# Electrochemical Sensors for Cortisol: A 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 several processes that cortisol is involved in in the body.**

**This review presents a systematic search of the literature on electrochemical cortisol sensing techniques that allow real-time measurement of cortisol in human biofluids. Several structural and performance-related parameters of sensors are being discussed, including the sensor stack layers, limit of detection (LoD), dynamic range, sensitivity, selectivity, reusability, redox probe usage, and the electrochemical detection technique used. The sensors here are primarily categorized based on the type of bioreceptors used: antibodies, molecularly imprinted polymers (MIPs), and aptamers. According to this review, cortisol aptasensors and the MIP-based sensors present, in general, superior stability and sensitivity over immunosensors. They also promise reversible binding, albeit limited research exists on sensors deploying such bioreceptors. Additionally, notable advancements in the field and their impact on the development of point-of-care (PoC) and wearable devices are discussed.**
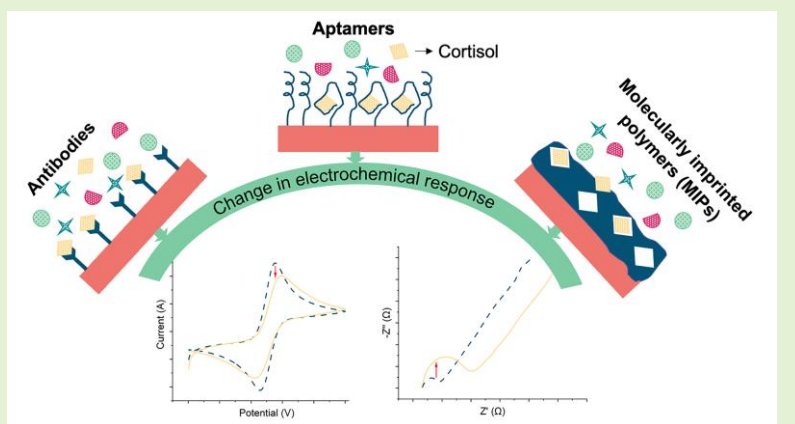
*Index Terms—* **cortisol, electrochemical sensing, antibodies, molecularly imprinted polymers, aptamers**

## I. INTRODUCTION

Cortisol, also known as the "stress hormone" plays a vital role in the body's stress response. Cortisol is involved in the homeostasis of the cardiovascular, immune, renal, skeletal, and endocrine systems [1] as well as the regulation of glucose levels, blood pressure, and carbohydrate cycles [2]. Cortisol secretion is controlled by the hypothalamic-pituitary-adrenocortical (HPA) axis [3], and it can be found in various body fluids (in the physiologically normal range of 20-250 ng/mL in blood and serum [4], 1-8 ng/mL in saliva [5], and 8-141 ng/mL in sweat [6]).

Sustained abnormal levels of cortisol have detrimental effects on various physiological processes. 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]. Cortisol levels in the body follow a circadian rhythm with the highest levels in the morning and significantly lower levels at night [7]. Studies suggest that not only genes and environmental factors (e.g. socioeconomic status [9], intake of caffeine [10], smoking [11], and exercise [12]) affect cortisol levels in individuals at different times of the day [13], but also daily social and emotional experiences can lead to systematic day-to-day changes in cortisol levels in

the body [14]. These highlight the importance of acquiring longitudinal data across different time points to better understand the causal relationship between experience, cortisol, and well-being [14]. Monitoring the diurnal rhythm of cortisol can help understand the influence of social, emotional, and environmental factors on the HPA axis functioning and disease processes [15]. Such a "continuous" monitoring paradigm requires a cortisol sensing technology that allows frequent measurements ideally in real-time and within a low-cost setting.

However, current commercially available cortisol measurement techniques (e.g. high-performance liquid chromatography (HPLC), enzyme-linked immunosorbent assay (ELISA), chemiluminescence immunoassay (CLIA), and surface plasmon resonance (SPR) [16]) require bulky laboratory equipment, dedicated space, and expertise as they require sample preparation and/or labeling steps, making them unsuitable for real-time or point-of-care (PoC) applications.

Electrochemical sensing is an alternative technique for cortisol measurement that is typically low-cost and rapid, with the potential to detect in a label-free setting. These sensors 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 [17]. The signal can be measured and processed by simple electronic instrumentation further reducing the cost of the entire analysis system.

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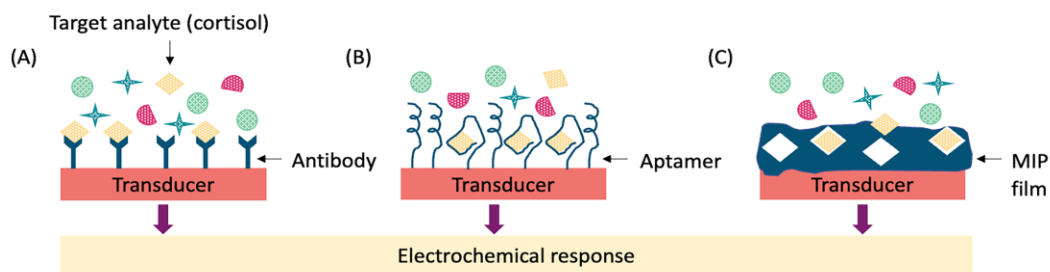
Fig. 1. Schematic representation of the operation of electrochemical sensors with (A) antibodies, (B) aptamers, and (C) MIPs as bioreceptors.

The electrochemical cortisol sensors typically employ one of the three 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 (as shown in Fig. 1A).

MIPs have been gaining popularity in cortisol sensor research partly due to their potential for regeneration. The selective recognition sites in MIPs are achieved through the polymerization of monomers in the presence of a template molecule (target analyte). This could be either a bulk polymerization of all agents in a few distinct steps involving a cross-linker as well [18], [19] or a one-step electro-polymerization [20], [21], [23], [24], [25], [26]. The template is subsequently removed 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 Fig. 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 [27]. Upon binding with the target analyte, aptamers undergo conformational changes (as shown in Fig. 1B), which results in changes in the electrochemical response of the electrode.

