance (R_{ct}) is the electron transfer resistance from one phase to another and is used to quantify the impedance increase [100]. Fig. 4 shows an increased –Z" from the blank Au (111) electrode to the XM210 coated Au electrode, and subsequently an increased R_{ct} value. This increased impedance suggests that a layer of antibodies has bound to the surface of the electrode [101]. A further increased R_{ct} value is observed as the blocking agent, ac-BSA is added to the surface and an even greater increase is seen when cortisol is added to the electrochemical assay. The increase in impedance from EIS between each layer formation of the electrochemical bioassay (see Fig. 4) suggests that the three components: 1) XM210 antibody 2) ac-BSA blocking agent and 3) cortisol have successfully been added to the surface of Au (111) working electrodes [94–96]. This obtained result is supported by CV data (see Fig. 3A). The current height peak decrease from CV and increase in R_{ct} from EIS both demonstrate layer formation on the surface of working electrodes.

Further, a similar trend as CV was observed when layer formation of hGH assay on Au (111) electrodes was assessed using EIS. Impedance was used to compare electrochemical changes between a) blank Au (111), b) Au (111) with Gh29 antibody on the surface, c) Au (111) with Gh29 and ac-BSA blocking agent and d) Au (111) with Gh29, ac-BSA and specific target antigen – rhGH sequentially added to the electrode surface. Obtained results from this experiment has been depicted in Fig. 5. The graphs in Fig. 5 indicates dramatic differences between each sequential layer formation on Au (111) electrode surfaces. There is a clear, distinct increase in R_{ct} as indicated by the width of the bell curve. A simple circuit model, consisting of inductors (Warburgs impedance), resistors (solution resistance and charge transfer resistance) and capacitors (double layer capacitance) was fitted to the data plots to calculate the R_{ct} value. The increase in R_{ct} is expected due to the greater general resistance as the amount of substance on the electrode surface increases a greater barrier is formed [94–96]. This barrier at the electrode/electrolyte interface is electron-dense and therefore disrupts electron transfer, requiring a greater voltage for charge transfer [102]. Moreover, observed result is in correlation to the obtained CV data (see Fig. 3B). The current height peak decrease from CV and increase in Rct from EIS both demonstrate layer formation on the surface of working electrodes.

3.5. *Analytical performances of impedimetric biosensors developed using auses*

Electrochemical techniques such as EIS are highly advantageous as they are fast and effective when compared to the standard ELISAs currently available [103,104]. There is no need for long incubation periods and EIS measurements can be incorporated into a POC for general use, whereas ELISA requires a trained technician to conduct the experiment [23,65]. Therefore, a bio-assay calibration data sets were obtained both for cortisol and rhGH via EIS on Au (111) electrodes as shown in Fig. 6.

Obtained results showed a clear positive correlation between 0 – 300 ng/mL ($R^2 = 0.983$) of cortisol as depicted in Fig. 6A, which indicates that the concentration of cortisol added to the electrochemical assay has a direct effect on the impedance observed. Moving forward with the direct assay modified AuSEs impedance spectroscopy was completed to obtain calibration data for a series of rhGH concentrations as well with a range of 0 – 30 ng/mL. Results from EIS of rhGH assay on Au (111) electrodes has been shown in Fig. 6B Obtained results portray a clear and strong relationship ($R^2 = 0.996$) demonstrated by observed data set, and further there was a significant difference between results at each concentration. The error associated with each reading comes from very small changes in the electrochemical cell. Impedance spectroscopy is known for its high level of sensitivity [81]. Miniscule factors such as

