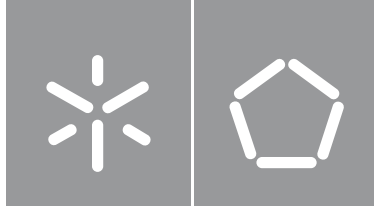


Universidade do Minho
Escola de Engenharia

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Carbon based cortisol sensor for chronic stress assessment



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**Carbon based cortisol sensor for
chronic stress assessment**

Doctoral thesis in
Materials Engineering

Work accomplished under the supervision of
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I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

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University of Minho, Guimarães, July 2026

Ana Raquel Ribeiro dos Santos

Abstract

Chronic stress has become a major global health challenge, highlighting the need for low-cost and portable tools capable of continuously monitoring cortisol, its main biological marker. This thesis focuses on the development of non-affinity electrochemical sensors based on screen-printed carbon electrodes (SPCEs), following a simple, material-driven approach that avoids the complexity of antibody- or aptamer-based systems.

Covalent functionalization of multi-walled carbon nanotubes with anhydride groups (MWCNT-ANI) was initially explored. thermogravimetric analysis (TGA) and x-ray photoelectron spectroscopy (XPS) confirmed successful modification, although the electrochemical response to cortisol remained limited.

A pyrene derivative (PY) bearing amide and carboxylic groups was subsequently investigated to promote interactions with cortisol while leveraging pyrene's strong affinity for carbon surfaces to enable straightforward SPCE modification. Two approaches were implemented to develop the PY-based platform. In the first approach, a hydroalcoholic solvent system was used, resulting in a sensor with a detection range from 3 to 500 nM, a limit of detection of 3 nM and a sensitivity of 2.21 $\mu\text{A}/\text{dec}$. Molecular dynamics (MD) and density functional theory (DFT) simulations were used to understand the sensing mechanism. The results showed that cortisol interacts with the PY layer via multiple hydrogen bonds, stabilizing the molecule and decreasing its intrinsic reduction peak at -1.1 V . In the second stage, an alkaline PY aqueous solution ($\text{pH} > 7$) was used to modify the electrode, resulting in improved surface uniformity and droplet confinement. This led to a clear improvement in analytical performance, with a sensitivity of 4.36 $\mu\text{A}/\text{dec}$. Hybrid carbon systems were also explored by combining PY with multi-walled carbon nanotubes (MWCNTs) and carbon black (CB). The PY/CB configuration reached a sensitivity of 4.13 $\mu\text{A}/\text{dec}$, supported by its high electroactive surface area and efficient electron transfer.

This work demonstrates that controlling the surface chemistry and morphology of carbon-based interfaces enables detection of cortisol at nanomolar levels using a straightforward, scalable fabrication process. These results support the development of low-cost wearable devices for real-time stress monitoring.

Keywords Carbon nanomaterials, Cortisol, Molecular modeling, Non-affinity electrochemical sensing, Pyrene derivatives.

Resumo

O stress crónico é um desafio de saúde global, evidenciando a necessidade de ferramentas de baixo custo e portáteis capazes de monitorizar continuamente o cortisol, o seu principal marcador biológico. Esta tese centra-se no desenvolvimento de sensores eletroquímicos não baseados em reconhecimento por afinidade, assentes em elétrodos de carbono impressos (SPCEs), com base numa abordagem simples centrada nos materiais, evitando a complexidade de sistemas com anticorpos ou aptâmeros.

A funcionalização covalente de nanotubos de carbono de paredes múltiplas com grupos de anidrido (MWCNT-ANI) foi inicialmente explorada. As análises por termogravimetria (TGA) e espectroscopia de fotoeletrões por raios X (XPS) confirmaram a modificação bem-sucedida, embora a resposta eletroquímica ao cortisol tenha sido limitada.

