TURUN YLIOPISTO

Electrochemical immunosensors for non-invasive and wearable cortisol and estradiol sensing

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The monitoring of steroid hormones such as cortisol and estradiol is important for understanding physiological processes related to stress, metabolism, and reproduction. Conventional hormone detection methods are typically based on single time-point measurements and rely on invasive sampling combined with laboratory-based analysis. As a result, they are incapable of continuous monitoring and cannot capture the dynamic fluctuations of hormone levels over time. This thesis reviews electrochemical immunosensors as an approach for non-invasive and wearable hormone sensing, with a focus on cortisol and estradiol detection. The sources in this thesis consist of scientific articles, review articles, research papers, and a textbook.

The working principles of biosensors, specifically antibody-antigen recognition and electrochemical detection, are described, and fabrication strategies based largely on carbon nanomaterials and surface modification are discussed. The key advantages of immunosensors include high selectivity and compatibility with miniaturization. Limitations include cross-reactivity between structurally similar steroids, sensitivity to environmental conditions, loss of activity over long-term usage, susceptibility to biofouling, and high production cost. Covalent and affinity-based immobilization provide superior stability and reproducibility compared to physical adsorption. Carbon nanomaterials offer high electrical conductivity, large surface area, mechanical flexibility, and compatibility with textile integration.

A comparison of recent studies shows that wearable electrochemical immunosensors for cortisol have been successfully demonstrated in biofluids such as sweat and saliva. The lowest detectable concentrations range from 10^{-5} to 10^{-2} pg/mL. In contrast, estradiol sensing remains largely limited to controlled laboratory conditions, with detection limits ranging from 0.3 to 10 pg/mL. The challenges are primarily driven by low analyte concentrations, along with limitations in device stability and calibration. Despite these limitations, electrochemical biosensors demonstrate strong potential for non-invasive hormone monitoring, with ongoing developments expected to improve their applicability in personalized healthcare, particularly in women's health applications.

Key words: electrochemical immunosensors, wearable biosensors, non-invasive monitoring, carbon nanomaterials, cortisol, estradiol

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Steroidihormonien kuten kortisolin ja estradiolin seuranta on tärkeää stressiin, aineenvaihduntaan ja lisääntymiseen liittyvien fysiologisten prosessien ymmärtämiseksi. Perinteiset hormonien määritysmenetelmät perustuvat tyypillisesti yksittäisiin aikapisteisiin ja edellyttävät invasiivista näytteenottoa sekä laboratorioanalyysiä. Tämän vuoksi ne eivät mahdollista jatkuvaa seurantaa eivätkä pysty kuvaamaan hormonitasojen ajallisia vaihteluita. Tässä kandidaatintutkielmassa tarkastellaan sähkökemiallisia immunosensoreita ei-invasiiviseen ja puettavan hormoniseurannan menetelmänä, keskittyen kortisolin ja estradiolin havaitsemiseen. Työn lähteinä on käytetty tieteellisiä artikkeleita, katsausartikkeleita, tutkimusjulkaisuja ja yhtä oppikirjaa.

Tutkielmassa kuvataan biosensoreiden toimintaperiaatteita, erityisesti vasta-aine-antigeeni-tunnistusta ja sähkökemiallista havaitsemista, sekä käsitellään hiilinanomateriaaleihin ja pintamuokkaukseen perustuvia valmistusstrategioita. Immunosensoreiden keskeisiä etuja ovat korkea selektiivisyys ja yhteensopivuus miniatyrisoinnin kanssa. Rajoituksiin kuuluvat rakenteellisesti samankaltaisten steroidien aiheuttama ristireaktiivisuus, herkkyys ympäristöolosuhteille, aktiivisuuden menetys pitkäaikaisessa käytössä, altistus biofouling-ilmiölle sekä korkea kustannus. Kovalenttinen ja affiniteettipohjainen immobilisointi tarjoavat paremman stabiilisuuden ja toistettavuuden verrattuna fysikaaliseen adsorptioon. Hiilinanomateriaalit tarjoavat korkean sähkönjohtavuuden, suuren pinta-alan, mekaanisen joustavuuden ja yhteensopivuuden tekstiili-integraation kanssa.

Viimeaikaisten tutkimuksien vertailu osoittaa, että puettavat sähkökemialliset immunosensorit kortisolin havaitsemiseksi on onnistuneesti osoitettu biofluideissa, kuten hiessä ja syljessä. Alhaisimmat havaittavissa olevat pitoisuudet vaihtelevat välillä 10^{-5} - 10^{-2} pg/mL. Sen sijaan estradiolin mittaus on edelleen pääosin rajoittunut kontrolloituihin laboratorio-olosuhteisiin, ja sen havaintorajat vaihtelevat välillä $0{,}3$ - 10 pg/mL. Keskeisiä haasteita ovat analyytin matalien pitoisuuksien lisäksi laitteen stabiilisuuteen ja kalibrointiin liittyvät rajoitukset. Näistä rajoituksista huolimatta sähkökemialliset biosensorit osoittavat merkittävää potentiaalia ei-invasiivisessa hormoniseurannassa. Jatkuvan kehityksen odotetaan parantavan niiden sovellettavuutta yksilöllisessä terveydenhuollossa, erityisesti naisten terveyteen liittyvissä sovelluksissa.

Avainsanat: sähkökemialliset immunosensorit, puettavat biosensorit, ei-invasiivinen seuranta, hiilinanomateriaalit, kortisoli, estradioli

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1 Introduction

Hormone monitoring plays a significant role in research and healthcare since hormones control many of our central physiological processes. Monitoring of female hormones has become increasingly important, as traditional research has historically been biased toward male physiology [1]. Steroid hormones such as cortisol and estradiol are of particular interest due to their involvement in circadian regulation, the menstrual cycle, fertility, and overall well-being. However, female hormones have been historically left out partly because their cyclical variation introduces complexity into datasets, and exclusion has often been used to simplify analysis [1]. This imbalance has led to challenges in personalized healthcare for women as well as in drug development.

Hormone concentrations fluctuate over time, contributing to variability in measured values. Cortisol follows a circadian rhythm, meaning it varies during the day, while estradiol changes over the duration of the entire menstrual cycle. Despite the dynamic patterns, traditional measurement methods are typically single time-point measurements that rely on invasive blood sampling and laboratory analysis [2,3]. In most cases, data for the hormone profiles is more clinically valuable than isolated measurements, as it captures physiological dynamics that would otherwise be missed. Continuous real-time monitoring of hormonal levels can therefore provide richer datasets for personalized healthcare and health management, while also offering improved insights into endocrine function for research applications.

Advancements in biosensor technology could offer a promising solution for continuous hormone monitoring. A biosensor is a device that combines a biorecognition element, to detect a specific analyte, and a transducer, to convert the signal [4]. Specifically, immunosensors that utilize antibody-antigen interactions can be used for hormone detection. This work focuses on cortisol and estradiol measurements. Electrochemical immunosensors, which convert the antibody-antigen binding event into a measurable electrochemical signal, can be miniaturized into wearable devices to specifically monitor these molecules. Wearable devices can be, for example, patches or watches that measure hormones from sweat, saliva, or interstitial fluid [3,5]. Carbon-based nanomaterials have shown promise for wearable electrochemical immunosensors due

to their electrical conductivity, high surface area, and mechanical properties compatible with such devices [6].