A number of review articles have already been published on cortisol biosensors over the past 10 years, further highlighting the increasing importance and the vast research and development effort dedicated to the topic by the community. Steckl and Ray (2018) [28] presented a review of 12 primary stress biomarkers, including cortisol, as well as the optical and electrochemical methods used for their detection. Zainol Abidin et al (2017) [29] reviewed aptamer-based cortisol sensors (aptasensors) while Singh et al (2014) [30] reviewed antibody-based electrochemical cortisol sensors (immunosensors). Additionally, Sekar et al (2020) [31] focused on the developments made in cortisol sensing towards wearable applications with an emphasis on sensors developed on fabrics and flexible substrates, while Khumngern et al (2023) [32] highlighted the progress made on wearable electrochemical immunosensors.

A majority of previously published reviews only cover cortisol sensors that employ a single type of bioreceptor. Yulianti et al (2022) [33] mainly focused on the advances in MIP-based cortisol sensors but also gave a brief summary of aptasensors and immunosensors. Karuppaih et al (2023) [34]

discuss the progress made in the electrochemical sensing of cortisol with all three types of sensors.

This work, for the first time, provides a systematic review that is carried out according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. It extensively reviews all three types of electrochemical cortisol sensors published over the past 10 years, with a specific focus on the reusability aspect of the sensors for real-time on-body and continuous cortisol monitoring applications.

## II. METHODS

A systematic search of the literature has been conducted to find articles that report an electrochemical cortisol sensor, published between 2011 and 2022. The following search terms were used 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 64 and 167 results in PubMed and the Web of Science, respectively. From these, only papers that met the following criteria were selected for inclusion in this review.

(1) Cortisol quantification was aimed for real-time measurement in PoC or wearable devices (thus, for example, immunoassays developed for ELISA were excluded).

(2) The work was aimed for cortisol detection in human biofluids. 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 biofluids were considered for inclusion.

(3) Only sensors with electrochemical transduction were considered (thus sensors that required optical transduction were excluded).

(4) The paper was a primary research paper, thus any abstract-only or review papers were excluded. All review papers that were found in the initial search are included in the introduction section (Section I) where differences with this work are highlighted.

After applying the above criteria, a total of 61 papers were shortlisted for full-text review and inclusion in this 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 specific definition of each parameter mentioned here is listed in Table S1 in the supplementary material for the sake of clarity, and to ensure fair comparison among included studies.

## III. RESULTS

The frequency of the biological receptors used in the included studies is as follows: (i) 34 papers used antibodies, (ii) 9 papers used molecularly imprinted polymers (MIPs), and (iii) 18 papers used aptamers. A narrative review of the sensors in each category is provided 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 key information extracted from the papers in each category for review is included in Table S3 (immunosensors), Table S4 (MIP-based sensors), and Table S5 (aptasensors) in the supplementary material.

The milestones and key progress achieved in cortisol electrochemical sensing over the past ten years are given in each subsection, while the notable advancements towards real-time quantification PoC and wearable devices are summarized in Section IV along with a discussion.

### A. Antibodies

#### A.1 Sensor structure

The most commonly used electrode base layer material in immunosensors is gold (Au). Alternative surface electrode materials include indium tin oxide (ITO), graphene, derivatives of graphene such as graphene oxide (GO), reduced graphene oxide (rGO), 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 flexible wearable immunosensing platforms, in addition to high electrical conductivity and low production cost [35], [36], [37], [38]. Conductive thread textiles have also been utilized for their flexibility, lightweight, and size [39].

While some of the electrode base layer materials, such as graphene, GO, rGO, CNTs, and CCY are nanostructures themselves, most sensors in this category also incorporated nanostructures on top of the base layer as depicted in Fig. 2. Nanostructures such as gold nanoparticles (AuNPs) [16], [39], [40] or zinc oxide nanoparticles (ZnO-NPs) [41], [42] 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 iron (III) oxide ($Fe_2O_3$) (IEP: 8.5) [35] and titanium dioxide ($TiO_2$) (IEP: ~6.5) [37]. Tin sulfide ($SnS_2$) [43] nanoflakes and molybdenum disulfide ($MoS_2$) [44] were additional semiconductor nanostructures used for their high carrier mobility, low cost, and good chemical stability.

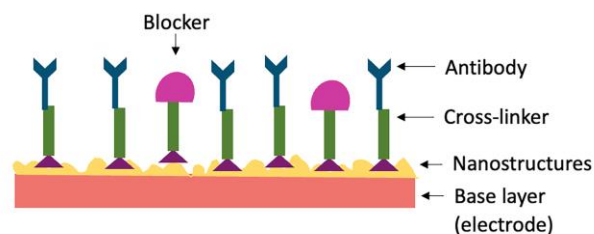Tin oxide ($SnO_2$) nanoflakes were also employed, where



Fig. 2. Structure of a typical immunosensor: the base electrode, nanostructures to improve sensitivity, cross-linkers, antibodies, and a blocker to prevent non-specific binding of the sensor.

hydrogen bonding between their surface hydroxyl groups and the carboxyl group of antibodies reportedly allows the adsorption of antibodies via non-covalent interactions [38]. Additionally, a graphene nanoplatelet–polymer (GRP-(poly(styrene)-block-poly(acrylic acid)) (PS-b-PAA) composite was developed to increase the conductivity and sensitivity of the sensor in [5]. Another polymer-based nanostructure was proposed in [45], where a conductive polymer, polypyrrole (PPy), was used to synthesize PPy nanotubes (PPy-NTs), owing to their high electric conductivity and biocompatibility. Further, nanostructures of functionalized poly(3,4-ethylene dioxythiophene) (PEDOT) derivatives, poly(EDOT-COOH-co-EDOT-EG3) nanotubes, were engineered in [46] to decorate the active channel areas of organic electrochemical transistor (OECT)-based sensors. The EDOT-COOH was used to immobilize anti-cortisol antibodies (Anti-CAb) and EDOT-EG3 to minimize non-specific binding on the sensor platform.