Posteriormente, foi estudado um derivado de pireno (PY) funcionalizado com grupos amida e carboxílicos, com o objetivo de promover interações com o cortisol, aproveitando a forte afinidade do pireno por superfícies de carbono, o que permite uma modificação simples dos SPCEs. Foram implementadas duas abordagens. Na primeira, utilizou-se um sistema hidroalcoólico, obtendo-se um sensor com intervalo de deteção de 3 a 500 nM, limite de deteção de 3 nM e sensibilidade de $2,21 \mu\text{A}/\text{dec}$. Simulações de dinâmica molecular e teoria do funcional da densidade foram utilizadas para compreender o mecanismo de deteção. Verificou-se que o cortisol interage com o PY através de múltiplas ligações de hidrogénio, estabilizando o PY e diminuindo o seu pico de redução ($-1,1 \text{ V}$). Numa segunda fase, a modificação do elétrodo foi realizada com uma solução aquosa alcalina de PY ($\text{pH} > 7$), resultando numa maior uniformidade da superfície e confinamento das gotas. Esta abordagem conduziu a uma melhoria do desempenho analítico, com uma sensibilidade de $4,36 \mu\text{A}/\text{dec}$. Foram também explorados sistemas híbridos de carbono, combinando PY com MWCNTs e com carbon black. A configuração com PY/carbon black apresentou sensibilidade de $4,13 \mu\text{A}/\text{dec}$, devido à elevada área eletroativa e à eficiente transferência de eletrões.

Este trabalho demonstra que o controlo da química e morfologia de interfaces baseadas em carbono permite a deteção de cortisol a níveis nanomolares através de um processo de fabrico simples e escalável, sustentando o desenvolvimento de dispositivos vestíveis de baixo custo para monitorização do stress.

Palavras-chave Cortisol, derivados de pireno, deteção eletroquímica não baseada em afinidade, modelação molecular, nanomateriais de carbono.

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Acronyms

3-MPA	3-mercaptopropionic acid
Ab	antibody
AC	alternating current
ACTH	adrenocorticotrophic hormone
AFM	atomic force microscopy
Ag/AgO NPs	silver/silver oxide nanoparticles
ALP	alkaline phosphatase
ANI	anhydride groups
Apt	aptamer
ATPES	3-aminopropyltriethoxysilane
ATR	attenuated total reflectance
AuND-LEG	gold nanodendrite-decorated laser-engraved graphene
AuNP	gold nanoparticle
B3LYP	Becke, 3-parameter, Lee-Yang-Parr
BE	binding energy
CA	chronoamperometry
CAR	cortisol awakening response
CB	carbon black
CC	chronocoulometry
CCY	conductive carbon yarn
CE	carbon counter electrode
CFME	carbon fiber microelectrode
CHELPG	charges from electrostatic potentials using a grid based method
CMA	4-carboxymethylaniline
CNC	cellulose nanocrystals
CNT	carbon nanotubes
CORT	cortisol

CRH	corticotropin-releasing hormone
CRP	C-reactive protein
Cu-BTC	copper benzene-1,3,5-tricarboxylate
CuPcTS	copper tetrasulfonated phthalocyanine
CV	cyclic voltammetry
D3BJ	Grimme's D3 dispersion with Becke–Johnson damping
DFT	density functional theory
DPV	differential pulse voltammetry
DSP	dithiobis (succinimidyl propionate)
DTSP	dithiobis(succinimidylpropionate)
e-RGO	electroreduced graphene oxide
EDL	electrical double-layer
EIS	electrochemical impedance spectroscopy
ELISA	enzyme-linked immunosorbent assay
ERGO	electroreduced graphene oxide
FSCV	fast-scan cyclic voltammetry
FTIR	fourier transform infrared
G-PANI	graphene-polyaniline
GCE	glassy carbon electrode
GF	graphene foam
GO	graphene oxide
GQD	graphene quantum dot
GR	glucocorticoid receptor
HPA	hypothalamic–pituitary–adrenal
HRP	horseradish peroxidase
IDE	interdigitated electrode
ITO	indium tin oxide
LIG	laser-induced graphene
LIG-IDE	laser-induced graphene interdigitated electrode

