Electrochemical Sensing of Cortisol: A systematic review

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Abstract: Cortisol, also known as the “stress hormone”, is secreted under the control of the hypothalamic pituitary adrenocortical (HPA) axis in response to psychobiological stress. Real-time and continuous monitoring of the cortisol levels throughout the day can provide the information necessary to identify any abnormalities in cortisol’s circadian rhythm that may disrupt the processes that cortisol is involved in in the body. This review presents a systematic search of literature on electrochemical sensing techniques for real-time measurement of cortisol in human biofluids. The sensors are here categorised based on the type of bioreceptors used: antibodies, molecularly imprinted polymers (MIPs), and aptamers. Several structural and performance related parameters of the sensors have been discussed, including the sensor stack layers, limit of detection (LoD), dynamic range, sensitivity, selectivity, reusability, redox probe usage, and electrochemical detection method used. A narrative review of the resulting 41 papers is presented focusing on the sensor structure and performance, and its unique features such as reusability to allow sustained measurement from bio-fluid. Additionally, notable advancements in the field and their impacts were summarized. Aptasensors and MIP-based sensors have shown superior stability and sensitivity over the traditional immunosensors. They also demonstrated reversible binding, and 5 papers presented reusable cortisol sensors using these bioreceptors.
Keywords: *cortisol, electrochemical sensing, antibodies, molecularly imprinted polymers, aptamers*

I. Introduction

Cortisol, an important glucocorticoid, plays a vital role in the body’s stress response. Also known as the “stress hormone”, cortisol secretion is controlled by the hypothalamic pituitary adrenocortical (HPA) axis [1]. Cortisol is involved in the homeostasis of the cardiovascular, immune, renal, skeletal, and endocrine systems [8] and plays an important role in the regulation of glucose levels, blood pressure, and carbohydrate cycles [9].

Clinical studies indicate that cortisol levels in the body follow a circadian rhythm with the highest levels in the morning and significantly lower levels at night [7]. Sustained abnormal levels of cortisol can have detrimental effects on various physiological processes. For example, it is well known that substantially high or low cortisol levels throughout the day are associated with Cushing’s syndrome and Addison’s disease, respectively [5]. Lower than normal levels of cortisol in the morning have been shown to be associated with high levels of sleepiness at awakening and poor health and exhaustion the previous day, while high cortisol levels in the evening have been shown to be related to symptoms of stress [10].

Although one-off cortisol measurements are already used widely in clinical practice and in stress and wellbeing research [2], it is evident that continuous monitoring of cortisol levels throughout the day can provide more accurate and comprehensive information about the diurnal variations of cortisol in the body. Such information may be used in various scenarios to enhanced diagnosis, allow the individual to self-monitor their stress level and take appropriate self-care measures, as well as for closed–loop personalised care and therapy for those suffering from cortisol related diseases such as Cushing’s syndrome and Addison’s disease. Such “continuous” monitoring paradigm requires a cortisol sensing technology that allow frequent measurements ideally in real-time and within a low-cost setting. However, all current technologies used for the quantification of cortisol (e.g. high-performance liquid chromatography (HPLC), enzyme-linked immunosorbent assay (ELISA), chemiluminescence immunoassay (CLIA), and surface plasmon resonance (SPR) [11]) require laboratory equipment, space, and expertise, making them unsuitable for real-time or point of care

(PoC) applications. Furthermore, they only offer a snapshot of the cortisol levels which does not provide information about variation in cortisol level in response to triggers.

Electrochemical biosensing techniques have been an area of significant research interest for medical diagnostics that facilitate real-time measurement. These biosensors are typically fabricated by immobilizing a biological receptor molecule on the surface of a suitable transducer that converts the interaction between the receptor and target analyte into a quantifiable electronic signal [12]. The biosensing of cortisol includes either optical and electrochemical transduction. The techniques to measure through optical transducers include chemiluminescence [13], colorimetry [14], fluorescence [15], and SPR [18], while electrochemical measurement techniques include electrochemical reaction [16] and immunoassay [17]. Optical detection techniques mostly employ benchtop analysers or external analysis software for the measurement. Although portable analysers have been reported for a few optical-based techniques[15], the electrochemical detection has the advantage of utilizing simple and low-cost electronic instrumentation. Techniques employing electrochemical detection allow for label-free, rapid, and real-time measurement, making them suitable detection mechanisms for real time measurements.

The electrochemical cortisol sensors typically employ one of the three following biological receptors or probes in their structure: antibodies, molecularly imprinted polymers (MIPs), and aptamers. Antibodies-based sensors (also called immunosensors) are arguably the most widely researched type of cortisol sensors. They rely on the formation of antibody-antigen complexes (Figure 1a).

MIPs have been gaining popularity in cortisol sensor research. MIPs have shown remarkable recognition properties that have been utilized in a variety of applications, including template-assisted synthesis, catalysis, and drug separations and as biomimetic sensors, receptor mimics, and antibody mimics [47]. The selective recognition sites in MIPs are achieved through polymerization of monomers in the presence of a template molecule (target analyte) and its subsequent removal from the polymer matrix. Once the template is removed, cavities of the same size bearing structural similarity to the template are left behind in the polymer matrix as illustrated in Figure 1c.

Aptamers are single-stranded nucleic acid sequences that can be made to have a selective affinity towards a certain biomarker, here cortisol. They are generated via a process called SELEX (systematic evolution of ligands by exponential enrichment). The process involves screening large combinatorial libraries of oligonucleotides by an iterative process of in vitro selection and amplification [58]. Upon binding with the target analyte, aptamers undergo conformational changes as shown in Figure 1b, which results in changes in the electrochemical response.