In most sensor stacks listed in Table S3, a cross-linker has been used to immobilize Anti-Cab (as shown in Fig. 2) on either the base layer or the nanostructures layer. The dithiobis(succinimidyl propionate) (DTSP) is 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 the DTSP. Alternative cross-linkers that were used include 1-pyrenebutyric acid N-hydroxysuccinimide ester (PBASE), 3-glycidoxypropyltrimethoxysilane (GOPTS), (3-Aminopropyl) triethoxysilane (APTES), poly(styrene-co-methacrylic acid) (PSMA), L-cysteine (L-cys), and 3- mercaptopropionic acid (3-MPA). In contrast, another antibody, protein A, was used to immobilize the Anti-Cab in [47], because of its affinity to the constant Fc part of a range of immunoglobin macromolecules, including Anti-Cab.

Following the immobilization of Anti-Cab, non-binding ligands are introduced to block the non-specific binding sites of the sensor. The role of such non-binding ligands in decreasing the propensity of biofouling caused by sample matrices [48] and in reducing steric hindrance effects [49] has been established. As such, the non-specific binding in cortisol immunosensors is reduced by using either bovine serum albumin (BSA) or ethanolamine (EA).

#### A.2 Electrochemical modality and label-free detection

Electrochemical sensing techniques such as cyclic voltammetry (CV), differential pulse voltammetry (DPV), and
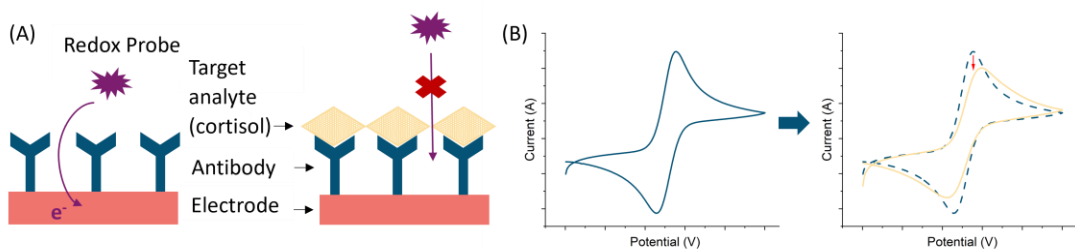
Fig. 3. Operation of immunosensors with external redox probes: (A) When cortisol binds to the antibody, mass tunneling of the redox probes to the electrode surface is reduced, decreasing the electron transfer between the redox probes and electrode surface. (B) This is depicted by the reduced current peak in the cyclic voltammogram.

chronoamperometry (CA) typically rely on the change of the redox status of an electroactive species. Since cortisol lacks a redox center, an auxiliary redox mediator/probe is often used in such sensors. As such, the potassium ferricyanide/ferrocyanide complex, $[Fe(CN)_6]^{3-/4-}$, was used as a redox probe with amperometric (CA) or voltammetric (CV and DPV) techniques for sensor readout [3], [16], [36], [38], [39], [40], [41], [43], [47], [50].

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 shown in Fig. 3. Hence, when cortisol concentration increases, the redox current decreases. On the other side, the binding of cortisol to the antibody increases the charge transfer resistance that can be detected using the EIS technique. Alternatively, an antibody tagged with ferrocene as a redox probe was demonstrated in [51]. A redox probe is not normally required in field-effect transistor (FET)-based sensors as they use current-voltage (I-V) characteristics of the FET to observe the binding status, facilitating label-free detection [45], [46], [52], [53], [54], [55], [56], [57], [58], [59]. In these sensors, chemical reactions at the top of the gate dielectric induce 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 allow label-free detection is those that rely on measuring the capacitive modulation of the interfacial properties of the sensor through EIS [5], [44], [60], [61], [62], [63], [64], [65]. 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 the target analyte to the bioreceptor is characterized by an increase in charge transfer resistance owing to the combined effects of capacitive charge storage and solution phase resistance.

### A.3 LoD, dynamic range, and sensitivity

The majority of the papers demonstrate a logarithmic dependency of the sensor signal on cortisol concentration except for [19], [39], [61], [63], [66], and [67], which demonstrated a linear response. Among the 34 papers presenting cortisol immunosensors, the lowest detection limits achieved were reported to be 0.005 fg/mL [35], 0.0088 fg/mL [46], 0.098 fg/mL [36], 0.3 fg/mL [16], 1.6 fg/mL [38], and 6 fg/mL [37]. The sensors in [35], [36], [37], and [38] had similar sensor structures of BSA/Anti-CAb/semiconductive

nanostructure/CCY. The above-mentioned sensors in [16], [35], [36], [37], [38], and [46] also achieved the widest sensor response ranges ranging from fg/mL to µg/mL, in addition to [60], which reported a response range from pg/mL – µg/mL. The maximum sensitivity achieved in [16], [35], [36], [37], [38], [46], and [61] is 10.85 µA/log (g/mL) [16]. Two different sensitivities were reported in [41] and [42] using different nanostructures namely zinc nanorods (ZnNRs) and zinc nanoflakes (ZnNFs). ZnNRs demonstrated a higher sensitivity (11.86 µA/log(M) and 3.078 kΩ/log(M)) compared to ZnNFs (7.74 µA /log(M) and 0.540 kΩ/log(M)). This was attributed to the high surface area-to-volume ratio of the NRs compared to the NFs. Furthermore, in [46], using polymeric nanotubes in an OECT device was proven to lead to signal amplification, whereby it was observed that the OECT device engineered with the nanotubes had a higher transconductance than the device without nanotubes. Conversely, attaching antibodies directly to single-walled carbon nanotubes (SWCNTs) was shown to result in sensors being five times more sensitive than having an extra layer of AuNPs in between [59]. This was attributed to the shorter distance between the antibodies and the transducing layer in the absence of the additional AuNPs layer.