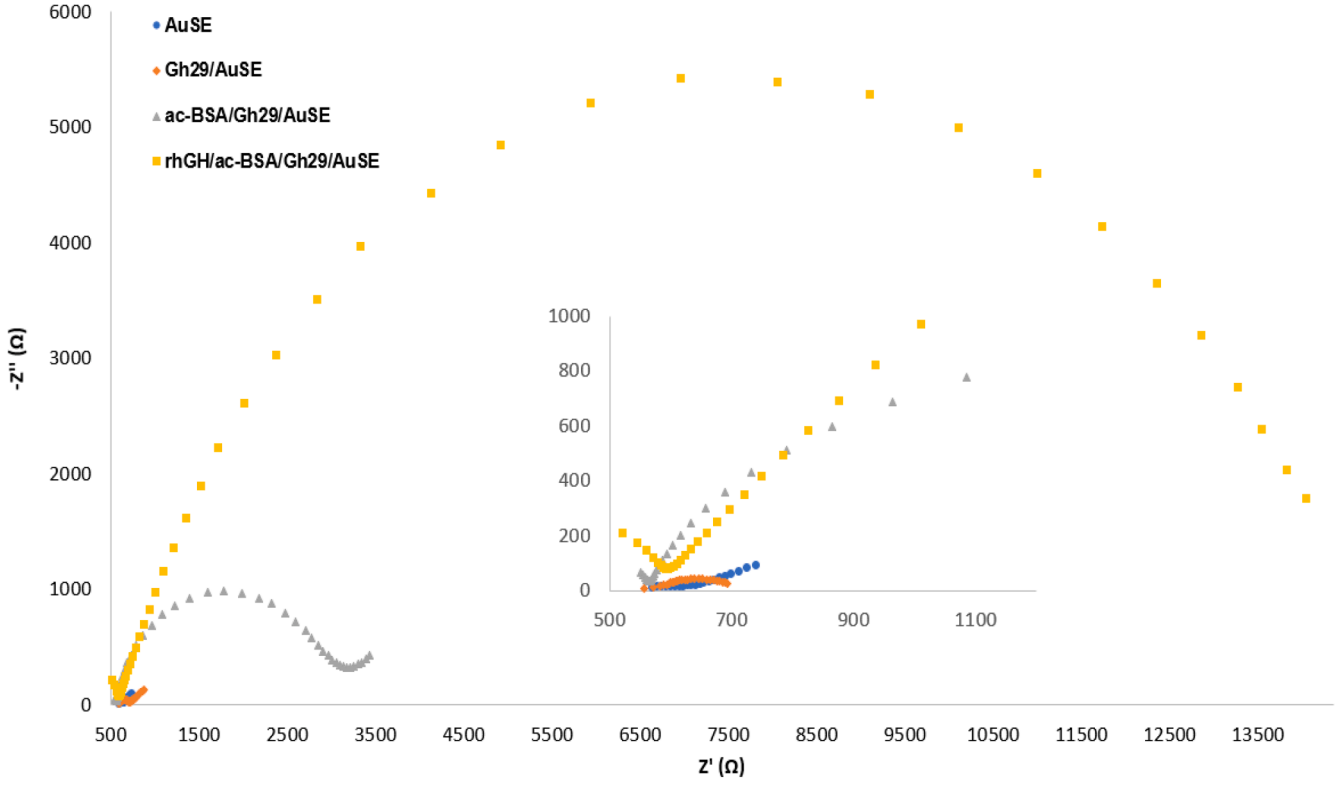


Fig. 5. Each layer of finalized GH bioassay on AuSEs analyzed *via* EIS. The graph shows blank AuSE (dark blue), Gh29 antibody-modified AuSE (light blue), AuSE with Gh29 and ac-BSA blocking agent (orange), AuSE with Gh29, ac-BSA and target antigen rhGH (green). Inset: Zoomed-in image showing impedance differences between the curves. All EIS measurements were conducted in 1 M PBS containing 5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$.