LOD	limit of detection
LSV	linear-sweep voltammetry
MB	methylene blue
MD	molecular dynamics
MIP	molecularly imprinted polymer
MOF	metal-organic framework
MWCNT	multi-walled carbon nanotube
MWCNT-ANI	multi-walled carbon nanotubes functionalized with anhydride groups
MWCNT-ANI _{lp}	MWCNT functionalized in the liquid phase
MWCNT-ANI _{vp}	MWCNT functionalized in the gas phase
MWCNT-Cu-PP	multi-walled carbon nanotubes–copper(II) porphyrin–polypyrrole nanocomposite
nF	non-Faradaic
nF-EIS	non-Faradaic electrochemical impedance spectroscopy
NP	nanoparticle
NPT	isothermal-isobaric
NVT	canonical
o-PD	o-phenylenediamine
OECT	organic electrochemical transistor
PANI	polyaniline
PB	Prussian Blue
PBASE-NHS	1-pyrenebutyric acid N-hydroxysuccinimide ester
PBS	phosphate-buffered saline
PCA	1-pyrene carboxylic acid
PDMS	polydimethylsiloxane
PEDOT	poly(3,4-ethylenedioxythiophene)
PEDOT:PSS	poly(3,4-ethylenedioxythiophene):poly(styrene sulfonate)
PET	polyethylene terephthalate
PGE	pencil graphite electrode
PI	polyimide
POC	point-of-care
poly-o-PD	poly(o-phenylenediamine)

PPA	1H-pyrrole propionic acid
PPAuNPs	polyaniline-protected gold nanoparticles
PPPM	particle-particle particle-mesh
PPy	polypyrrole
PSS	poly(styrene sulfonate)
PTFE	polytetrafluoroethylene
PY	pyrene derivative
PyTS	pyrene-tetrasulfonic acid sodium salt
RE	reference electrode
RuPEG	ruthenium complex bearing pyrene chains
S-G	sulfur-doped graphene
SA	sodium alginate
SDS	sodium dodecyl sulfate
SEM	scanning electron microscopy
SPCE	screen-printed carbon electrode
SPGE	screen-printed graphene electrode
SWV	square-wave voltammetry
TGA	thermogravimetric analysis
UV-Vis	ultraviolet–visible spectroscopy
VMD	visual molecular dynamics
WE	working electrode
WHO	World Health Organization
XPS	x-ray photoelectron spectroscopy

List of Symbols

A	absorbance
I_p	peak current intensity
M_i	molar mass of component i
N_A	Avogadro's number
N_i	number of molecules of component i
R	correlation coefficient
R^2	coefficient of determination
ΔA	change in the chronocoulometric signal A
ΔE	interaction energy (molecular dynamics simulations)
ΔR	change in resistance
ΔZ	change in impedance
$\Delta Z $	change in magnitude of impedance
ϵ	molar extinction coefficient
ν	scan rate
ω_i	mass fraction of component i
$\pi-\pi$	$\pi-\pi$ stacking interactions between aromatic rings (e.g., pyrene and carbon surfaces)
ρ_i	density of component i
ρ_{mix}	mixture density
ρ_{sim}	simulated density
c	concentration

$h\nu$ photon energy (where h is Planck's constant and ν is frequency)

l optical path length

m_i mass of a single molecule of component i

Chapter 1

Introduction

1.1 Motivation

According to the World Health Organization, chronic workplace stress has emerged as the epidemic of the 21st century [1, 2], leading to burnout syndrome, affecting millions of people all around the world. The sudden emergence of the COVID-19 pandemic has contributed significantly to this health crisis [3–5] and has also highlighted the importance of mental health.

Chronic stress has wide-ranging effects, impacting both physiological and psychological functions. These include hypertension, diabetes, arthritis, inflammatory diseases, and an increased risk of cardiovascular disorders, as well as anxiety, depression, decreased productivity, concentration, and decision-making ability [6]. The impact is particularly pronounced in high-demand, high-risk professions, including healthcare workers, surgeons, air traffic controllers, pilots, police officers, military personnel, and executives. Governments and companies incur substantial costs associated with healthcare treatments, sickness absence, and absenteeism, ranging from billions of euros annually in some countries [7, 8], while indirect costs associated with presenteeism and reduced productivity are incalculable.