The FET-based sensors in [52], [54], and [55] achieved the narrowest dynamic ranges that did not cover the cortisol range of the sensor's target biofluid. However, they achieved LoDs that covered the lowest normal cortisol levels in the biofluid.

### A.4 Selectivity

The three most commonly tested interferents for testing sensor selectivity in immunosensors are progesterone [35], [36], [37], [38], [43], [52], [54], [68], cortisone [16], [35]. [36], [37], [38], [39], [45], [46], [52], [54], and corticosterone [36], [38], [39], [43], [45], [46], [52], [54]. These hormones (and the other less frequently tested hormones such as prednisolone [45], [46], testosterone [37], [38], [43], [63], and β-estradiol [43]) are steroid hormones and are structural analogs of cortisol. Other interfering molecules such as glucose [16], [38], [40], ascorbic acid [5], [16], [39], [40], lactic acid [16], and urea [36], [38] were also tested because they are electroactive species that are physiologically coexisting with cortisol in large quantities and may cause interference [16]. The reported percentage changes in the electrochemical response of interferents ranged between < 0.5% (for 2 µM cortisone, corticosterone, and prednisolone) compared to 3% by 270 pM cortisol [45] and < 20% (which was reported to be below the limit of blank for IL-6 [65] and EtG [44]) compared to 20-80% and 25-45% change by

cortisol, respectively.

None of the immunosensors reported in the included 34 studies here demonstrated reusability or potential of regenerating the sensor for multiple uses.

### B. Molecularly imprinted polymers

#### B.1 Sensor structure

The MIP films are typically fabricated via electropolymerization of monomers on a conductive surface. The pyrrole monomer is the most popular choice [20], [21], [23], [24], [26]. Pyrrole monomers were electropolymerized in the presence of the template molecule, cortisol, on inkjet-printed carbon electrodes in [20] and on screen-printed carbon electrodes in [21], [23], and [24]. 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 over-oxidation of the PPy film by performing CV in PBS at the potential range from -0.2 to +0.8V for 20 to 25 cycles [20], [21], [23], [24], [26]. PPy was also chosen as the polymer scaffold of the MIP film in [26], which had an rGO layer functionalized with β-cyclodextrin (β-CD) on a GCE. The β-CD was employed to enhance the sensor performance by providing complimentary recognition sites in conjunction with the MIP.

The electropolymerization of poly(o-phenylenediamine) (poly(o-PD)) was also used to form MIP layers on GCE [22], [25]. In [22], GCEs were coated with nickel nanoclusters (NiNCs) loaded onto nitrogen-doped CNTs prior to the deposition of the MIP layer, while MIP films were doped with AuNPs to increase the sensitivity of the sensor in [25].

Additionally, the MIP-based sensor in [18] was fabricated by first depositing a carbon nanotube/cellulose nanocrystal (CNC/CNT) nanoporous conductive film on a PDMS base. A prepolymer mixture (consisting of glycidyl methacrylate (GMA), ethylene glycol dimethacrylate (EGDMA), 4,4′-azobis(4-cyano valeric acid) (ACVA), and cortisol as the template molecule) was then deposited and allowed 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). The same layer-by-layer assembly was replicated on cotton textiles instead of PDMS in [19]. In this work, a conductive polyaniline (PANI) film was deposited additionally on top of CNC/CNT film and the poly(GMA-co-EGDMA) MIP film was decorated with AuNPs.

#### B.2 Electrochemical modality and label-free detection

All except two papers report the use of redox probes. The most popular redox probe has been $[Fe(CN)_6]^{3-/4-}$ [21], [22], [24], [25], followed by Prussian blue (PB) [20], [23], and hexacyanoferrate (HCF) [26]. All were coupled with voltammetric (CV, DPV), and amperometric (CA) techniques. The only label-free MIP sensors are reported in [18] and [19] where changes in double layer capacitance of the sensor (measured through CV) were determined instead of the faradic current. The double-layer capacitance was shown to decrease as the cortisol concentration increased.
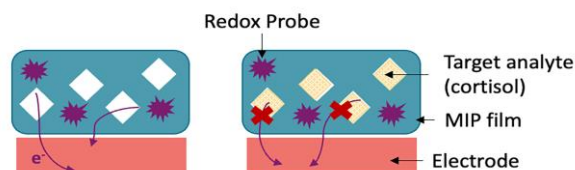


Fig. 4. Operation of MIP-based sensors with embedded redox probes: when cortisol binds to the cavities in the MIP film, the electron transfer between redox probes and the electrode is hindered.

#### B.3 LoD, dynamic range, and sensitivity

All papers in this section demonstrated a logarithmic dependence of sensor output on the cortisol concentration, with the exception of [18] and [19] which showed a linear dependence.

All the papers showed low enough detection limits that matched the lowest level of normal cortisol level in the biofluids, with the lowest being 0.86 fg/mL [22].

While most of the papers reported wide dynamic ranges with the widest being 0.1 ng/mL – 10 µg/mL [23], the dynamic ranges of 3.63 fg/mL – 362.5 pg/mL [22], 10 – 66 ng/mL [18], and 9.8 – 49.5 ng/mL [19] were not wide enough to cover the normal cortisol range in the target biofluids: saliva, sweat, and sweat, respectively.

From the reported sensitivities in this section, a relatively high sensitivity of 9.47 µA/ log (nM) was achieved by optimizing synthesis parameters via computer modeling, resulting in a 1.5-fold increase in their original sensitivity [24].