A key challenge in hormone sensing arises from the in vivo measurement environment. Target analytes such as cortisol and especially estradiol are present at low concentrations, requiring highly sensitive detection methods [7,8]. Measurements in complex biofluids also come with additional complications, including biofouling and signal drift, which can affect sensor accuracy and stability over time [1]. These factors make reliable device calibration and long-term signal reproducibility difficult to achieve in real-world conditions.

The biorecognition element in immunosensors causes further limitations. While antibodies provide high selectivity, their usage introduces challenges related to cost, limited shelf life with potential loss of activity over time, especially under challenging environmental conditions [9].

Additional limitations arise from translating the systems into wearable formats. Particular challenges include withstanding continuous mechanical deformation while maintaining stable performance [6]. Wearable formats also have challenges to consider with user compliance, since problems can be caused by incorrect usage, inconsistent wear or differences in behavior across users [1].

This thesis will review electrochemical immunosensors for non-invasive and wearable sensing of cortisol and estradiol, utilizing carbon nanomaterials as the substrate. The aim is to evaluate how continuous hormone monitoring can improve the understanding of hormonal functions and support personalized healthcare, to examine the working principles as well as preparation methods of immunosensors, and to assess strategies for non-invasive and wearable hormone sensing technologies.

ChatGPT AI was used in the making of this thesis for the following reasons: searching for reference documents with keywords as well as proofreading and linguistic refinement.

2 Hormones and analytical background

In order to design and evaluate biosensors for hormone detection, it is essential to understand both the biochemical nature of the target molecules and the analytical challenges associated with their measurement. This chapter provides the necessary background on hormone function and the analytical considerations for their detection, focusing on cortisol and estradiol.

Hormones are biochemical messengers that are produced by endocrine tissues and glands [4]. They travel through the bloodstream to deliver signals that regulate and control many different physiological processes such as metabolism, stress, and reproduction. The concentration of hormones fluctuates throughout the day.

Importantly, in most hormonal diagnostics, the pattern of hormonal fluctuation is considered more important than values at a single time point [1]. Measurements done at a single point of time do not accurately reflect the dynamic hormonal cycle and therefore lack diagnostic value. This highlights the value and importance of continuous monitoring and measurement of hormones over the entire cycle.

Among different classes of hormones, steroid hormones share a common four-ring carbon structure and are derived from cholesterol [7]. Cortisol and estradiol both belong to this class, and their structures are shown in Figure 1. In addition to structural similarity, both occur at extremely low concentrations in the body and exhibit dynamic variation, making their accurate detection analytically challenging.

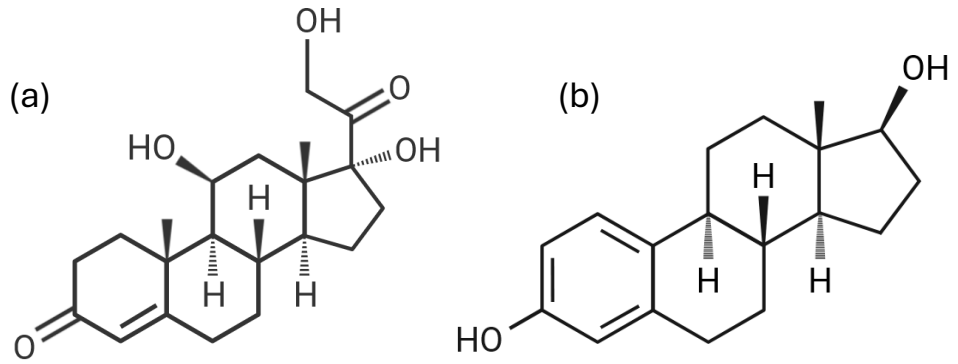

Figure 1. (a) Cortisol structure (b) Estradiol structure (Created with BioRender)

2.1 Cortisol and estradiol as biomarkers

Steroid hormones such as cortisol and estradiol circulate in blood primarily bound to carrier proteins, with only a small fraction existing as free, biologically active hormone. For cortisol, approximately 90% is protein-bound, while only 5-10% is free [7]. Estradiol follows a similar pattern, with the majority bound and only a small portion free. Total hormone levels reflect overall endocrine function and remain the clinical standard for diagnostic purposes. However, free hormone levels are more relevant for real-time physiological monitoring, stress tracking, and understanding biologically active hormone dynamics as they represent the fraction available to bind to receptors and elicit physiological responses.

Cortisol is the primary stress hormone and is regulated by the hypothalamic-pituitary-adrenal (HPA) axis. It follows a 24-hour circadian rhythm, with typical levels ranging from approximately 50-163 ng/mL in serum [7]. Levels peak shortly after waking up and decrease gradually throughout the day, reaching their lowest point around midnight, as represented by Figure 2a.

Abnormal cortisol levels may be an indicator of various endocrine disorders and stress-related conditions. Elevated cortisol is associated with Cushing's syndrome, chronic stress, and depression. Low cortisol levels may be caused by Addison's disease or adrenal insufficiency [7]. In addition to clinical diagnostics, cortisol levels are increasingly tracked for general well-being, stress management, and athletic performance.

Estradiol, also known as 17β-estradiol, E2, or oestradiol, is the primary form and most potent estrogen in women during reproductive years and is primarily controlled by the hypothalamic-pituitary-gonadal (HPG) axis [10]. Estradiol levels fluctuate in a cyclical pattern throughout the menstrual cycle, with typical levels ranging from 20-750 pg/mL [11]. During reproductive years serum estradiol concentrations are lowest in the follicular phase (20-350 pg/mL), rise during ovulation and the midcycle peak (150-750 pg/mL) and decrease again during the luteal phase (30-450 pg/mL) as illustrated in Figure 2b. After menopause, serum estradiol levels fall to 20 pg/mL or below.

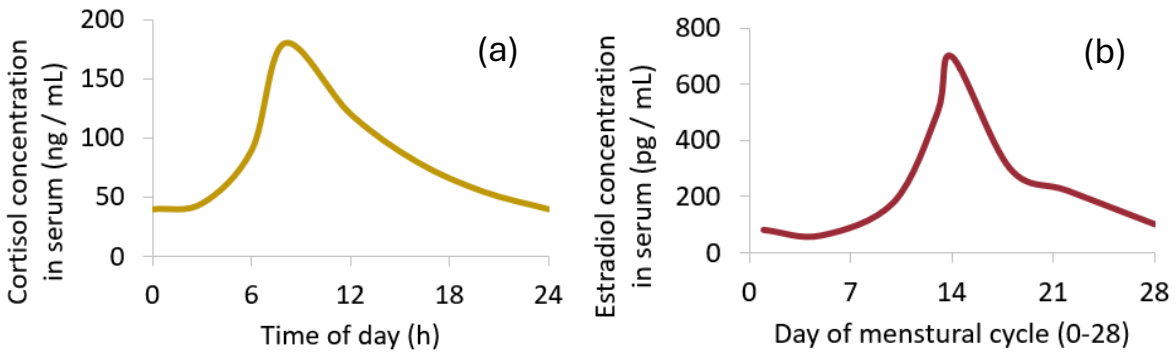

Figure 2. (a) Cortisol fluctuation over a 25-hour cycle (b) Estradiol fluctuation over a 28 day menstrual cycle (Created with Excel)

Abnormal estradiol levels may indicate endocrine, reproductive, or metabolic disorders. High estradiol levels are commonly associated with obesity, liver problems, mood changes, and libido changes [10]. Hypoestrogenism, meaning abnormally low levels of estrogen, can typically be caused by menopause, stress, and extreme dieting or exercising. Low estradiol may lead to functional hypothalamic amenorrhea (FHA) [12].