This paper provides a systematic review of literature on electrochemical cortisol biosensors that utilise electrochemical transduction techniques. There are a few review articles published on related topics, for example Steckl and Ray [66] present a review on 12 primary stress biomarkers, including cortisol, and the optical and electrochemical detection methods used for their detection. Zainol Abidin et al [67] reviewed aptasensor -based cortisol sensors while Singh et al. [64] published a review in 2014 that is focused on the use of antibody-based electrochemical cortisol sensors (immunosensors). Sekar et al. [68] focuse on the developments made in cortisol sensing towards wearable applications. This paper instead provides an overview of the recent advances in developing electrochemical cortisol sensors that include all three categories of recognition elements (antibody, aptamer, MIP) used in the sensor structure.

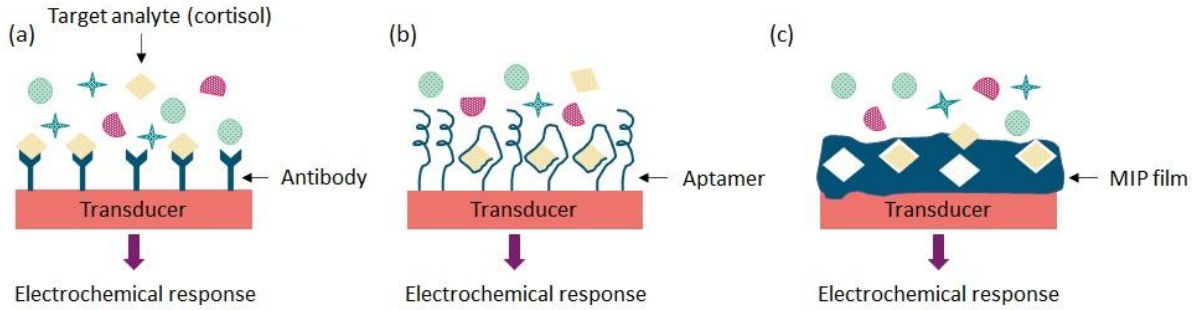

Figure 1: Schematic representation of the working mechanism of electrochemical sensors with (a) antibodies, (b) aptamers, and (c) MIPs as bioreceptors.

II. Methods

A systematic search of literature has been conducted to find articles that report an electrochemical cortisol sensor, published between 2011 and 2021. We used the following search terms in PubMed and the Web of Science: hydrocortisone or cortisol, sensor or measure or quantify or detect, and antibody or molecularly imprinted polymer or aptamer. The search yielded 52 and 140 results in PubMed and the Web of Science, respectively. Among these, the papers that meet the following criteria were selected for inclusion in this review.

(1) Cortisol quantification was aimed for biosensing platforms (thus, for example, immunoassays developed for ELISA were excluded)
(2) The work was aimed for the cortisol detection in human biofluids (thus animal studies were excluded). As cortisol can be found in detectable quantities in several biofluids, including blood, sweat, saliva, urine, and interstitial fluid (ISF), papers focusing on any of the listed bio fluids were considered for inclusion.
(3) Either antibodies, MIPs, or aptamers were used as the biorecognition element in the construction of the sensor
(4) An electrochemical transduction method was used (thus sensors that required optical transduction were excluded)
(5) The paper was a primary research paper (thus review papers were excluded)

After applying the above criteria, a total of 41 papers were selected for full-text review and inclusion in the review. The key information extracted from the papers are the following: the biological receptor or the probe used, the limit of detection (LoD), sensor dynamic range, electrochemical detection technique, redox probe type (if used), sensor sensitivity, sensor response time, selectivity towards cortisol, and the reusability of the sensor. The definition of each parameter is listed in Table 1.

Key word	Description
Biological receptor/probe	The biorecognition element for the specific recognition and capture of the target analyte
Limit of detection	The lowest concentration of the target analyte that gives rise to a signal that is significantly larger than that of a blank
Dynamic range	The concentration range over which the sensor can reliably (concentration perturbation results in a distinguishable sensor response above the noise level, which is the sensor response from blank sample) identify and report concentration perturbations
Detection method	The technique employed to readout the sensor (e.g. cyclic voltammetry, differential pulse voltammetry, electrochemical impedance spectroscopy)
Redox probe	A molecule that has been used in the sensing technique that possesses electroactivity and can undergo oxidation/reduction to facilitate the measurement
Sensitivity	The indication of how much the output of the sensor changes with changes in concentration of the target analyte

Response time	The time taken from the onset of change in the cortisol concentration to when the sensor output reaches 90% of the final value
Selectivity	The ability of the sensor to detect the target analyte with specificity in the presence of interferents and/or structural analogues
Reusability	The ability of the sensor to be regenerated for multiple uses

Table 1: Short descriptions for the key information extracted from the literature review

III. Results and discussion

The frequency of the biological receptors used in the included studies are as follows: (i) 27 papers used antibodies, (ii) 11 papers used aptamers, and (iii) 3 papers used molecularly imprinted polymers (MIPs). An overview of papers in each bioreceptor category is given in this section along with tabulated data summarizing the above key information. A narrative review of the sensors in each category is given focusing on the sensor structure (the various layers of the sensors, their purpose and composition), the usage of redox probe and the measurement techniques employed, LoD, dynamic range, and the sensitivity, selectivity, and reusability of the sensor. The milestones and key progress achieved in cortisol electrochemical sensing over the past ten years is given in each subsection, while the notable advancements and their impacts on ?? are summarized in subsection IIIb along with a discussion.

IIIa. Antibodies

Table 2 – Antibody Table – v2

III.a.1- Sensor structure

The most commonly used electrode base layer material in cortisol immunosensors is gold (Au). Alternative surface electrode materials included indium tin oxide (ITO), graphene, derivatives of graphene such as reduced graphene oxide (rGO), and derivatives of carbon, such as glassy carbon, carbon nanotubes (CNTs), and carbon yarns. While CNTs have been used due to their superior electrical properties and mass production, conductive carbon yarns (CCY) have shown potential for integration with fabrics to achieve a flexible wearable immunosensing platforms, in addition to their high electrical conductivity and low production cost [25, 36, 45].