This highlights the need for continuous monitoring of stress through measurable physiological indicators. These include vital signs, such as blood pressure and heart rate, as well as biochemical markers, particularly stress-related hormones such as serotonin, epinephrine, and cortisol. Among these, cortisol is widely recognized as a key biomarker of chronic stress, reflected in sustained elevations and a flattened diurnal rhythm [9–13].

Conventional methods for cortisol detection, such as enzyme-linked immunosorbent assay (ELISA), typically require complex instrumentation, skilled personnel, and time-consuming, costly procedures. The measurement of cortisol in non-invasive biofluids such as saliva and sweat represents a promising alternative for continuous monitoring. Electrochemical sensors have emerged as powerful tools in POC diagnostics due to their sensitivity, portability, and rapid response. Numerous electrode materials, designs, and detection formats have been reported. However, several limitations persist, including the reliance on complex biorecognition elements, complex fabrication processes, and limited scalability.

The development of material-based electrochemical sensors offers a promising low-cost approach for real-time monitoring of cortisol. These systems exploit the intrinsic properties of functional materials, including electrocatalytic or electrochemical activity, efficient electron transfer, and tunable surface chemistry. Among them, pyrene derivatives are particularly attractive, as they can be immobilized on electrode surfaces via simple π – π stacking with carbon-based materials, such as graphene or carbon nanotubes. Chemical modification of pyrene enables the introduction of functional groups that specifically interact

with target analytes, including cortisol, combining straightforward surface functionalization with molecular selectivity.

1.2 Objectives

This work aims to develop a PY-based electrochemical sensor for cortisol detection, using a cost-effective, straightforward approach that avoids the use of expensive biorecognition elements and complex chemical functionalization.

Therefore, several objectives were established. Preparation and characterization of PY-modified electrodes, followed by the evaluation of their electrochemical response toward cortisol detection. A key focus of this work is the optimization of experimental conditions that affect sensor performance, including PY concentration, solvent, drop-casting parameters, SPCE pre-treatment, incubation time, and electrochemical measurement settings (potential window, CV, and DPV).

In addition, the analytical performance of the developed sensor will be evaluated for sensitivity, linear range, and limit of detection. The interaction mechanisms between cortisol and the PY-modified surface will also be investigated through theoretical approaches, including MD and DFT simulations, to elucidate the fundamental sensing mechanism.

Finally, PY-modified electrodes further modified with carbon-based materials, such as MWCNTs and CB, will be developed and characterized. Multi-walled carbon nanotubes functionalized with reactive anhydride groups (MWCNT-ANI) will also be prepared and evaluated as alternative sensing platforms. Finally, the performance of the optimized sensor will be assessed in artificial sweat and benchmarked against state-of-the-art cortisol sensing systems.

1.3 Thesis framework

The thesis follows a materials-oriented progression (Figure 1.1), moving from the study of functionalized MWCNTs to the development of hybrid carbon interfaces. The work begins with the investigation of multi-walled carbon nanotubes functionalized with anhydride groups (MWCNT-ANI) as an initial sensing platform. It then advances to the systematic development of PY interfaces, with particular attention to morphological control via solvent selection and molecular-level validation using computational methods. In a final stage, the PY is integrated into hybrid carbon architectures by combining it with either CB or MWCNTs, to improve analytical performance and establish a consistent framework for non-affinity cortisol sensing.