While both cortisol and estradiol are steroid hormones, their dynamics and physiological mechanics differ significantly. Cortisol is regulated by the HPA axis and follows a 24-hour circadian rhythm, while estradiol is regulated by the HPG axis and fluctuates across the menstrual cycle, typically four weeks. Depending on the cycle phase, cortisol is present at 100 to 1000 times higher concentrations in blood serum, making it much easier to detect in comparison to estradiol. As a result, rapid response times and efficient electron transfer are critical requirements for cortisol sensors. In contrast, sensitivity, selectivity, and minimizing background noise are key in estradiol sensor design.

2.2 Analytical methods for cortisol and estradiol

Cortisol and estradiol can be measured from different matrices, and the most suitable one largely depends on the analyte as well as the intended application. Serum, obtained through invasive blood sampling, is the clinical standard for hormone measurements due to established diagnostic reference ranges and standardized analytical methods. In contrast, saliva, and sweat allow non-invasive sampling, while interstitial fluid (ISF) requires minimally invasive methods such as microneedles [2]. These biofluids are

more suitable for continuous wearable monitoring due to easier access and measurement.

A key distinction between these matrices is what they measure. Serum captures total hormone levels, both bound and free, whereas alternative matrices such as saliva, sweat, and ISF reflect only the free, biologically active fraction [7]. This distinction is important for sensor design, as free hormone concentrations in these fluids are substantially lower than total hormone concentrations in serum. Even with these lower concentrations, several electrochemical immunosensors have successfully demonstrated cortisol detection in sweat and saliva, showcasing the applicability for real-time monitoring [3, 5,13,14].

Despite the advantages of non-invasive matrices, blood sampling remains the clinical standard. However, it is invasive, unsuitable for continuous monitoring, and the procedure itself can be stressful, potentially leading to temporarily elevated cortisol levels [2]. Non-invasive methods such as saliva, sweat, and ISF (minimally invasive) offer advantages in ease of collection, patient comfort, and suitability for wearable monitoring. However, these matrices are less standardized in clinical practice, have more variable reference ranges, and are more susceptible to interference and variability in sampling conditions compared to serum.

Cortisol is typically present in ng/mL concentrations in serum, saliva, and sweat, allowing reliable detection with relatively moderate limits of detection (LOD). LOD represents sensor sensitivity and gives the lowest concentration of the analyte that can be measured and separated from background noise. In contrast, estradiol is present at pg/mL levels in non-invasive fluids [7].

Traditional quantification of cortisol and estradiol relies on laboratory-based techniques. Mass spectroscopy (MS) is the current gold standard for both cortisol and estradiol measurement, and liquid chromatography (LC) is commonly used together with MS (LC-MS/MS) for highly sensitive and specific results [15]. LC-MS/MS can distinguish hormones from interfering compounds and structurally similar steroids [7]. Especially due to estradiol's low-circulation concentrations, measurements commonly use high-performance liquid chromatography (HPLC) and other LC-based techniques

for high analytical sensitivity [8]. While these methods are very efficient and able to achieve high sensitivity, they require bulky and expensive equipment, trained personnel, and complex sample preparation. This limits possibilities for easy on-site measurements and most importantly limits continuous measurements.

Different immunoassays, such as enzyme-linked immunosorbent assays (ELISA) or chemiluminescent assays, are widely used due to their simplicity and accessibility [8,16]. However, these techniques may produce inaccurate measurements, especially at low concentrations. This is largely due to structurally similar steroid hormones causing cross-reactivity and interfering with the results. Fluorescence-based assays are also commonly used for rapid hormone detection, but they may not achieve accurate measurements due to background interference [7,8].

In summary, traditional laboratory-based methods offer high sensitivity but require invasive sampling, bulky equipment, and trained personnel, making them unsuitable for continuous monitoring. Non-invasive and minimally invasive matrices enable wearable sensing but introduce challenges such as lower analyte concentrations, greater variability, and susceptibility to interference. These limitations highlight the need for electrochemical immunosensors that combine the selectivity of antibody-antigen recognition with the sensitivity and miniaturization potential required for continuous, real-time hormone monitoring.

3 Biosensors and electrochemical detection

This chapter focuses on biosensors and their role in addressing key limitations in hormone detection, particularly selectivity and sensitivity in complex biological matrices. Selectivity refers to distinguishing the target analyte from structurally similar molecules, which is especially critical for steroid hormones such as cortisol and estradiol due to their similar chemical structures and low physiological concentrations. Sensitivity describes the ability of a sensor to detect small changes in analyte concentrations, which is also essential for hormones with low concentrations. Biosensors address these challenges by integrating biorecognition elements with physicochemical transducers. This chapter will introduce the fundamental principles of biosensors and immunosensors, followed by an overview of different immunoassay formats. Finally, electrochemical detection methods and their advantages, limitations, and suitability for non-invasive sensing platforms are discussed.

A biosensor is an analytical device used to selectively detect an analyte and convert it into a measurable signal. Biosensors consist of three main components: a biorecognition element, a physicochemical transducer, and a signal generator, as illustrated in Figure 3. The biorecognition element selectively interacts with the target molecule. The transducer converts the interaction into a signal. Finally, the signal generator amplifies and interprets the output [17]. Hormones are typically measured with immunosensors.

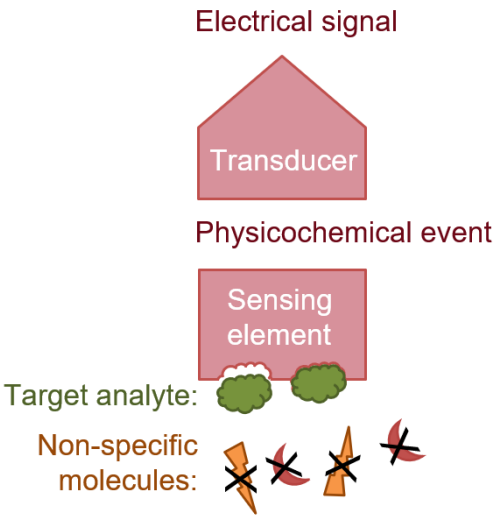

Figure 3. Biosensor elements, only the target analyte binds (Created with PowerPoint)

3.1 Immunosensors

An immunosensor is a specific type of biosensor that relies on antigen-antibody binding for target recognition. Antibodies are essential molecules of the body's immune system. They are proteins capable of highly specific binding to the target molecule. Antibodies are Y shaped molecules and have binding sites called paratopes at both ends of the Y shape. These binding sites can only interact selectively with the corresponding epitope, a specific part of the antigen. Antigens are molecules that antibodies can bind to, and that can trigger immune responses when the binding occurs. The binding happens through non-covalent forces, such as hydrogen bonds, Van der Waals forces, and electrostatic interactions [4]. Figure 4 illustrates a simplified representation of an antibody and antigen, including their paratope and epitope that bind selectively.