While some of the electrode base layer materials, such as graphene, rGO, CNTs, and CCY are nanostructures themselves, most sensors in this category also incorporated nanostructures on top of the base layer. Nanostructures such as gold nanoparticles (AuNPs) [11,46] or zinc oxide nanoparticles (ZnO-NPs) [17,41] were used to enhance charge transfer by increasing the surface area of the sensor, resulting in improved sensor performance. Nanostructures also act as efficient immobilizing matrices. For example AuNPs facilitate the immobilization of antibodies via thiol bond formation with cross-linkers. ZnO-NPs on the other hand enable direct immobilization of antibodies by physical adsorption via electrostatic attraction. The electrostatic attraction occurs because of the difference in isoelectric points (IEPs) of ZnO (9.5) and antibodies (4.5), which provides ZnO with positively charged surfaces for the adsorption of negatively charged antibodies. Other metal oxide nanostructures used in cortisol immunosensors were Fe_2O_3 (IEP: 8.5) [25] and TiO_2 (IEP: ~6.5) [45]. Tin sulfide (SnS_2) [37] nanoflakes and molybdenum disulfide (MoS_2) [27] were additional semiconductive nanostructures used for their high carrier mobility, low cost, chemical stability, and electrical properties. Additionally, Khan et al. [5] developed a graphene nanoplatelet–polymer (GRP-(poly(styrene)-*block*-poly(acrylic acid)) (PS-*b*-PAA) composite to increase the conductivity and sensitivity of the cortisol sensor. Another polymer-based nanostructure was proposed by Kim et al. [34], Here, a conductive polymer, polypyrrole (PPy) was used to synthesize PPy nanotubes (PPy-NTs), owing to its mechanical and chemical properties, along with its biocompatibility.

In most sensor stacks listed in table I, a cross-linker has been used to immobilize anti-cortisol antibody (Anti-CAb) on either the base layer or the nanostructures layer. The dithiobis(succinimidyl propionate) (DTSP) has been the most commonly used cross-linker here. DTSP forms a self-assembled monolayer (SAM) on the Au electrode surface via thiol bonds. The DTSP-SAM then facilitates the immobilization of Anti-CAb by covalent bonding of the amine group of the antibody with

the succinimidyl group of DTSP. Alternative cross-linkers that were used include 1-pyrenebutyric acid N-hydroxysuccinimide ester (PBASE), 3-glycidoxypropyltrimethoxysilane (GOPTS), and poly(styrene-*co*-methacrylic acid) (PSMA).

Following the immobilisation of Anti-Cab, the non-specific binding sites to the ?? are normally blocked using either bovine serum albumin (BSA) or ethanolamine (EA).

III.a.2- Redox probe and measurement technique

The electrochemical sensing techniques such as cyclic voltammetry (CV), differential pulse voltammetry (DPV), and chronoamperometry (CA) typically rely on the change of the redox status of an electroactive species. Since cortisol lacks a redox centre, an auxiliary redox mediator is often used in such sensors. In fact, eight out of the 27 papers [11, 17, 36, 37 41, 42, 43 46] that employed antibodies as their recognition element employ potassium ferricyanide/ferrocyanide complex [$Fe(CN)_6^{3-/4-}$] as a redox mediator with voltametric techniques (CV and DPV)) for sensor readout. Vabbina et al. [4] use the same redox mediator but couple this with electrochemical impedance spectroscopy (EIS). The binding of cortisol molecules to the antibodies, hinders the mass and electron transfer between the redox probe and the electrode, reducing the redox current in voltammetry, as show in Figure 2. As such, when cortisol concentration increases, the redox current decreases as shown in [35] . On the other side, the binding of cortisol to the antibody increases the charge transfer resistance that can be detected using the EIS technique demonstrated in [35].

A redox probe is not normally required in a FET-based sensors as they use the current-voltage (I-V) characteristics of the FET to observe the binding status [24, 28, 32, 34, 38, 39, 44]. In these sensors, chemical reactions at the top of the gate dielectric induces a change in the gate dielectric characteristics (such as the FET's threshold voltage) which modulates the I-V characteristics. Another class of sensors that do not use redox probes are those that rely on measuring the capacitive modulation of the interfacial properties of the sensor measured through EIS [5, 26, 27, 29, 30, 31, 40, 62]. Such interfacial properties arise from the accumulation of the target analyte molecules at the electrical double layer (EDL) that modulates the dielectric constant. The capacitive modulation resulting from the binding between the analyte and receptor can be captured using non-faradaic EIS. Typically, in such non-faradaic systems, binding of target analyte to bioreceptor is characterized by an increase in charge transfer resistance owing to the combined effects of capacitive charge storage and solution phase resistance.

III.a.3- LoD, dynamic range, and sensitivity

The majority of the papers demonstrate a logarithmic dependency of the sensor signal to cortisol concentration with a maximum sensitivity achieved was 10.85 µA/log (g/mL) [11] using a sensor that was based on xyz. The lowest detection limits was achieved in [25], [36], and [45] to be 0.005, 0.098, and 0.3 fg/mL, respectively. All three sensors had a similar structure (BSA/Anti-CAb/semiconductive nanostructure/CCY). These were also above mentioned were the sensors that also achieved the widest sensor response ranges ranging from fg/mL to µg/mL. Vabbina and others in [17] and [41] reported two different sensitivities using different nanostructures, namely zinc nanorods (ZnNRs) and zinc nanoflakes (ZnNFs). ZnNRs demonstrated a higher sensitivity (12 kΩ/M and 3 kΩ/M) compared to ZnNFs (8 and 0.5 kΩ/M). This was attributed to ??.