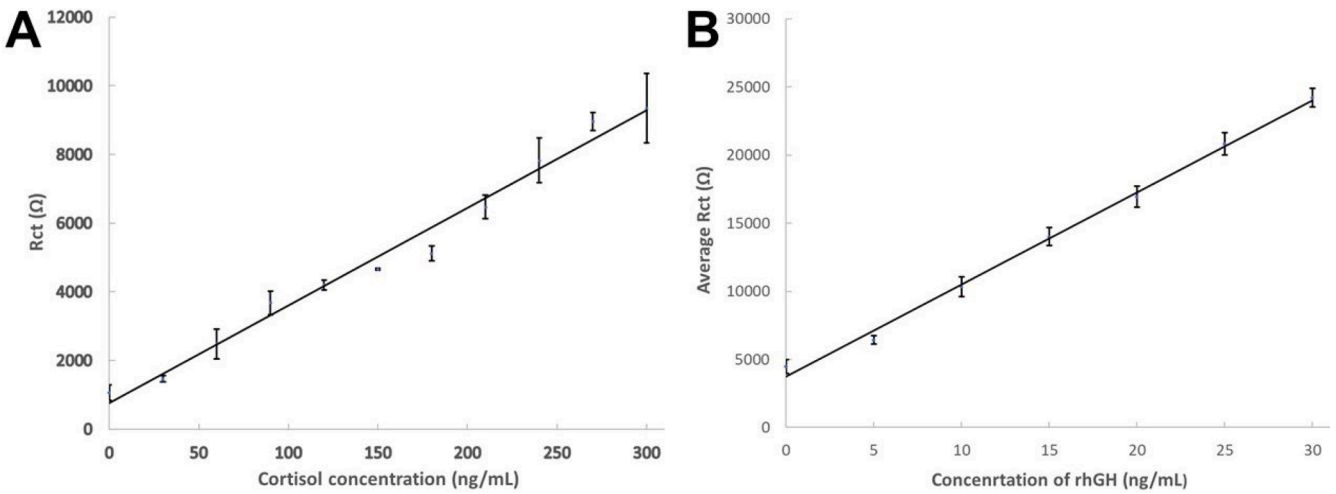


Fig. 6. (A) Average R_{ct} from impedance taken from three repeats of EIS measurements of **(A)** cortisol assay and **(B)** rhGH on Au (111) electrodes at various concentrations of either. Error bars are representative of ± one standard deviation.

electrode quality and variation of distance (down to nanoscale) between electrodes for different cell configurations can show massive variance between readings [105].

3.6. Analytical performances of impedimetric biosensors developed using SPGEs

Thus far, manufacturing of the sensor chips is costly due to the expensive Au (111) electrode platform. For this reason, it would be desirable to reduce the cost of the sensors by replacing this component (Au (111) electrode) with a cheaper alternative. Screen-printing technology, now responsible for the mass production of electrodes for biosensors [106], provides a means of dramatically reducing the cost of biosensor devices [107]. Screen-printing is a well-established method for the inexpensive production of disposable electrodes [18,69]. Therefore, for the first-time SPGEs based alternative approach was adopted for the development of low-cost biosensors for the quantification of both cortisol and hGH. SPGEs consists of Au working electrode, Ag reference electrode and a Pt counter electrode on a miniature (est. 1 × 3 cm) ceramic surface. Therefore, using same protocol as biosensor developed for cortisol using AuSEs, a calibration data collected for cortisol detection was obtained over SPGEs, which includes XM210 and ac-BSA, at various concentrations of cortisol on SPGEs (see Fig. 7A). Similarly, calibration data generated for hGH electrochemical assay over SPGEs has been shown in Fig. 7B

Fig. 7A indicates a distinct, strong relationship between the change in the concentration of cortisol and the representative impedance. There is a clear linear correlation (coefficient value $R^2 = 0.979$) showing a strong trend between Rct and increased cortisol concentration for the concentration range 0–360 ng/mL. Further, as observed in Fig. 7B, a relationship between impedance and an increase in rhGH concentration was established (0–30 ng/mL). The correlation between Rct and rhGH concentration exhibits a strong relationship ($R^2 = 0.982$). Calibration data is established by fitting a simple circuit model, consisting of inductors (Warburgs impedance), resistors (solution resistance and charge transfer resistance) and capacitors (double layer capacitance) which was fitted to the data plots to calculate the Rct value is plotted against a range of concentrations for cortisol (0–300 ng/mL) and rhGH (0–30 ng/mL). The accumulation experiments in this paper validate the development of a novel electrochemical SPGE sensor biochip (biosensor), using XM210 and Gh29 antibodies, for the quantitative detection of cortisol and hGH respectively. Further sensing performances of the developed biosensors have been summarized and compared with commercially available ELISA in Table 2. Interestingly, through the