#### B.4 Selectivity

Glucose [18], [20], [22], [23], [24], [25], [26] and lactate [19], [20], [21], [24], [26] were the most tested interferents in MIP-based sensors, owing to their abundance in biofluids. The selectivity assay performed in [21] calculated the change in the current peak in response to each interferent at 100 nM with respect to the change in the current peak elicited by 100 nM cortisol. It was shown that lactate and progesterone had a cross-reactivity of 1.5% and 11.4%, respectively, while prednisolone had a cross-reactivity of 18.3%, which was a reduction from its 100% interference in ELISA.

#### B.5 Reusability

Four of the papers that reported MIP-based sensors demonstrated reusability [18], [19], [21], [25]. The sensors developed in [18] and [19] were regenerated via electrochemical cleaning by running CV in the range of 0.9 V to -0.9 V in PBS for 15 cycles. It was shown that the sensors could be regenerated 10 and 15 times, respectively.

In [21], the bound cortisol molecules in the MIP-based sensor 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. 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.

Lastly, the sensor developed in [25] was regenerated three times by rinsing it with ethanol. The relative standard deviation (RSD) value for the fourth successive regeneration increased here, and this was attributed to the possible degradation of the imprinted cavities.

## C. Aptamers

### C.1 Sensor structure

The base electrode materials used in aptasensors include Au, carbon, glassy carbon, and graphene.

Various nanostructures were used to accelerate electron transmission rates and increase the surface area to volume ratio and hence enhance surface reactivity and the electrical conductivity of the sensor. Examples include ZnO [4], [69], multi-walled carbon nanotubes (MWCNTs) [70], MWCNTs with ordered mesoporous carbon (CMK-3) and silver nanoparticles (AgNPs) [71], silicon nanowires (SiNWs) [1], gold nanowires (AuNWs) [72], gold nanorods (AuNRs) conjugated with aptamers [73] (before immobilization on electrodes), graphene quantum dots (GQDs) [74], and samarium molybdate flower-like nanoparticles (SmM-NPs) [75]. A combination of two or more nanostructures was used in some works, for example, a combination of nitrogen-doped carbon quantum dots (N-CQDs) and functionalized graphene (FG) was utilized to form 3D electron transmission channels on the electrode surface in [76]. Further, polyacrylonitrile (PAN) nanofibers and carboxylated poly(3,4-ethylene dioxythiophene) (PEDOT) were used on the sensing channel of the FET-based sensor in [77].

Aptamers are usually synthesized with modifications that facilitate their immobilization on the sensing platform. The most 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, 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 [4], [69]. Another route for the immobilization of thiolated aptamers was reported on nanometre-thin indium (III) oxide ($In_2O_3$) channels of a FET, which were modified with a crosslinker, 3-maleimidobenzoic acid N-hydroxysuccinimide ester (MBS) [78]. Here, MBS cross-linked amine-terminated silanes (APTES and trimethoxy(propyl)silane (PTMS)) on the $In_2O_3$ surface with the thiolated aptamers. Thiolated aptamers were also immobilized over a gold nanowire (AuNW) composite [72], gold nanorods (AuNRs) [73], and flat Au electrodes [67], [79] via thiol bond formation.

The second most common modification was the addition of amino groups to the aptamers to enable the formation of amide

bonds with carboxy groups activated by 1-ethyl-3-(3-dimethylamino propyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS) on GCE [71], [75] and with tetrakis(4-carboxyphenyl) porphyrin (TCPP) [80]. Amino-modified aptamers were further utilized to covalently immobilize via amide bond formation with an ester group of 1-pyrene butyric acid N-hydroxysuccinimide ester (PBSE) [74] and with silane triethoxysilylpropylsuccinic anhydride (TESPSA) on a silica surface [1]. Additionally, amino groups attached to the aptamers were exploited for their conjugation with PEDOT-PAN-NFs via the formation of an amide bond between the amino group and the carboxylic acid group of the PEDOT [77].

Another notable modification was reported in [70], where a cortisol-specific biotin-modified-aptamer was conjugated with magnetic nanoparticles (MNP) (via biotin-streptavidin binding between streptavidin-coated MNP and biotin-modified aptamer).

In summary, while crosslinkers such as MBS, TCPP, and PBSE were used in some papers (as shown in Fig. 5A), aptamers were mostly attached to the electrode surface via either aptamer modifications (e.g. attachment of thiol or amino groups as illustrated in Fig. 5B) or silanization (via chemicals such TESPSA and APTES) of the electrode surface followed by EDC and NHS activation.

After aptamer immobilization, the non-specific binding sites in some papers were blocked with BSA, EA, or mercaptoethanol (2ME). Other papers did not report such a blockage step [1], [4], [69], [70], [74], [75], [76], [77], [78], [81], [82], [83].

### C.2 Electrochemical modality and label-free detection

The majority of the aptasensors reported label-free detection, where no redox probes were deployed. Redox probes were generally not used in sensors that relied on the EIS technique for the sensor readout [4], [69], where the system was shown to become increasingly capacitive with increasing cortisol concentration. Similar to the FET-based immunosensors, the FET-based aptasensors allowed label-free detection as well [1], [77], [78], [80], [81], [82], [83]. Notably, it was shown in [70] that the addition of metalloporphyrin on the working electrode can catalyze the electrochemical reduction of cortisol, allowing redox probe-free measurement.