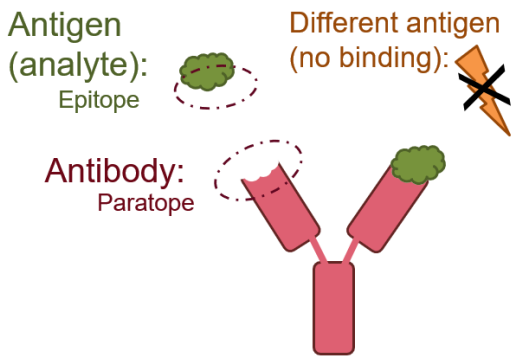

Figure 4. Simplified antibody-antigen structure and selectivity (Created with PowerPoint)

The main advantage of using antigen-antibody binding as the recognition method is selectivity, as antibodies can only bind with the specific target molecule. Antibodies reduce inaccurate measurements and allow for sensitive measurements even in samples with low concentrations, which makes them suitable for cortisol and estradiol measurements, as their concentrations require high selectivity [7]. In addition, the ability to measure antigen-antibody binding as electrical signals is compatible with miniaturization and low-power continuous wearable technologies [17].

Limitations of using antibody-antigen recognition for cortisol and estradiol sensing in steroid sensing applications include cross-reactivity, as steroid hormones share similar three-dimensional structures and may have overlapping binding sites [8]. This can lead to non-specific binding and reduced selectivity. Although certain antibodies have been developed to exhibit lower cross-reactivity, for example estradiol-antibodies with

improved selectivity [18], the problem is still present. In contrast, sensing methods without biorecognition elements generally suffer from reduced selectivity and non-specific signal interference.

Another key disadvantage with antibody-antigen approaches is their sensitivity to environmental conditions such as pH and temperature, and they might become unstable or inactive over long-term usage and storage [9]. Biosensors are also susceptible to biofouling, a phenomenon where proteins attach to the sensor surface, leading to reduced accuracy and working duration [1]. Production costs for immunosensors can also be relatively high due to the cost of antibody production, limiting scalability in comparison to using synthetic recognition elements, such as aptamers [9].

3.2 Immunoassay formats

Immunoassays are analytical methods, where antibodies are used to selectively detect the analyte based on epitope-paratope binding. The binding event generates a signal, which is converted into a measurable signal reflective of analyte concentration. Immunoassays can be divided into two main categories based on signal generation: labeled and label-free methods.

In labeled immunoassays, the signal is produced using a label, such as enzymes, fluorophores, chemiluminescent compounds, or nanoparticles. Enzymes are commonly used because they catalyze reactions that generate measurable signals, such as current changes. Immunoassays are often performed in microplate formats, such as ELISA in conventional enzyme plates [15]. Here, antibodies are attached (i.e., immobilized) to the solid plate surface, and enzyme labels generate a measurable signal that is read using a plate reader. In label-free immunoassays, the signal is generated directly from changes at the sensor interface, such as slower electron transfer, increased mass, or a thicker biomolecule layer [4].

Immunoassays can also be classified by their assay format, which depends largely on the characteristics of the target molecule. For larger molecules such as proteins, immunometric (sandwich) immunoassays are commonly used. In this format, the

analyte is bound between two antibodies, which bind to different epitopes on the analyte, as shown in Figure 5a. One of the antibodies is typically immobilized onto a surface, while the other one is free and carries a label in labeled formats, or is detected directly in label-free formats [4]. However, immunometric assays are not applicable to small molecules such as steroid hormones, since they typically possess only a single epitope.

For small molecules, the most widely used approach is therefore a competitive immunoassay. In this format, as shown in Figure 5b, the analyte competes with a labeled or label-free tracer for limited antibody binding sites. The tracer is a chemically modified molecule, that optionally carries the label, and is still able to bind to the antibody as the target analyte [4]. The labels are typically enzymatic, fluorescent, or chemiluminescent. As the analyte concentration increases, there are fewer opportunities for tracers to bind to antibodies. As a result, the measured signal decreases, meaning the signal is inversely proportional to the concentration of the analyte in the sample.

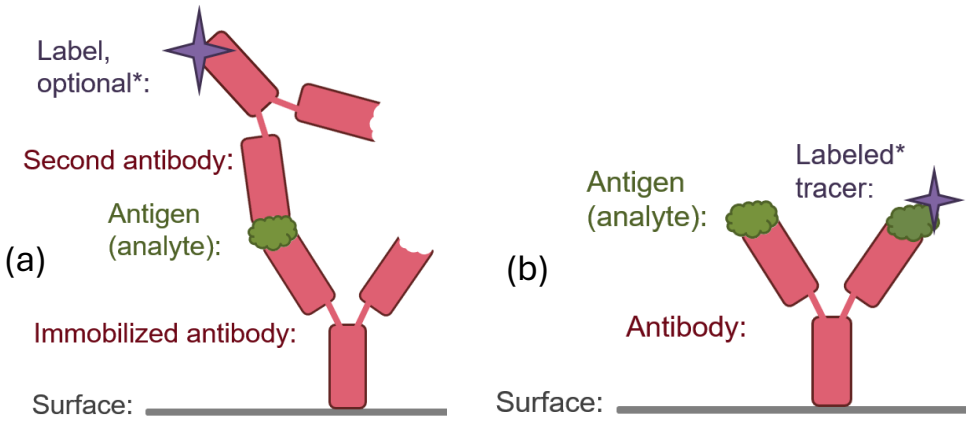

Figure 5. (a) Labeled Immunometric immunoassay (b) Labeled Competitive immunoassay * Labels used only in labeled format, label-free versions omit label and detect surface changes directly (Created with PowerPoint)

Advanced alternative immunoassay formats have also been developed for more sensitive and selective measurements. Anti-immunocomplex immunoassays, for example, use antibodies that are able to specifically detect the complex formed between the analyte and antibody, rather than the free analyte. This format has been applied in digital immunoassays, where individual binding events are detected and counted for high sensitivity using dark-field microscopy [19]. However, this approach is

limited by its need for specialized instrumentation and demanding handling. It is also not compatible with miniaturization or wearable platforms, making it unsuitable for continuous hormone monitoring, unlike electrochemical detection methods.

3.3 Electrochemical detection methods

Electrochemical transduction methods are particularly suitable for detection of cortisol and estradiol in immunosensors, due to high sensitivity, compatibility with miniaturization and point-of-care wearable applications, as well as low cost. In electrochemical immunosensors, the antigen-antibody binding interactions at the biorecognition element are converted into measurable changes in current, potential, or impedance by the transducer [17].