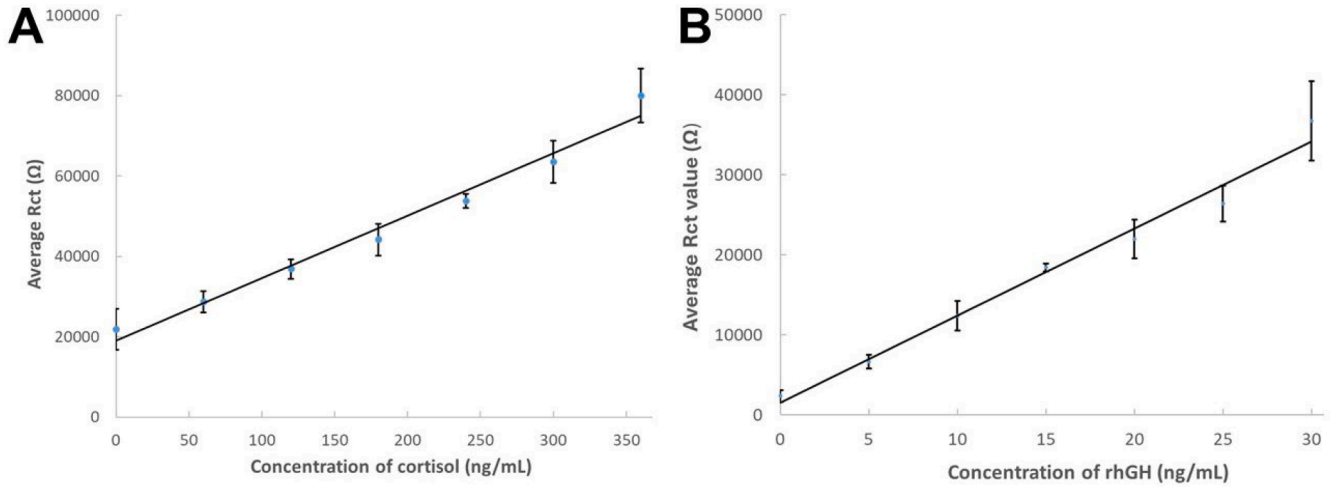


Fig. 7. (A) Graphical representation of the average R_{ct} from the impedance of various cortisol concentrations and **(B)** rhGH concentrations, from direct label-free electrochemical immunoassay on SPGEs. EIS was conducted between frequency range 0.1–10,000 Hz and the electrolyte used was 5 mM potassium ferri/ferrocyanide. Error bars are representative of ± one standard deviation.

Table 2
Sensing performances of the developed biosensors for the quantitative detection of cortisol and hGH compared to commercially available ELISA.

Detection method	Transducer	Antigen-Antibody Binding Time	Sensing Principle	Quantification/ Detection Range	Limit of Detection	Refs.
Cortisol						
Electrochemical biosensor	AuSE	60 min	EIS	30–300 ng/mL	30 ng/mL	This work
Electrochemical POC biosensor	SPGE	60 min	EIS	60–360 ng/mL	60 ng/mL	This work
ELISA	–	60 min	Colorimetric	25–300 ng/mL	25 ng/mL	This work
hGH						
Electrochemical biosensor	AuSE	60 min	EIS	5–30 ng/mL	5 ng/mL	This work
Electrochemical POC biosensor	SPGE	60 min	EIS	5–30 ng/mL	5 ng/mL	This work
ELISA	–	90 min	Colorimetric	1–100 ng/mL	1 ng/mL	This work

substitution of electrode type, the overall cost of the biosensors is dramatically reduced as Au (111) cost an average price of approximately £17 per electrode whereas SPGEs are roughly £3 per electrode.