Of the aptasensors that reported labeled detection, mainly $[Fe(CN)_6]^{3-/4-}$ was employed along with voltammetric techniques [71], [74], [75], [76]. Depending on the original orientation of the aptamers on the electrode surface and the 3D conformational change it undergoes, the way the current responded to a change in cortisol concentration differed. For
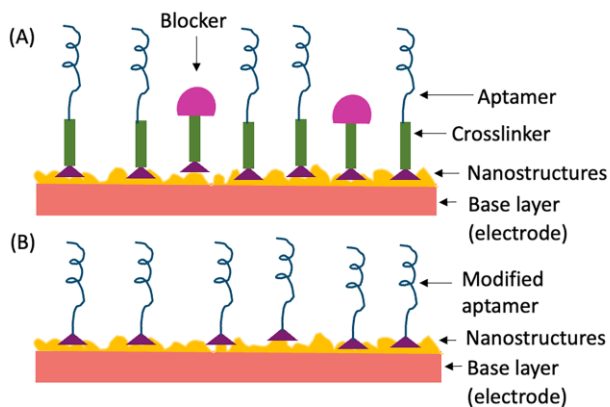


Fig. 5. Schematic depicting the layers in a typical aptasensor, where the aptamers were immobilized: (A) via a cross-linker and (B) via added functional groups, such as thiol or amino group.
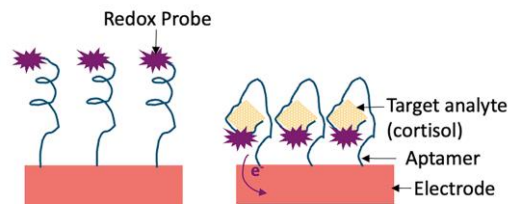


Fig. 6. A working mechanism of aptamers tagged with redox probes: Aptamers bind with the cortisol, undergoing conformational changes bringing the redox probes closer to the electrode surface, facilitating the electron transfer between the redox probes and electrode surface.

instance, in [74], the binding of cortisol to the aptamer is shown to cause a structural change that 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 the sensor reported in [71]. Here, the initial cortisol recognition and capture happens externally with antibody-AuNPs conjugates and when the cortisol/antibody-AuNPs were introduced to the sensor, the immunocomplex was captured by the aptamers forming an insulating barrier for the electron transport, decreasing the current.

Alternatively, aptamers were also tagged with methylene blue (MB) in [72] and [79]. The binding of cortisol to the aptamers brings the MB closer to the electrode surface resulting in an increased current, as illustrated in Fig. 6. In [67], the aptamer was loaded with magnetic nanoparticles conjugated with multiple MB moieties, which resulted in a signal-on (sensor output signal increased with increasing cortisol concentration) assay with the ability to distinguish between a blank sample versus a 1 µg/mL cortisol in serum, unlike with aptamers tagged with a single MB label.

### C.3 LoD, dynamic range, and sensitivity

The sensor response in the majority of the papers in this section was shown to depend logarithmically on cortisol concentration with linear logarithmic expressions. However, a linear relationship was also reported [67], along with the derivation of a closed nonlinear logarithmic relationship [81]. Additionally, a four-parameter fit typically used for affinity-based assays including ELISA, to fit the calibration curve, was applied in [69].

The two widest dynamic ranges reported were 0.36 ng/mL – 3.63 µg/mL [81], [82] and 4 pg/mL – 3.63 µg/mL [77], and the lowest detection limit was 16.3 fg/mL [75]. 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 [1], [75], and [76], which had low enough LoDs but limited dynamic ranges. For instance, the FET-based sensor reported in [1] 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.

### C.4 Selectivity

The interferents most tested in this category of sensors were cortisone [1], [70], [74], [77], [80], [81], [82], progesterone [1], [67], [69], [70], [73], [75], [76], [78], [79], [80] and corticosterone [70], [74], [77], [80]. Although no standard way or standard concentration level of these molecules was 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 and compared this with the sensor response to a fixed cortisol concentration.

Notably, a truncated aptamer (with 14 bases) was shown to result in better selectivity for some interferents compared to when the parent aptamer (with 61 bases) was used [74]. As such, 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 30.4% for the truncated aptamer.

### C.5 Reusability

Four reported aptasensors demonstrated mechanisms for the regeneration of the sensors to achieve sensor reusability [1], [71], [72], [79]. In [72], the sensor was regenerated by exposing it to 1x PBS with 1M sodium chloride (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 a low-pH solution of highly concentrated salt. The FET-based aptasensor in [1] was also regenerated by exposing the sensor to 2 M NaCl. However, the possible number of regenerations was not reported here. Additionally, in [71], the sensor maintained 94% of its initial response after 8 days of testing (8 regenerations by rinsing with a PBS solution of pH 7.4 after each measurement). Lastly, [79] reported that their developed sensor could be reused five times, but the method used for the regeneration was unclear.

## IV. DISCUSSIONS

### A. Antibody vs aptamer vs MIP sensors: current state and practical limitations

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. However, antibodies are still the more popular choice as the recognition element. This is mostly attributed to the extensive usage of antibodies as capture probes in the design and fabrication of biosensors for over 70 years and partly due to the advancements in antibody technology with the introduction of recombinant antibody fragments which are smaller, more stable, and easily modified to have highly oriented immobilization on the sensor surface [84]. Although immunosensors are well-established, antibody production requires animals and suffers from batch-to-batch variations [27]. To this end, MIPs exhibit advantages over antibodies, such as their inherent stability in extreme (temperature and pH) conditions, long shelf-life, and low cost [43]. 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 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. However, there is not enough research on MIP-based electrochemical sensing (only 9 out of the 61 papers for the literature review were MIP-based), and achieving homogeneity in morphology and binding affinity in MIP-based sensors remains a complex task on the road to reproducible sensors [85].

Aptamers also present distinct advantages over antibodies. This 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 [86]. They are also thermally stable and more specific compared to antibodies [86]. Thermal denaturation of aptamers is reversible and the versatility of aptamers in labeling and modification with functional groups allows for simpler immobilization and signaling [87]. For instance, aptamers can be modified with a 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 low immunogenicity compared to antibodies, since oligonucleotides are less likely to cause immune reactions [88]. 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 the temporal variation of pH may be substantial [56]. Additionally, aptasensor can be developed to have a large charge storage capacity [4], making them suitable for prolonged and continuous biosensing with higher sensitivity than that of immunosensors. Aptamers are about 5 times smaller (27 kdA) than the cortisol antibody (150 kDa) [4]. This directly contributes to the steric hindrance effects (SHE) - where aptasensors have less SHE than immunosensors. One important attribute that makes aptamers particularly advantageous for the detection of small molecules such as cortisol, is their ability to undergo significant conformational changes bringing the target-bound aptamers closer to the transducing surface and increasing the biosensor signal [87]. 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 the Debye screening effect in ionic liquids [89]. Hence, the use of aptamers brings the biorecognition event of the analyte within the Debye screening length, allowing for the sensitive

quantification of cortisol. However, even with the many advantages, the field of aptasensing is still very new and the discovery of new aptamers with high affinity for other biomarkers is a complex task [90] with the current SELEX technologies being time-consuming and labour-intensive [91]. A qualitative comparison of LOD and maximum dynamic range among the three categories of sensors is presented in Fig.7. It is clear that a wide range of LoD (and maximum range) can be achieved in any of the three categories of sensors. Notably, the lowest LoDs (and highest maximum dynamic range) have been achieved using immunosensors. However, given a particular application and a target biofluid, all three types of sensors demonstrated suitability.