A typical electrode consists of a working electrode (WE), a reference electrode (RE), and a counter electrode (CE). The working electrode is the functional component of the electrode, and the part that is chemically modified with the biorecognition layer. The RE provides a stable and known potential while the CE completes the circuits by allowing current to flow. Signal generation in electrochemical immunosensors is often based on redox reactions and electron transfer occurring near the WE. These processes are often monitored using redox probes or mediators, compounds that can undergo reversible oxidation and reduction reactions at the electrode surface, which are measured electrochemically. Redox probes or mediators include methylene blue [5], ferrocyanide [3], or hydroquinone [15,20].

Electrochemical immunosensors are typically classified into three categories based on their detection techniques: voltammetric, amperometric, and impedimetric methods. Voltammetric methods measure current as a function of an applied potential. These methods are used for their high sensitivity, low noise, and compatibility with multiplex detection, meaning the detection of multiple analytes within a single sample [17]. Common voltammetric methods include cyclic voltammetry (CV), differential pulse voltammetry (DPV), square wave voltammetry (SWV), and photoelectrochemical (PEC) detection. Each method has different advantages and is used in different scenarios depending on the suitability.

CV is the most fundamental electrochemical method and is often used to inspect sensor surface modification. In immunosensors, CV is a useful tool for checking whether antibody immobilization has been successful. It is done by sweeping the voltage back and forth while the current is measured, producing a curve showing electron behavior. A typical application is observing the redox peak currents after antibody attachment [21]. Successful antibody immobilization leads to a decrease in redox peak currents and an increase in charge transfer resistance, since the biomolecule layer hinders electron transfer at the electrode surface [5].

DPV is a widely used method thanks to its high sensitivity. DPV applies and measures the difference in small voltage pulses over a slowly increasing baseline. DPV achieves high sensitivity by minimizing background noise and highlighting slight changes in electron behavior. The resulting values can be interpolated with a calibration curve to quantify analyte concentration. In immunosensors, analyte binding typically results in a decrease in peak current, due to increased resistance at the sensor interface. Signal direction may vary depending on whether a label-free or labeled format is used [22].

SWV is a fast and precise electrochemical method that applies a square-wave potential and records current at two points in each cycle. This minimizes background noise, achieving precise measurements. For example, SWV can be applied in competitive immunoassay formats to detect redox-active labels. Immunosensor binding events typically cause changes in current intensity, while signal direction is again dependent on the use of labels [20].

In PEC, light excitation and electrochemical measurement are combined to achieve high sensitivity with little background noise. This is done by illuminating a photoactive electrode to produce a signal. The signal changes when binding events occur. In immunosensors, binding events typically lead to a decrease in photocurrent due to reduced electron transfer efficiency at the electrode surface [23].

In amperometry, the electrode is held at a fixed potential, and the resulting current is measured to determine analyte concentration. The measured current is proportional to the analyte concentration. The target analyte is often quantified by comparing the results with a calibrated plot generated with standard solutions. Binding reactions in

immunosensors typically cause a time-dependent change in current, increasing or decreasing depending on whether electroactive species are generated or consumed at the electrode interface [15].

Impedimetric methods apply an AC signal and measure the resulting impedance. Impedance is a property that describes the resistance to electron transfer. Electrochemical impedance spectroscopy (EIS) is a widely used impedimetric method that enables label-free detection. Because binding events slow electron movement at the electrode surface, the analyte can be measured without the requirement of additional labels. Antigen-antibody binding typically results in an increase in charge transfer resistance, since the electron movement is being slowed [13].

4 Materials and surface modification

This chapter highlights the importance of material selection and surface modification strategies for the fabrication of electrochemical immunosensors. Carbon-based nanomaterials are first discussed in detail, due to their widespread use and suitable properties for wearable biosensors. Then, surface modification approaches for enhancing sensor performance are described. Finally, surface functionalization is considered with antibody immobilization onto the electrode to achieve efficient and stable biorecognition.

Carbon-based materials such as graphene, carbon fibers, and carbon yarn are widely used in electrochemical immunosensors due to their structural and chemical properties; high electrical conductivity, large surface area and chemical stability [6,24]. Carbon nanomaterials can also be integrated with flexible materials while maintaining these properties, making them well-suited for applications in wearable immunosensors. Understanding the properties of carbon nanomaterials is essential when considering if they can be adapted for estradiol sensing, since many cortisol sensors already rely on them.

4.1 Carbon-based materials

Graphene is a carbon material consisting of a single layer of carbon atoms arranged in a two-dimensional hexagonal lattice, as illustrated in Figure 6a. The structure gives graphene its high electrical conductivity, mechanical strength, and large surface-to-volume ratio [6]. The large surface area is ideal for the immobilization of antibodies on its surface. The more antibodies can be attached to it, the more sensitive the immunosensor will be. The high conductivity means it can transfer electrons quickly, allowing for fast electrochemical response times in immunosensors. Graphene can also be modified for more stable immobilization, making it an optimal material for immunosensors.

Carbon nanotubes (CNTs) consist of rolled sheets of graphene into cylindrical nanostructures and can be single-walled (SWCNT) as shown in Figure 6b or multi-walled (MWCNT) as shown in Figure 6c. In addition to the properties of graphene, the

one-dimensional CNT structure can transport electrons quickly along the nanotube axis. This enhances the electrochemical response times of immunosensors [6]. While the toxicity of CNTs is still under study, their potential for integration into wearable sensors makes them promising candidates for estradiol monitoring. When developing new sensing technologies for estradiol and other hormones, considering both performance and biocompatibility is increasingly important for sustainability.

Carbon fibers (CF) are graphitic carbon structure filaments in the micrometer range. The fibers also share the properties of graphene. When bound together in bundles they form carbon yarn (CY). Carbon yarn is very flexible and durable, making it suitable for use in wearable immunosensors [3,14].

Carbon nanofibers (CNF) are like CFs but in nanoscale instead of micrometer-scale. They can be combined with CNTs to further improve their properties [22].

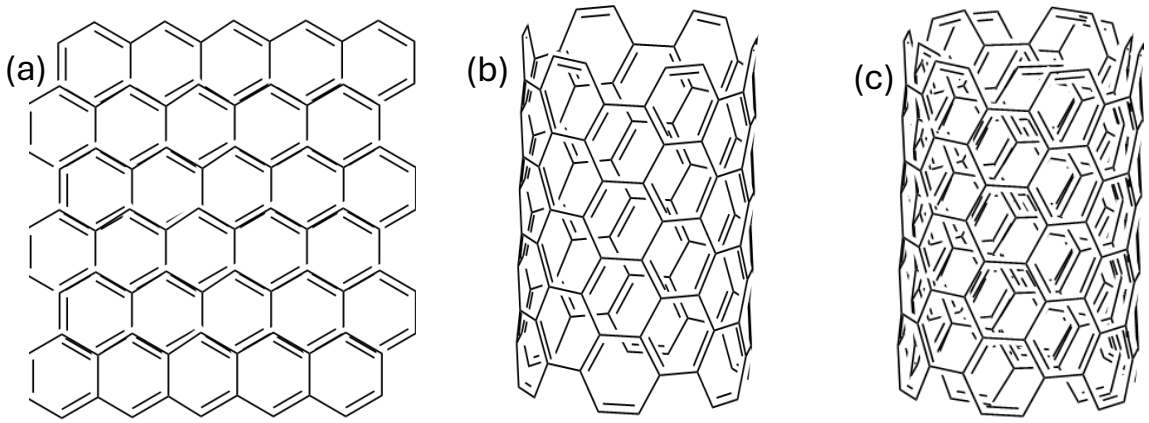

Figure 6. (a) Graphene (b) Single-walled carbon nanotube (c) Multi-walled carbon nanotube (Created with ChemDraw)