The FET-based sensors achieved the narrowest dynamic ranges that did not cover the cortisol range of the sensor's target biofluid [24] [32] [38] However, they achieved acceptable had LoDs that covered the lowest normal cortisol levels in their target biofluid.

III.a.4- Selectivity

The three most commonly tested interferents for testing sensor selectivity in antibody-based sensors are progesterone [24, 25, 32,33, 36, 37, 45], cortisone [11, 24, 25, 32, 34, 36,45], and corticosterone [24, 36, 32, 34 37]. These hormones, (and the other less frequently tested hormones such as prednisolone [ref?], testosterone[ref?], and β-estradiol [ref?]) are steroid hormones that are structural analogues of cortisol. Othet interfering molecules such as glucose [11, 46] ascorbic acid [5,11, 46],

lactic acid [11], and urea [11] were also tested because they are electroactive species that are physiologically coexisting with cortisol in large quantities and may cause interference.

None of the antibody-based sensors reported in the included 27 studies here demonstrated reusability or potential of regenerating the sensor for multiple use.

IIIb. Molecularly Imprinted Polymers

Table 3 – MIP Table – v2

III.b.1- Sensor structure

The MIP films are typically fabricated via electropolymerization of monomers on a conductive surface. The pyrrole monomer has been used in two out of the three MIP-based cortisol sensors [49,50], making this the most popular choice. In these papers, pyrrole monomers were electropolymerized in the presence of the template molecule, cortisol on carbon electrode [49] and screen printed carbon electrodes (SPCE) [50]. The elution of cortisol from the matrix resulted in surface recognition cavities that were complementary to the shape and size of the cortisol molecule. The elution was achieved through overoxidation of PPy film by performing CV in PBS at the potential range from -0.2 to +0.8V for 20 to 25 cycles.

Additionally, the MIP-based sensor presented by Mugo et al. [48] was fabricated by first depositing a carbon nanotube/cellulose nanocrystal (CNC/CNT) nanoporous conductive film on a PDMS base. The MIP layer was then fabricated by depositing a prepolymer mixture (consisting of glycidylmethacrylate (GMA), ethylene glycol dimethacrylate (EGDMA), 4,4′- azobis(4-cyanovaleric acid) (ACVA), and cortisol as the template molecule) and allowing it to polymerize in the oven, as opposed to electropolymerization. Similar to the previous papers, cortisol template molecules were removed from the poly (GMA-co-EGDMA) film using an electrochemical cleaning method (CV in the potential range +0.9V to -0.9V at 0.1V/s for 15 cycles, in PBS).

III.b.2- Redox probe and measurement technique

The $[Fe(CN)_6]^{3-/4-}$ [50] and Prussian blue [49] have been used as redox probe coupled with the CV [50] and chronoamperometry [49] techniques, respectively. Similar to the antibody based sensors, the current decreases with increasing cortisol concentration, because the binding of cortisol to the cavities in the MIP film inhibits the electron transfer from the redox probe to the electrode surface.

Mugo et al. [48] did not use any redox probe and evaluated the electrochemical response of the sensor by performing CV and then determining the double layer capacitance change by taking the ratio of current to the scan rate. The capacitance of the sensor was shown to decrease as the cortisol concentration increases, since the charge transfer resistance increases with the binding of cortisol with the MIP cavities.

III.b.3- LoD, dynamic range, and sensitivity

All the papers presented in this section had low enough detection limits to match the lowest level of normal cortisol level in the biofluids, with the lowest being 1 pM [50]. While the dynamic ranges of the sensors reported in [49] (0.3 – 3625 ng/mL) and [50] (0.0004 – 3625 ng/mL) were wide, the dynamic range of the redox probe free MIP sensor presented in [48] (10 – 66 ng/mL) was not wide enough to cover the normal cortisol range in the target biofluid (sweat).

The highest sensitivity achieved was of 60.3 nA/ log nM in artificial sweat (AS) [49]. This was higher than the sensitivity of the same sensor in PBS (38 nA/log nM) [49]. The sensitivity of reox-free sensor reported by Mugo et al. [48] was the lowest among all 1.9/ (ng/mL).

III.b.4- Selectivity

Glucose [48,49] and lactate [49,50] were the most tested interferents in MIP-based sensors. Manickam et al. [50] demonstrated that lactate and progesterone had a cross-reactivity of 1.5% and 11.4% with cortisol detection respectively, while prednisolone had a cross-reactivity of 18.3%, which was a reduction from its 100% interference in ELISA.

III.b.5- Reusability

The MIP-based sensors developed by Mugo et al. [48] and Manickam et al. [50] demonstrated reusability. In [48], a blank and a 35 ng/mL cortisol standard were tested by the same sensor (3 sensors were tested this way) every 3 days for over 30 days – demonstrating 10 regeneration cycles. The sensors were stored at room temperature and electrochemically cleaned after every use by running CV in the range of 0.9 V to -0.9 V in PBS for 15 cycles (same as the initial elution of the template molecule from the MIP film).

In [48], the bound cortisol molecules were removed through the over-oxidation of the PPy matrix. This electrochemical cleaning was achieved by running CV in the potential range between -0.2 and 0.8 V for 25 cycles in PBS, a step that is similar to the initial elution of the template molecule. The sensitivity of the sensor remained over 90% after seven cycles of cleaning/rebinding (7 regenerations), after which, the adhesion of the polymer to the electrode weakened.

IIIc. Aptamers

There are x papers in this category for review.

Table 4 – Aptamers Table – v2

III.c.1- sensor structure

The base electrode materials used in this category of cortisol sensor include Au, carbon, glassy carbon, and graphene.