### B. Suitability for on-body measurement

Immunosensors are reported as suitable candidates for single-use real-time testing platforms such as PoC devices [5], [39], [47], [58], [61], [63]. An example of a wearable device was also proposed in [58] where cortisol immunosensors were embedded into a skin sticker.

Wearable form factors for on-body cortisol measurement have been mainly developed using MIP-based sensors [18], [20], [23] and aptasensors [4], [69], [77], [78], where the sensors were fabricated on stretchable patches to be used on the skin for sweat cortisol sensing. However, a majority of these papers report a single-time cortisol measurement [18], [20], [23], [77], [78].

#### B.1 Sensor reusability and calibration

Sensor reusability is a crucial characteristic for the development of personal health monitoring devices, such as wearable sensors that allow frequent time-resolved measurements. The possibility of reusing a sensor after a regeneration step was shown in four MIP-based sensors and four aptasensors as explained in detail in previous sections and summarised in Table I.

Currently, all the reported regeneration techniques require additional reagents (e.g. PBS or NaCl) to be manually introduced on the sensor surface. Further studies are necessary to automate these, for example through incorporating the reagents into the sensing platforms followed by an automated or externally and remotely controlled release to allow in situ regeneration. A promising technique is presented in [92] where solid-state palladium electrodes are used to control local pH to establish a pH-activated regeneration in situ.

Continuous on-body cortisol monitoring, without needing sensor regeneration was reported in [4] and [69]. However, the response of the sensor shows dependence on the direction of the changes giving two or more sensor outputs per single cortisol concentration.

Another notable advancement towards continuous measurement is the development of platforms with multiple sensing electrodes on a single substrate. These could be used to allow time-resolved measurements or measurements from multiple samples. A multiplexed platform consisting of an array of 16 cortisol aptasensors fabricated on silicon nanowire-based FETs has been reported in [1], for simultaneous measurement of different concentrations of cortisol or potentially time-resolved measurements.
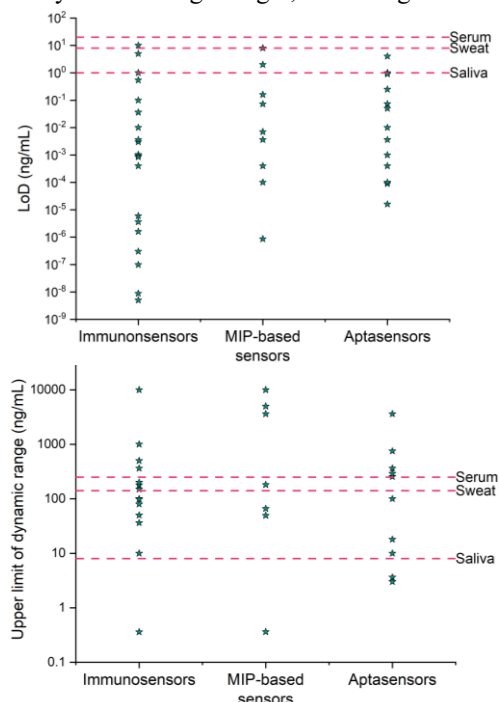


Fig. 7. The LoD and the upper limit of the dynamic range reported in the three categories of sensors. The lower and upper range of cortisol in biofluids are also illustrated, respectively, for comparison.

TABLE I
Regeneration details for the sensors that demonstrated reusability

| Receptor | Method | Wash buffer | # of reg.* | The factor limiting the number of reg. | Ref |
|---|---|---|---|---|---|
| MIP | CV | PBS | 10 | NR | [18] |
| | CV | PBS | 15 | NR | [19] |
| | CV | PBS | 7 | MIP film adhesion weakening | [21] |
| | Rinse | EtOH* | 3 | MIP film degradation | [23] |
| Apt* | Rinse | NaCl | 3 | Aptamer degradation | [72] |
| | Rinse | NaCl | NR | NR | [1] |
| | Rinse | PBS | 8 | NR | [71] |
| | NR | NR | 5 | NR | [79] |

*Apt: Aptamer EtOH: ethanol, reg: regenerations, NR: not reported

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 and how calibrations may be carried out conveniently. For instance, [93] demonstrated that there is a correlation between the occurrence of a fault in the glucose sensor with the sensor's double-layer capacitance. Such a correlation introduces the possibility for an in-situ faulty sensor detection or potentially sensor calibration, where sensor sensitivity may be predicted in situ through easily measurable sensor characteristics such as impedance.

### B.2 Label-free (redox-probe free) sensing

The detection techniques that require redox probes generally run at higher input voltages, have a slower response time and depend on electron transfer kinetics between the electrode and redox probe [60]. Furthermore, the need for an external redox probe is not ideal for on-body cortisol measurement applications, as it would require user intervention for the addition of the redox probe before taking the measurement. To address this, a number of recent studies employed antibodies that are already tagged with redox probes [51], MIP films with embedded redox probes [20], [23], [26], and aptamers tagged with redox probes [67], [72], [79] to obviate the need for an external redox probe.