4.2 Surface modification techniques

Surface modification of electrochemical immunosensors is performed to enhance sensor properties such as electron transfer, antibody immobilization, and sensitivity [6]. Surface modification can provide a higher density of accessible adsorption surface sites for hormones, which increases the likelihood of binding events between antibodies and hormones. This is why surface modification is critical for low analyte concentrations of cortisol and especially estradiol. Carbon-based materials like graphene, CNTs, and

CNFs are often modified to improve these properties and improve interaction with target analytes.

Computational studies give important insight into antibody-antigen binding interactions at the molecular level, which can be used to further enhance surface modification techniques. Density functional theory (DFT) simulations have shown how estradiol can adsorb onto graphene surfaces through intermolecular interactions, and these simulations have been studied computationally to find the most efficient configurations [25]. Further studies are needed to analyze more surface-modified materials, and to take real-world conditions into account, but DFT and analysis with computational methods can greatly help with the development of wearable immunosensors.

The main approach to surface modification is nanostructuring, where the goal is to increase surface area, add adsorption sites, and improve conductivity [6]. This can be done by integrating different carbon nanomaterials such as carbon nanofibers with graphene planes. This adds to the possible adsorption sites, increasing the likelihood of binding events, leading to lower detection limits.

Another widely used method is enhancing carbon substrates by integrating nanostructured metal oxides such as SnO_2 nanoflakes and ZnO nanorods into them [3,14]. Nanostructures add potential antibody immobilization sites by increasing the structure area. Hydrothermal synthesis is commonly used for nanostructuring, where crystalline nanostructures are created from solutions. An example of this is growing vertically aligned ZnO nanorods on carbon yarn. The process is carried out at 90 °C for 6 hours, and results in uniform ZnO nanorod arrays on the carbon yarn [14].

Metallic nanoparticles such as gold nanoparticles (AuNPs) can also be incorporated to enhance the electrical properties of the sensor [5]. This can be done by depositing the AuNPs onto pre-cleaned electrode surfaces using drop-casting. The liquid solution containing the nanoparticles is dropped onto the electrode surface and allowed to dry [5]. This leaves behind a nanostructured coating that increases the surface area and improves electrode transfer.

Surface modification also includes the fabrication and modification of different nanocomposite systems. Nanocomposite systems can be materials composed of two or more components with different functional roles. For example, complex nanocomposite systems for estradiol detection have been developed, where $ZnIn_2S_4$@NH_2-MIL-125(Ti) composite is used as the matrix and combined with quantum dots that provide signal amplification [23]. This demonstrates the effectiveness of combining nanomaterials to improve sensor properties.

4.3 Antibody immobilization

For the immunosensors to be functional, antibodies need to be immobilized on the sensor surface. Antibody immobilization is a process where the biological recognition element is attached to the transducer surface. With successful immobilization, the antibody maintains its biological activity, meaning it can still bind with the target antigen. The immobilization strategy determines how effectively the antibody can capture the analyte and generate a signal for the detection methods to measure.

Different immobilization techniques include physical adsorption and covalent immobilization. Physical adsorption is the simplest method, where antibodies are immobilized to the sensor surface through weak interactions such as hydrophobic and van der Waals interactions, or electrostatic forces [4]. There may be instability with this approach since the interactions are weak and the orientation of the antibodies is random. This can reduce binding efficiency and weaken the signal.

Electrostatic immobilization is a form of physical adsorption, where antibodies are attached to the surface through charge-based interactions. An example of this is immobilizing anti-cortisol antibodies onto ZnO nanorods grown on carbon yarn, where the difference in isoelectric points between ZnO (~9.5) and the antibody (~4.5) allow for the antibodies to be immobilized through electrostatic forces. In this example, the electrode is incubated for two hours in the antibody solution, with a concentration of 80 µg/mL. The electrode is then rinsed to remove unbound antibodies, and non-specific binding sites are blocked using bovine serum albumin (BSA). The electrode is stored at a low temperature of 4 °C to preserve antibody activity while not in use [14]. While this method is simple and preserves biological activity of antibodies, the electrostatic

interactions are relatively weak and can lead to reduced long-term stability, especially in a real operating environment.

Covalent immobilization leads to a more stable attachment. With this technique, chemical bonds are formed between the antibody and sensor surface. Antibody orientation can be controlled with covalent attachment [9]. In one example, AuNP-modified electrodes are activated using a coupling reagent (e.g., AnteoBind) that allows binding between the electrode surface and antibodies. The electrode is then incubated for 12 h at 4 °C in an antibody solution with a concentration of 100 µg/mL. After the antibody attachment, the non-specific binding sites are blocked using a 1% BSA solution [5]. This method provides a strong and stable antibody attachment, while preserving biological activity and ensuring effective orientation of the antibodies.

In addition to direct covalent immobilization, affinity-based approaches such as biotin-streptavidin interactions are commonly used for antibody attachment [9]. In this example, a functionalized electrode surface is prepared by modifying it with streptavidin, which binds specifically to biotin. This is done by incubating the electrode with streptavidin for one hour. Anti-estradiol antibodies are then immobilized onto the electrode by modifying the anti-estradiol antibody solution with biotin and incubating this antibody solution with the electrode for 15 minutes. The nonspecific binding sites are then blocked using a 5% BSA solution combined with biotin, over an incubation period of 40 minutes [15]. This method also allows for strong and oriented antibody attachment.

An advanced immobilization technique is soft plasma polymerization (SPP) where low-energy cold plasma is used to deposit antibodies onto the electrode surface. The antibody solution is introduced into a plasma zone as a gel, where it undergoes partial polymerization and forms a thin antibody coating on the electrode surface. The process is performed in temperatures under 40 °C, to preserve the biological activity of antibodies as well as achieve uniform deposition [22]. The result of this method is a stable sensor capable of high sensitivity and a low detection limit. The method is also simple and quick.

Overall, different immobilization methods have their strengths and weaknesses, and the method needs to be chosen based on the application and goal. Physical adsorption methods are simple and cost-effective, and do not use harsh chemical reagents that could damage components. These methods are often used for cortisol immunosensors, where the focus is achieving rapid electron transfer to detect the quickly changing cortisol levels. However, these interactions are more susceptible to signal interference, desorption, and reduced long-term stability [9]. Due to this, there is little applicability for estradiol detection where significantly higher sensitivity is required.