Various nanostructures were used to increase the surface area to volume ratio and hence enhance surface reactivity and the electrical conductivity of the sensor. Examples include nanoporous ZnO [6, 54], MWCNTs [53] , MWCNT with ordered mesoporous carbon (CMK-3) and silver nanoparticles (AgNPs) [52], silicon nanowires (SiNWs) [8] and gold nanowires (AuNWs) [4], Gold nanorods conjugated with aptamers [57] (before immobilization on the electrodes) and graphene quantum dots (GQDs) [16].

Aptamers are usually synthesized with modifications that facilitate their immobilization on the sensing platform. For example, in Fernandez et al. [53], a cortisol specific biotin modified-aptamer was conjugated with magnetic nanoparticles (MNP) (via biotin-streptavidin binding between streptavidin coated MNP and biotin modified aptamer). Another common modification of aptamers was the attachment of thiol groups to allow simple immobilization of aptamer on the surface via thiol bond formation. For instance, in [6, 54] the thiolated aptamers were immobilized on a ZnO coated nanoporous polyamide (PA) substrate, where they form a SAM due to the positive polar end group of ZnO. Thiolated aptamers were immobilized over a gold nanowire (AuNW) composite [4] and gold nanorods (AuNRs) [57] via thiol bond formation. Amino group has also been added to aptamers to enable the formation of amide bonds with carboxy groups activated by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS) on GCE [52] and with tetrakis(4-carboxyphenyl) porphyrin (TCPP) [51]. Amino-modified aptamers were also used to covalently immobilize via amide bond formation with ester group of 1-pyrenebutyric acid N-hydroxysuccinimide ester (PBSE) [16], and with silane triethoxysilylpropylsuccinic anhydride (TESPSA) on silica surface in [8].

While crosslinkers such as TCPP and PBSE were used in some papers, aptamers were mostly modified to attach to the nanostructure-modified electrode surfaces or silanized (via chemicals such TESPSA and APTES) electrode surface with the aid of EDC and NHS activation.

After aptamer immobilization, the non-specific binding sites in some papers were blocked with BSA, EA, or Mercaptoethanol (2ME).

III.c.2- Redox probe and measurement technique

The majority (8 out of 11) of the reported cortisol aptasensors do not use redox probes. Redox probes were not used in sensors that relied on EIS for the sensor readout [6, 54], where the solution

resistance decreases as the system became increasingly capacitive with increasing cortisol concentration. Similar to the FET-based immunosensors, the FET-based aptasensors do not utilize redox probe as they observe the I-V characteristics of the FT to achieve sensor readout [8, 51, 55, 56]. Additionally, Fernandez et al. [53] utilized a custom ink which was a conjugate of multi-walled carbon nanotubes and a metalloporphyrin-based ink (MWCNT-Cu-PP) to print the working electrode. Metalloporphyrin can act as enzyme mimics and catalyze the electrochemical reduction of cortisol, and hence it allowed a redox probe-free measurement.

Of the remaining three reported aptasensors, two employed $[Fe(CN)_6]^{3-/4-}$ as redox probe [16,52] along with voltametric techniques. Depending on the original orientation of the aptamers on the electrode surface and the 3D conformational change it undergoes, the way current responded to change in cortisol concentration differed. In [16], the binding of cortisol to the aptamer is shown to cause a structural change which involves the detachment of the aptamers (which were lying horizontally on the electrode surface) from the (GQDs modified) electrode surface. This exposed the electrode surface more and allowed the transfer of electrons from the redox probe to the electrode surface, leading to an increase in the voltammetry current. Unlike this, the current decreased with increasing cortisol concentrations in [52]. Here, the initial cortisol recognition and capture in [52] happens externally with antibody-AuNPs conjugates and when the cortisol/antibody-AuNPs were introduced to sensor, the immunocomplex was captured by the aptamers forming an insulating barrier for the electron transport, decreasing the current. Alternatively, in the work presented by Singh et al. [4], the aptamer was tagged with methylene blue (MB) as the redox probe. The binding of cortisol to the aptamers brings the MB closer to the electrode surface resulting in an increased current.

III.c.3- LoD, dynamic range, and sensitivity

The widest dynamic range and the smallest detection limit were reported to be 1 nM – 10 µM [55,56]. and 0.09 pg/mL [52] respectively. The sensor developed in the former paper exhibited a sensitivity of -75 mV/decade and the latter 7.7 µA/log(mg/mL). All the papers that reported LoD and dynamic range in this category matched the normal range of cortisol levels in the biofluid that the sensor was aimed to test, except for the sensor developed in Klinghammer et al. [8]. Here the the FET-based sensor reached saturation at around 0.3 µg/dL, requiring a 10x – 20x dilution of saliva samples to get measurements for concentrations above 0.3 µg/dL.

III.c.4- Selectivity

The interferents most tested in this category of sensors were cortisone [8, 16, 51, 53, 55, 56], progesterone [8,51, 53, 54, 57], and corticosterone [16, 51, 53]. Although no standard way or standard concentration level of these molecules were used in assessing the selectivity of the sensors, all papers that tested for selectivity compared the response of the sensor to a fixed concentration of interferants (ranging from X to Y nM) and compared this with the sensor response to a fixed cortisol level (ranging from x to y nM. This makes a fair comparison between the reported sensors difficult. In general the relative percentage variation in sensor response to interferants were reported to be between XX (X molecule compare to Z nM of cortisol) and YY% (y nM of y molecule compare to W nM of cortisol).

Sharma et al. [16] demonstrated that using a truncated aptamer (with 14 bases) resulted in a better selectivity for some interfenrants compared to when the parent aptamer (with 61 bases) is used. The percentage change (with respect to cortisol) in peak current was reported to be 32, 30, and 18% for triamcinolone, cortisone, and corticosterone, respectively for the parent aptamer, while the same was 2.6, 3.6, and 30% for the truncated aptamer.