The most popular choices for redox probe-free measurement are the use of non-faradaic EIS and FET-based I-V curves to characterize the sensor output. One of the main drawbacks of such techniques is that nonspecific bindings could decrease the sensitivity of the sensors. In particular, in immunosensors, it has been reported that aggregations among the antibodies lead to a decreased availability of antigen-binding sites, and reduced sensitivity [63].

### C. An integrated cortisol-sensing microsystem

Electrochemical sensing offers the advantage of real-time measurement and the potential for miniaturization to allow POC and wearable applications – applications widely referred to in the majority of included studies. However, most reported cortisol electrochemical sensors utilize bulky external potentiostats to carry out the electrochemical readout. To allow portability and system miniaturization, these could be replaced with portable or fully-integrated potentiostats similar to those reported in literature for other sensing applications [94], [95], [96], [97].

It is imperative that the development of sensing microsystems that allow both real-time and continuous cortisol measurement involves the integration of more than the cortisol sensor and the instrumentation electronics. Wireless telemetry (e.g. with smartphone), energy source, and reliable access to biofluid, are some other key components and aspects of such a system. Over the past decade, there has been substantial progress on developing mobile-based healthcare, compact energy sources, as well as customized wireless electronic systems as recently reviewed in other publications [98] [99]. The authors believe these are currently at a more mature technological level, and the current technological bottleneck towards developing a continuous cortisol sensor microsystem are the lack of a reliably re-usable cortisol sensor and robust in-situ biofluid sampling and preparation techniques.

### C.1 Challenges of measurement in saliva and sweat

Sweat and saliva are both valid and accessible choices of biofluids for non-invasive PoC cortisol measurement. Cortisol analysis in sweat has been more widely explored for such applications due to its fewer biofouling effects compared to saliva. To avoid the risk of biofouling, centrifugation of saliva samples is carried out to remove mucins.

An alternative to centrifuging the saliva samples was proposed in [100] where saliva was collected from the mouth by suction into a pipette-like saliva sampling device that includes a filter membrane to remove mucins, bubbles, and food particles from the sample before it reaches the sensor.

One main limitation to measuring endogenous cortisol levels in naturally secreted sweat is the low volume of sweat collection. The collection of sweat for cortisol sensing may involve sweat stimulation (e.g. by exercise [18] and heat [101] which can induce physiological and psychological stress, affecting the level of cortisol. To address these challenges, a highly permeable sweat-wicking porous PVA hydrogel was proposed in [20] to collect natural perspiration secreted from the fingertip.

Another challenge in developing wearable sensors for sweat-based analysis is the replenishment of biofluid and the lack of data on how the flow rate of biofluid would affect the sensor performance. Evaporation of the sampled biofluid using an integrated absorbent pad has been presented in [40] to allow replenishment. Tests with different flow rates indicated that at lower than 2 µL/min, the output current of the sensor reduces. This is likely due to poor contact between the sweat and the working electrode. Decreasing the volume of the microfluidic device inlet has been suggested to help improve signal performance at low flow rates [40].

### D. Market impact and commercialization potentials

There is increasing global attention on better understanding and managing mental health and stress-related disorders. Cortisol, the main stress hormone, is at the centre of such scientific investigations. However, the lack of commercially available and low-cost techniques for real-time cortisol measurement has been a major hindrance in collecting data

during such clinical studies. Furthermore, technologies that empower individuals to monitor their stress levels, potentially through measuring cortisol levels in real-time, are becoming extremely attractive, as evident in ever-increasing research carried out in developing such systems.

Inexpensive materials and techniques such as inkjet printing on paper [70], polyimide films [80], or roll-to-roll rotary screen printing on PET substrates [23] have recently been demonstrated to fabricate disposable tests for cortisol sensing. To ensure reproducibility and scalability for commercialization, automatization of sensor fabrication processes is a key requirement. The fabrication of sensors typically consists of several manual steps such as pipetting/deposition of functional layers, incubating, and washing which can add variability and affect the reproducibility of the sensors. Automatization of such processes would solve these limitations and pave the way for large-scale production [102].

## V. CONCLUSION

This paper presents a systematic review of the literature on all electrochemical cortisol sensors (employing aptamers, antibodies, and MIP as bioreceptors) for cortisol detection in human biofluids. A total of 61 primary research papers were included in the review with a special focus on on-body and re-usable sensing.

A detailed comparison between electrochemical sensors for cortisol shows that although all three categories (immunosensors, aptasensors, and MIP-based sensors) are capable of single-time measurement of cortisol in all valid biofluids (saliva, blood, and sweat), the immunosensors offer almost no prospect for multiple measurements. Instead, reusability has already been demonstrated in several aptasensors and MIP-based cortisol sensors, which together with their superior stability, could pave the way to the much-desired yet unmet goal of continuous cortisol sensing on the body. However, a majority of wearable cortisol sensors reported in literature are still limited to one-time measurement, due to challenges in regenerating the sensors in situ. More research into automating sensor regeneration could lead to a breakthrough — new sensors capable of truly time-resolved measurements from whole biofluids. An alternative, promising approach to achieve time-resolved measurement is through developing new sensor platforms that embed multiple sensing electrodes that can be activated at controlled times.

Continuous on-body cortisol measurement depends upon advancements in cognate areas of instrumentation electronics and biofluid sampling and preparation microsystems. Although these are research areas that have undergone substantial progress over the past two decades, a closer collaboration between researchers in the various disciplines involved could bring all these to fruition. An integrated sensing microsystem capable of continuous cortisol measurement on the body would not only provide accurate data to support clinical diagnostics, but also equip researchers with a unique low-cost tool to study various conditions affecting cortisol or being affected by cortisol. It may also allow individuals to monitor some aspects of their mental health (e.g. stress levels) and well-being.

## SUPPLEMENTARY MATERIAL

Supplementary information including tables listing detailed specifications of sensors reported in the 61 included studies are available at the following link https://zenodo.org/records/10076125.

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