In contrast, covalent and affinity-based immobilization methods provide stronger and more stable attachment, leading to less interference, as well as improved reproducibility, making them more suitable for estradiol detection [9]. Regardless of the immobilization method, antibody immobilization as well as use of blocking agents (e.g., BSA) generally increases charge transfer resistance. This effect is often used to confirm successful surface functionalization through electrochemical methods but also highlights the need to optimize immobilization while maintaining sufficient electron transfer.

5 Non-invasive and wearable sensors

This chapter explores the current state of non-invasive and wearable hormone sensing technologies, focusing on electrochemical immunosensors for cortisol and estradiol. It evaluates how advances in materials and device design enable sensitive detection of hormones, while also highlighting the limitations that restrict translation into real-world applications.

Non-invasive sensing refers to methods that measure biomarkers without penetrating the skin to draw blood for example. Hormones can be measured non-invasively from biofluids including sweat and saliva, while ISF requires minimally invasive methods such as microneedles [2]. Common non-invasive approaches include disposable test strips and laboratory-based immunoassays such as ELISA [16]. However, these methods are typically limited to single time-point measurements and do not enable continuous monitoring over extended periods.

In contrast, wearable biosensors are devices worn on the body that are designed for continuous monitoring, meaning real-time or repeated tracking of biomarker levels over hours or days. This is particularly valuable for hormones like cortisol, which follows a circadian rhythm, and estradiol, which fluctuates across the menstrual cycle. In comparison, single time-point measurements are not able to show these patterns. Wearable hormone sensors are commonly designed as sweat patches [13], smartwatches, skin-interfaced electrodes [5], or textile- and garment-based devices [14]. Many of these utilize electrochemical detection methods combined with immunosensors, allowing continuous measurement without the need for invasive sample collection or laboratory-based analysis.

Flexible and conductive materials such as carbon nanomaterials are used in designing electrochemical immunosensors for wearable applications. Their combination with antibody-based recognition elements allows for selective biomarker detection while maintaining mechanical stability during use. These properties are particularly important in wearable devices designed for continuous and non-invasive hormone monitoring in biofluids.

5.1 Cortisol and estradiol sensor applications

For evaluation of the current state of wearable and non-invasive hormone sensing, relevant studies on cortisol and estradiol detection are compared in Table 1 and discussed in this section.

Table 1 Comparison of electrochemical cortisol and estradiol sensing platforms for non-invasive and wearable applications

Analyte	Materials	Recognition Mechanism	Detection Method	Limit of Detection [pg/mL]	Sample Type	Reference / Year published
Cortisol	PET / ITO / AuNPs	Anti-cortisol antibody immunosensor	CV, **DPV**, EIS	5.8×10^2	Sweat and saliva	[5] / 2026
Cortisol	CCY / SnO_2	Anti-cortisol antibody immunosensor	CV, **DPV**	1.6×10^{-3}	Sweat	[3] / 2023
Cortisol	PDMS / LBG / $Ti_3C_2T_x$ MXene	Anti-cortisol antibody immunosensor	CV, **EIS**	1	Sweat	[13] / 2021
Cortisol	CF / ZnONR	Anti-cortisol antibody immunosensor	CV, **DPV**, EIS	9.8×10^{-5}	Sweat	[14] / 2020
Estradiol	GCE / CNT / CNF	Laccase enzyme-based biosensor	**DPV**	7.1×10^3	River water	[22] / 2025
Estradiol	AuNPs	Digital anti-estradiol antibody immunoassay	Fluorescence, **DFM**	1	Standard E2 solution	[19] / 2024
Estradiol	$ZnIn_2S_4$@NH_2-MIL-125(Ti) / PDA / QD	Anti-estradiol antibody immunoassay	**PEC**	0.3	Controlled lake water	[23] / 2020
Estradiol	SPE / MWCNT / THI / AuNPs	Anti-estradiol antibody immunoassay	CV, **DPV**	10	Clinical serum	[21] / 2018
Estradiol	Au electrode / Cu_2S	Anti-estradiol antibody immunoassay	CV, EIS, **SWV**	7.5	Standard E2 solution	[20] / 2014
Estradiol	SPCE	Anti-estradiol antibody immunoassay	CV, **Amperometry**	0.77	Clinical serum and spiked urine	[15] / 2012

| PET = polyethylene terephthalate, ITO = indium tin oxide, AuNPs = gold nanoparticles, CCY = conductive carbon fiber, PDMS = polydimethylsiloxane, LBG = laser-burned graphene, CF = carbon fiber, ZnONR = ZnO nanorod, GCE = glassy carbon electrode, CNT = carbon nanotube, CNF = carbon nanofiber, PDA = polydopamine, QDs = quantum dot, SPE = screen-printed electrode, MWCNT = multi-walled carbon nanotube, THI = thionine, SPCE = screen-printed carbon electrode, CV = cyclic voltammetry, DPV = differential pulse voltammetry, EIS = electrochemical impedance spectroscopy, DFM = dark-field microscopy, PEC = photoelectrochemical detection, SWV = square wave voltammetry |

Table 1 compares the materials, recognition mechanisms, detection methods, the sample type, and the LOD. Notably, none of the estradiol studies are wearable formats, but rather sensors still in development stages applied in laboratory environments, while research on cortisol wearable immunosensors is much further.

Several wearable electrochemical immunosensors for cortisol detection show exceptionally low limits of detection under controlled laboratory conditions. For example, ZnO nanorod integrated carbon fiber sensors and SnO_2-modified carbon yarn electrochemical immunosensors report very low LODs of 9.8×10^{-5} pg/mL and 1.6×10^{-3} pg/mL respectively, while maintaining high selectivity and reproducibility [3,14]. The excellent performance of these immunosensors represents idealized and controlled measurements even though validated using real sweat samples. Even though the results do not fully represent real-sensing environments, these studies showcase the potential in wearable carbon-based electrochemical immunosensors.

In comparison, sensors designed for practical use report higher detection limits. For example, a PET / ITO / AuNP-based sensor achieves a higher LOD of 5.8×10^{2} pg/mL [5]. The sensor still reports good stability and reproducibility in real sweat and saliva samples. Similarly, a $Ti_3C_2T_x$ MXene based wearable patch immunosensor achieves a LOD of 1 pg/mL, while enabling non-invasive and real-time cortisol measurements from sweat [13]. In comparison with the studies that achieve extremely low LODs, these studies highlight the challenges in maintaining sensitivity and performance in complex biological environments.

The performance of wearable cortisol sensors is strongly influenced by the material choice for the electrode. Carbon-based substrates are widely used in these sensors due to their mechanical properties such as flexibility, conductivity as well as compatibility with textile integration. Electrochemical performance is also enhanced through nanostructuring with metal oxides such as ZnO and SnO_2 by increasing the electroactive surface area and improving antibody immobilization [3,14]. Additionally, materials such as $Ti_3C_2T_x$ MXene improve electron transfer rates, enabling rapid signal transduction required for real-time cortisol monitoring [13].

In contrast, estradiol sensing platforms utilize a broader range of materials, with the aim of improving analytical sensitivity, rather than mechanical integration. These materials include metal nanoparticles such as AuNPs and quantum dots utilized to improve electron transfer and catalytic activity for low detection limits [19,21,23]. These systems are not designed to be mechanically durable and are implemented in rigid electrode configurations, limiting suitability for flexible or wearable applications.