III.c.5- Reusability

Three reported aptasensors [4,8, 52] demonstrating mechanisms for regeneration of the sensors to achieve sensor reusability. Singh et al. [4] regenerated their developed sensor by exposing the sensor to 1xPBS with 1M NaCl at pH 4.5 for 15 minutes. The sensor was regenerated three times, after which the aptamer regeneration efficiency decreased. This was attributed to the repetitive exposure of the aptamers to low pH solution of highly concentrated salt. The aptasensor developed by Klinghammer et al. [8] was regenerated by exposing the FET-based sensor to 2 M NaCl. However,

the possible number of regenerations was not reported. Finally, Huang et al. [52] showed that following a regeneration step (rinsing with a PBS solution of pH 7.4) after each measurement the sensor maintained 94% of its initial response after 8 days of testing (8 regenerations).

IIIb. Recent advances in cortisol sensing

IIIB.1 Comparison between three cortisol recognition elements

One of the most substantial recent advancements that has brought about multiple improvements in cortisol sensing is the use of aptamers and MIPs as bioreceptors. Although antibody based sensors are well-established, the antibody production requires animals and suffer from batch-to-batch variations [58]. MIPs on the other hand have advantages over antibodies, such as their inherent stability in extreme (temperature?, pH?, humidity?) conditions, long shelf-life, and low-cost [37]. In particular as antibodies are sensitive to temperature and prone to denaturation, the stability of MIPs, which allows them to be stored and transported at room temperature makes them an attractive option as antibody mimics. MIPs also allow a binding with the target analyte that is typically reversible (as described in Section III.b.5), which makes them a suitable sensing technology to achieve continuous measurement of biofluid.

Aptamers also have superior advantages over antibodies. That includes their negligible batch-to-batch variations because their production is based on in-vitro chemical synthesis that can be made extremely accurate and reproducible [59]. They are also thermally stable and more specific compared to antibodies [59]. Thermal denaturation of aptamers is reversible and the versatility of aptamers in labelling and modification with functional groups allow for simpler immobilization and signalling [60]. For instance, aptamers can be modified with thiol group at one end for simple immobilization on surfaces, foregoing the need for crosslinkers and they can also be modified with redox probes, obviating the need for an external redox mediator. Moreover, aptamers have been shown to have lower immunogenicity compared to antibodies, since oligonucleotides are less likely to cause immune reaction [61]. Owing to their chemical stability under a variety of buffer conditions and pH fluctuations, aptasensors provide another layer of stability for the detection of cortisol in sweat and saliva where temporal variation of pH may be substantial [39].

Additionally, aptasensor can be developed to have a large charge storage capacity [6], making them suitable for prolonged and continuous biosensing with higher sensitivity than that of immunosensors. Aptamers are also advantageous in reliably detecting small molecules such as cortisol, due to their ability to undergo significant conformational changes bringing the target bound aptamers closer to the transducing surface and increasing the biosensor signal [65]. Because of their folding ability and shorter length, they also make the better choice of bioreceptor for FET-based sensors. FET-based sensors present the challenge of Debye screening effect in ionic liquids, preventing its potential from extending further than the Debye length of that liquid. Hence, the use of aptamers brings the biorecognition event of the analyte within the Debye screening length, allowing for the sensitive quantification of cortisol.

IIIB-2 Cortisol sensing devices for on-body cortisol measurement

Real-time testing devices for cortisol sensing have been reported in the form of disposable tests with inexpensive materials and techniques such as inkjet printing electrodes on paper [53] or polyimide films [51] that utilize all three types of bioreceptors. Wearable form factors for on-body cortisol measurement were developed using MIP sensors [48, 49] and aptasensors [6, 54], where the sensors were fabricated on stretchable patches that were used on the skin for sweat cortisol sensing. The materials of these stretchable patches were PDMS and Ecoflex [48], Ecoflex and polyurethane [49], and polyamide [6,54]. The antibody-based immunosensors are reported as promising candidates for single-time real-time testing platforms such as POC devices. However, their susceptibility to denaturation upon exposure to unfavourable temperature makes them unsuitable for prolonged sensing and continuous monitoring [6].

In addition to disposable testing strips/patches, platforms with multiples sensing chips are becoming a research topic of interest. For example, Klinghammer et al. [8] presented a multiplexed platform consisting of an array of 16 single-use cortisol aptasensors fabricated on silicon nanowire-based FETs for the real-time measurement of salivary cortisol levels. The device has the potential for sample analysis from different patients or multiple samples from the patient in a time-resolved manner.

Pali et al. [54] and Ganguly et al. [6] present a continuous cortisol sensor based on aptasensors that monitor cortisol levels continuously, albeit in an accumulative way (i.e. increasing cortisol levels) over an 8h period, without sensor regeneration. It is not clear how decreasing levels of cortisol can be measured with this technique. Ganguly et al [6], present a cortisol aptasensor that is able to follow the changes in cortisol concentration (when dosed with a 128 ng/mL of cortisol standard at t= 1, 2, 4, 6, 8, and 9 hours). The sensor was also shown to be responsive to changes in concentration from both low to high (1 – 256 ng/mL) and high to low (256 – 1 ng/mL) trends without saturation. However, the response of the sensor has shown dependence on the direction of the changes and kept decreasing from 1 – 256 – 1 ng/mL. For instance, 16 ng/mL of cortisol in the upward trend had a different sensor output than that of the downward trend. This gives two possible sensor outputs per concentration for the calibration in the accumulative mode of operation of the sensor.