4. Conclusions

To conclude, studies towards the development of two POC devices for the quantitative detection of both cortisol and rhGH have been completed. Laboratory-based ELISA techniques were chosen to validate the binding relationship between the antibody and specific antigen (XM210 and cortisol or Gh29 and rhGH). In addition, to evaluate that the XM210 and Gh29 antibodies used to immobilize over the electrodes provide reliable and distinguish affinity towards cortisol and rhGH respectively in the presence of other hormones or horse blood proteins specificity studies were conducted before developing the biosensors using ELISA.

To focus on the POC aspects of the biosensor, electrochemical techniques of CV and EIS were chosen to a) validate layer formations of each component of the direct assay onto AuSEs and b) create a calibration, ideally comparable to the ranges found in blood samples of healthy human (both adult and child). Calibration data sets were achieved from the direct, label-free bioassay used on Au (111) electrodes, with a strong correlation for detection ranges between 30 and 300 ng/mL for cortisol and 5–30 ng/mL for rhGH.

Further work was carried out to scale down reaction volumes and vastly reduce biosensor cost by approximately £14 per sensor chip through incorporating SPGEs for EIS calibration. This is more useful for a POC device as it eliminates the need for bulky equipment. SPGEs (which are roughly £3 per SPGE) were used as a sensor platform, replacing Au (111) electrodes (approximately £17 per electrode), for immobilization of primary antibody, blocking buffer and either cortisol or rhGH respectively. Data from SPGE experiments exhibited a strong correlation between EIS and a) cortisol concentration for range 0–360 ng/mL and b) rhGH concentration for range 0–30 ng/mL. Considering cortisol assay, the preliminary detection range spanned from 60 to 360 ng/mL, which is in line to reference ranges according to South Tees Hospitals NHS Foundation Trust (43–224 ng/mL) [108]. For hGH, biosensor exhibited a strong correlation for the detection range 5–30 ng/mL, which is also able to detect any increase in the healthy range for all humans (0–10 ng/mL) according to South Tees Hospitals NHS Foundation [109].

In addition, our future studies will focus on the non-specific binding study and recovery index for the SPGEs-biosensors developed for the quantitative detection of cortisol and rhGH concentrations level when exposed to biological samples. Further, we also intend to evaluate the stability of the developed POC electrochemical biosensors. Besides, we also aim for the autonomation of the device which would include real-time data processing suitable for the use and understanding by non-experts. Ideally for POC use, this user-interface would incorporate Rct values to provide a “too much/too little” response, aiding in the diagnosis of diseases such as Addison’s disease, Cushing’s syndrome, tumours in the pituitary and adrenal glands, as well as a useful physiological indicator to high-stress situations. Further, developed POC biosensors can support 6th generation (6G) sensing technology in several ways including for advanced computational approaches (e.g. artificial intelligence (AI), machine learning (ML) and Big Data) enabled POC biosensors for ultra-highly sensitive detection of biomarkers (cortisol and hGh) and internet of things (IoT) integrated POC biosensors for the real time data transmission and remote monitoring [110]. Moreover, POC biosensors integrated with 6 G sensing technology can be useful in precision medicines and personalized health monitoring including for pain management in a personalized manner [110,111]. For example, our developed POC biosensors enabled with AI-driven analytics algorithms will provide exact and real-time quantitative levels of biomarkers (cortisol and hGH) in the patients. Further, detected quantitative levels of biomarkers can be used for pain management in a personalized manner by tailoring treatment according to the specific needs and conditions of the patients.

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CRediT authorship contribution statement

Rachel Ashton: Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Mohammad Rizwan:** Writing – review & editing, Validation, Supervision, Methodology. **Christopher Gwenin:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

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Data availability

Data will be made available on request.

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