Development for estradiol detection is generally at a much earlier stage compared to cortisol detection, especially in wearable and real-sample applications. Most studies are performed under very controlled environments and use standard solutions or spiked samples. For example, studies on a Cu_2S-based immunosensor and a digital immunoassay for estradiol sensing are validated using standard solutions [19,20]. Similarly, a PEC-based immunosensor is validated with spiked environmental samples [23].

Only a few estradiol sensors have been validated in clinical samples. An older study done in 2012 about a SPCE-based immunosensor demonstrates a low limit of detection of 0.77 pg/mL in serum and urine [15], demonstrating that achieving high sensitivity for estradiol detection has been possible for quite some time. Similarly, a slightly more recent study from 2018 on a microfluidic paper-based immunosensor validates the sensor in serum while maintaining the potential for point-of-care monitoring [21]. Despite these advancements, wearable estradiol sensors for practical applications are in an early stage. Studies remain limited to controlled conditions and simplified samples.

While pg/mL-range LODs have been achieved for estradiol monitoring, similarly to cortisol sensing, the inherently low physiological concentrations of estradiol make reliable detection and distinction from background noise challenging. Reliable sensing requires detection limits well below the physiological concentrations to distinguish from background noise. A challenge also remains in designing sensors that work reliably in real-world applications, overcoming issues such as biofouling, signal shifting, and device calibration.

Alternative detection methods can achieve high sensitivity, such as a digital immunoassay that counts individual gold nanoparticles with dark-field microscopy. This study allows for estradiol detection at pg/mL levels, but has its own limitations [19]. While it eliminates background noise by observing single-molecule binding events, it is not compatible with miniaturization or real-time monitoring due to the need for complex machinery and sample preparation.

In addition to antibody-based systems, alternative recognition methods have been studied. For example, a CNF / CNT-based biosensor utilizes the enzyme laccase as a biorecognition element for rapid measurements while being low cost and without the need for complex preparation steps [22]. This method is simpler than immunosensors, since it does not rely on antigen-antibody interactions, but instead detects estradiol through an enzyme-catalyzed electrochemical reaction that generates the measured signal. However, compared to immunosensors, enzyme-based sensors exhibit lower selectivity and higher detection limits, as reflected by the relatively high LOD of 7.1×10^3 pg/mL in this study. While these sensors offer a simpler and cheaper alternative, they remain less established for hormone detection due to worse performance.

Overall, the comparison of cortisol and estradiol sensing platforms highlights how the performance of the devices is linked to material selection as well as application context. Cortisol sensors benefit from mechanically adaptable carbon-based platforms together with nanostructuring strategies to enable stable performance in sweat-based systems. In contrast, estradiol sensors are more focused on signal-amplifying materials to achieve high analytical sensitivity and are still demonstrated in rigid laboratory-based configurations. There is a clear separation in cortisol sensing systems that are optimized for real-time on-site monitoring, and estradiol sensing systems optimized for sensitive measurements in controlled conditions. While both cortisol and estradiol can be measured at low concentrations using electrochemical methods, only cortisol translates into wearable formats currently. This is largely due to challenges with sensitivity and selectivity with the ultra-low concentrations of estradiol, in comparison to cortisol.

6 Conclusions

This thesis reviewed electrochemical immunosensors for cortisol and estradiol detection, as well as reviewed the application of carbon-based materials in non-invasive and wearable sensing platforms. The analysis highlights that electrochemical sensor performance is primarily determined by three main factors: material selection, antibody immobilization strategy, and electrochemical transduction method.

Continuous hormone monitoring captures the complex data of dynamic hormonal patterns such as circadian rhythms in cortisol and cyclical variations in estradiol, which single measurements cannot obtain. The data of hormone fluctuations provides deeper insight into individual variability and deviations from normal physiological patterns, improving the understanding of endocrine function. Such data also supports personalized healthcare through more accurate diagnosis and individual hormone pattern analysis. Continuous hormone monitoring could also help understand the variability in female hormone cycles better, contributing to more inclusive healthcare.

Biosensors are analytical devices that convert biological recognition into measurable signals, while immunosensors use antibody-antigen interactions for selective analyte detection. The selectivity of immunosensors is a key advantage for hormone sensing, however cross-reactivity with structurally similar molecules remains a limitation. Sensor performance depends on several factors, such as electrode materials, immobilization strategies, and detection methods. Carbon-based nanomaterials such as graphene, CNTs, and CNFs are widely used in wearable immunosensors due to their mechanical flexibility for wearable applications, high surface area for antibody immobilization, and electrical conductivity for efficient electron transfer. Surface modification and nanostructuring with metal oxides or nanoparticles further enhance electrochemical activity and stability.

Antibody immobilization on the sensor surface is typically achieved through physical adsorption, covalent bonding, or affinity-based interactions, each based on different forces. These interactions influence antibody orientation, biological activity, and long-term stability of the biorecognition layer. Electrochemical detection such as CV, DPV, and EIS are particularly compatible with miniaturization and wearable formats, while

offering high sensitivity and low cost, making them suitable for continuous and real-time measurements.

Non-invasive and wearable sensing technologies are achieved through integrating selective biorecognition elements, such as antibodies, with flexible and durable electrochemical sensor platforms, operating in biofluids such as sweat, saliva or ISF. Reliable measurements are dependent on stable immobilization and active signal transduction. Currently, detection limits in systems for both cortisol and estradiol measurements are in the pg/mL range. This is generally sufficient for physiological monitoring for cortisol, which has concentrations of ng/mL. However, for estradiol which often exists at low pg/mL levels, sensors must achieve detection levels well below the physiological concentrations of the hormone for reliable detection in real samples. This is achievable for estradiol in controlled laboratory conditions, but translating the same performance to wearable formats remains a challenge.

From a materials and design perspective, carbon-based electrodes combined with nanostructuring is the most promising strategy for achieving wearable electrochemical immunosensors, as they balance mechanical durability with high electrochemical performance. Long-term stability is still limited by degradation of the immobilized antibodies and biofouling caused by biological matrices. Calibration drift, variability between users, as well as physiological conditions such as sweat rate also remain significant challenges for accurate hormone sensing.

Future strategies for non-invasive electrochemical immunosensors should prioritize robust surface modification methods for stable and correctly oriented immobilization of antibodies, strategies to combat biofouling such as antifouling coatings that allow for a reliable signal over extended use, and efficient calibration approaches such as algorithm-based referencing and correction on the device. Overall, wearable cortisol sensing with electrochemical carbon-based immunosensors is approaching practical applicability, while estradiol sensing still requires advances in sensitivity, and integrating those advancements with platforms suitable for wearable applications through the integration of carbon nanomaterials for example. Ultimately, advancing non-invasive electrochemical immunosensors for hormones such as cortisol and

estradiol and interpreting the data correctly is a crucial step towards addressing gaps in hormone research, which would enable more accurate, continuous, and personalized monitoring of female physiology in clinical and everyday settings.

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