In the same work, surface charge variations of the aptasensor (aptamer-ZnO) were compared with that of an antibody-based sensor (antibody-crosslinker-ZnO) in the entire physiological range of the sweat pH (i.e., pH 2, 4, 6, and 8) showing that the aptasensor was stable between pH 4-6 (most commonly occurring pH of sweat) and not susceptible to agglomeration. The zeta potential variations of the immunosensor stack were also measured to be almost half of that of the aptasensor stack, proving that the aptasensor had a larger charge storage capacity making it suitable for prolonged and continuous biosensing with improved resolution compared to that of the immunosensor. Furthermore, comparing EIS measurements of both sensors indicated that the output signal of the cortisol aptasensor was almost 3 times larger than that of the immunosensor, which was attributed to the larger charge storing capacity and size-based matching for the aptamer. Aptamer being around 5 times smaller (27 kdA) than the cortisol antibody (150 kDa) reduces the steric hinderance effects in the aptasensor — antibodies being bulkier means that it can slow down or prevent cortisol from efficiently finding the desired bond site.

IIIB-3 Biofluid sampling

Sweat and saliva are both valid and accessible choices of biofluids for non-invasive, point of care cortisol measurement. Cortisol analysis in sweat has been more widely explored for such applications than due to its fewer matrix and biofouling effects compared to saliva. Majority of the papers that tested saliva required centrifugation of the samples to remove mucins, which is not and ideal for low-cost PoC applications. An alternative to centrifuging the saliva samples was proposed by Vanitha et al. [15] where saliva was collected from the mouth by suction into a pipette-like saliva sampling device. The device takes the sample through a filter membrane made of low-density polyurethane foam with a pore size of 0.3 µm to remove mucins, bubbles, and food particles before directing it onto the sensor.

One main limitation to measuring endogenous cortisol level in naturally secreted sweat is the low volume sweat collection. The collection of sweat for cortisol sensing may involve sweat stimulation (e.g. by exercise and heat) which can induce physiological and psychological stress, affecting the level of cortisol. To address these challenges, Tang et al. [49] developed a highly permeable sweat wicking porous PVA hydrogel that is capable of collecting natural perspiration secreted from the fingertip for the quantification of stress-free endogenous levels of cortisol levels. The electrodes for the sensor were fabricated by screen-printing, followed by the deposition of a PPy MIP layer. A stretchable version of the same sensor was also developed, ensuring the conformal contact of the sensor to curved body surfaces. A soft substrate with Ecoflex and polyurethane was fabricated, and electrodes were printed with stretchable inks. It was demonstrated that the epidermal patch displayed similar analytical performance as the fingertip-based cortisol sensor.

Another challenge of developing wearable sensors for sweat applications include the mixing of old and new biofluid and the lack of data on how the flow rate of biofluid would affect the sensor performance. Naik et al. [46] presented a low-cost microfluidic architecture with printed electrodes and an integrated absorbent pad for sweat evaporation. The microfluidic sensor was coupled with a developed synthetic skin, and tested at sweating flow rates between 1 and 5 µL/min. The results indicated that for flow rates equal to or greater than 2 µL/min, the sensor response is similar. Lower flow rates resulted in reduced current response likely due to poor contact between the sweat and the working electrode, given the limited volume of sweat within the sensor at any time. This led to the conclusion that the microfluidic device needs to be optimized to decrease the internal volume from the device inlet (e.g. by printing the electrodes closer to the inlet and reducing channel size) to the sensor electrodes to improve signal performance at low flow rates

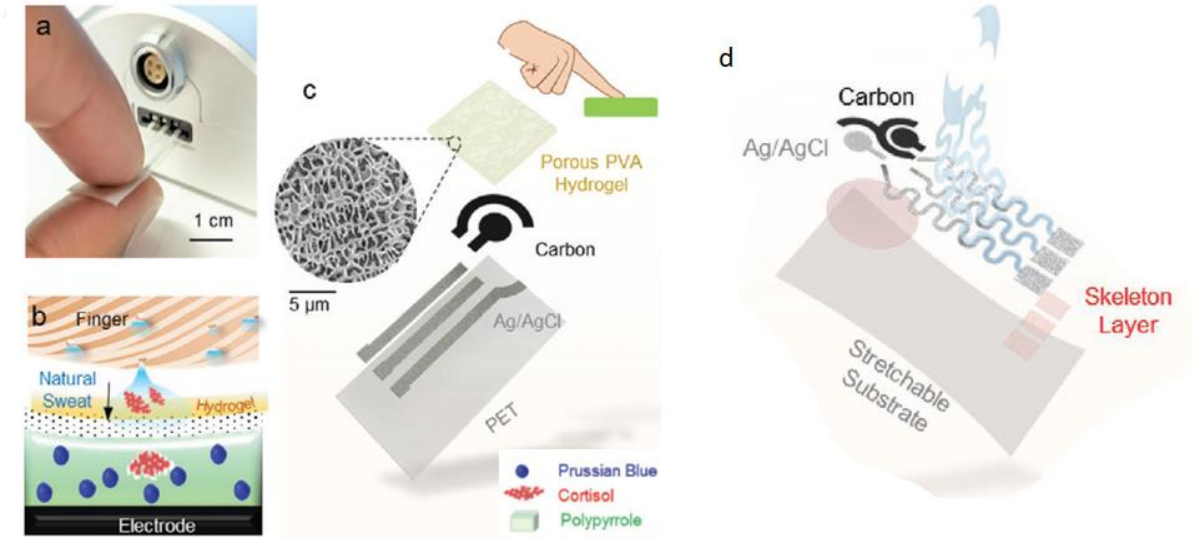

Figure 1: (a) illustration of the sensing mechanism, where the finger sweat diffuses through the hydrogel onto the MIP film on the electrode, and (c) illustration demonstrating the printed electrodes of the fingertip cortisol sensor, with the cryogenic scanning electron microscopy image of the porous PVA hydrogel (inset) (d) structure of the stretchable sensor with electrodes printed from the stretchable inks (Tang et al. [49])..

IV. Conclusion and future work

This paper presents a systematic review of literature on electrochemical sensors for cortisol detection in human biofluids. A total of 41 primary research papers are included in this review. Thesde have used either antibodies, aptamers, or MIPs as the cortisol recognition element. The reported sensors were categorised based on the type of bioreceptor used, and key information were extracted into tables focusing on the sensor structure (the layers of the sensors and its composition), the usage of redox probe and the measurement techniques employed, LoD, dynamic range, and sensitivity, selectivity, and reusability of the sensors.

Sensor reusability is an extremely attractive property in the road for personal health monitoring devices and sustainability. The possibility to reuse the sensors via regeneration was shown in two MIP-based sensors and three aptasensors. However, since additional reagents (PBS or NaCl) are required for sensor regenerations, further studies are required on methods to incorporate the reagents into the sensing platforms, especially when the device is in constant contact with the body or biofluid (e.g. in wearable devices), to allow for a truly continuous monitoring.

Further research may also be required to study methods for sensor calibration, especially in the case of reusable sensors where the sensitivity of the sensors may change over time. It is also imperative to study the biofouling effects from the sample, which may affect how frequently a calibration step would

be needed and how calibrations would be carried out conveniently, and if there are alternatives to the user performing calibrations. For instance, Ghoreizhizadeh et al. [65] demonstrated that there is a correlation between the sensitivity of glucose sensors with their double layer capacitance. Such a correlation introduces the possibility for a potential self-calibration algorithm where sensor sensitivity may be predicted in-situ through easily measureable sensor characteristics such as its impedance.

While electrochemical sensors offer the advantage of being suitable for miniaturizing and integration with microelectronics, most cortisol electrochemical sensors also utilize an external potentiostat to apply the necessary voltages and to measure the resulting currents for sensor readout. Even though portable potentiostats have been reported, a fully integrated sensor platform that consists of all the necessary electrodes and functionalization along with embedded readout electronics would pave the way for on-body operations, in addition to opening extra possibilities for PoC sensing platforms. Finally, to ensure reproducibility and scalability for commercialization, automatization of sensor fabrication processes would be ideal.

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Appendix

Abbreviation	Definition	Abbreviation	Definition
Sensor Stack			
Ab	Antibody	MCH	Mercaptohexanol
Ag, AgNP	Silver, silver nanoparticle	MNP	Magnetic nanoparticle
Anti-CAb	Anti-cortisol antibody	MWCNT	Multi-walled carbon nanotubes
Apt	Aptamer	2ME	Mercaptoethanol
APTES	3-(aminopropyl)triethoxysilane	NHS	N-Hydroxysuccinimide
Au, AuNP, AuNW, AuNR	Gold, gold nanoparticle, gold nanowire, gold nanorod	PA	Polyamide
BSA	Bovine serum albumin	PB	Prussian blue
CCY	Conductive carbon yarn	PBASE, PBSE	1-pyrenebutyric acid N-hydroxysuccinimide ester
CMK-3	Ordered mesoporous carbon	PDMS	Polydimethylsiloxane
CNC	Cellulose nanocrystal	PET	Poly(ethylene terephthalate)
CNT	Carbon nanotube	PI	Polyimide
Cu	Copper	PMA	1-Pyrenemethylamine hydrochloride
d-BSA	Denatured bovine serum albumin	Poly(GMA-co-EGDMA)	Poly(glycidylmethacrylate-co-ethylene glycol dimethacrylate)
DSP/DTSP	Dithiobis(succinimidyl propionate)	PP	Porphyrin
EA	Ethanolamine	PSMA	Poly(styrene-*co*-methacrylic acid)
EGFET	Extended gate field effect transistor	PPy	Polypyrrole
Fc	Ferrocene	Pt	Platinum
FET	Field effect transistor	PVA	Polyvinyl alcohol
G	Graphene	rGO	Reduced graphene oxide
GA	Glutaraldehyde	SiNW	Silicon nanowire
GCE	Glassy carbon electrode	SnS_2	Tin sulphide
GOPTS	3-glycidoxypropyltrimethoxysilane	SPE, SPCE	Screen-printed electrode, screen-printed carbon electrode

GQD	Graphene quantum dots	SWNT	Single-walled carbon nanotubes
GRP	Graphene nanoplatelets	TCPP	Tetrakis(4-carboxyphenyl) porphyrin
IDE	Interdigitated electrode	TESPSA	Triethoxysilylpropylsuccinic anhydride
ITO	Indium tin oxide	TiO_2	Titanium dioxide
Lg-FET	Liquid gate field effect transistor	ZnO, ZnONPs,ZnONSs, ZnONRs	Zinx oxide, zinc oxide nanoparticles, zinc oxide nanostructures, zinc oxide nanorods
Detection Method			
CV	Cyclic voltammetry	CA	Chronoamperometry
DPV	Differential pulse voltammetry	EIS	Electrochemical impedance spectroscopy
SWV	Square wave voltammetry	I-V	Current – voltage characteristics
Selectivity			
AA	Ascorbic acid	LA	Lactic acid
ALDO	Aldosterone	LAC	Lactate
APAP	Acetaminophen	MP	Methoxyprogesterone
CA	Cholic acid	Na	Sodium
CORT	Corticosterone	NE	Norepinephrine
DHEA	Dehydroepiandrosterone	NPY	Neuropeptide Y
DOC	21-hydroprogeterone	NSE	Neuron specific enolase
E	Cortisone	P	Progesterone
EGFR	Epidermal growth factor receptor	PRDL	Prednisolone
EPI	Epinephrine	PRED	Prednisone
EtG	Ethyl Glucuronide	PSA	Prostate specific antigen
E2	B-Estradiol	T	Testosterone
GLU	Glucose	TAC	Triamcinolone
IL-1β	Interleukin 1 beta	TYR	Tyrosine
IL-6	Interleukin 6	UA	Uric acid
K	Potassium	17α-OHP	17-α-hyrdroxyprogesterone
Cort	Cortisol		

Table 5: Abbreviations used in the description of the sensor stack, detection method, and selectivity of the sensors and their definitions

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