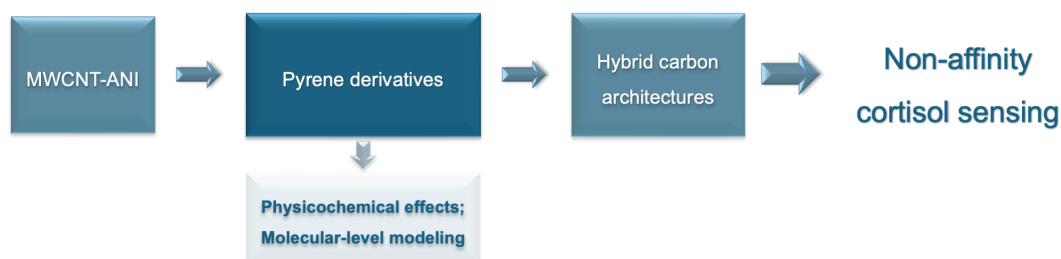


Figure 1.1. Overview of the thesis framework and materials progression.

1.4 Thesis outline

This thesis is organized into six chapters.

Chapter 1 introduces the motivation for this research, outlines its objectives, and presents the structure of the thesis along with the author's contributions.

Chapter 2 provides a comprehensive overview of the state of the art, including the biological role of cortisol, current methods for its detection, and relevant studies reported in the literature.

Chapter 3 presents an exploratory study on carbon nanotubes covalently functionalized with anhydride groups for electrochemical cortisol sensing, investigating their potential as a sensing platform.

Chapter 4 focuses on the development of PY-based electrochemical sensors for cortisol detection and is divided into three main parts. The first part describes the initial fabrication of pyrene modified electrodes, their evaluation for cortisol sensing, and MD analyses to provide insights into the molecular interactions between pyrene derivatives and cortisol. The second part investigates alternative solvent systems to improve pyrene deposition on the electrode surface and systematically examines other modification parameters, including applied potentials and incubation conditions, to enhance the sensor's electrochemical response. The third part presents the analytical evaluation of the optimized sensors, including sensitivity, low-concentration detection, and further molecular-level insights (DFT calculations) into the interactions between pyrene and cortisol.

Chapter 5 explores strategies to enhance pyrene-based sensors using additional carbon-based materials, aiming to improve their electrochemical response and reproducibility.

Finally, Chapter 6 summarizes the main findings, highlights the contributions of this work, and discusses future research directions for the development of low-cost, scalable cortisol-sensing platforms.

1.5 Author contributions

1.5.1 International journal publications

Santos, A. R., Bento, F., Faria, B., Paiva, M. C., Silva, A. F., Viana, A. S., & Viana, J. C. "Pyrene derivative-based electrode for biomarker detection". (Submitted to Small Methods - under review)

Santos, A. R., Bento, F., Paiva, M. C., Silva, A. F., & Viana, J. C. "Explorative study of carbon nanotubes covalently functionalized with anhydride groups for electrochemical cortisol Sensing". (in preparation)

Santos, A. R., Bento, F., Faria, B., Paiva, M. C., Silva, A. F., & Viana, J. C. "Pyrene derivative-based electrode for cortisol detection". (in preparation)

Santos, A. R., Bento, F., Paiva, M. C., Silva, A. F., & Viana, J. C. "Aqueous pyrene modification of carbon electrodes for cortisol sensing". (in preparation)

Santos, A. R., Bento, F., Paiva, M. C., Silva, A. F., & Viana, J. C. "Hybrid carbon-based SPCE/PY Electrodes for cortisol electrochemical Sensing". (in preparation)

1.5.2 International conferences

1.5.2.1 Panel communications

Santos, A. R., Bento, F., Paiva, M. C., Silva, A. F., & Viana, J. C. "Desenvolvimento de um sensor de cortisol baseado em elétrodos impressos com nanotubos de carbono modificados com um derivado de pireno", XXVI SIBAE Congress 2024, Faculdade de Ciências da Universidade de Lisboa, Lisbon, Portugal, 19–23 May 2024.

Santos, A. R., Bento, F., Faria, B., Paiva, M. C., Silva, A. F., & Viana, J. C. "Electrochemical detection of cortisol using carbon nanotubes (CNT) modified with a pyrene derivative", EUROSENSORS XXXVII, Politechnika Wroclawska, Wroclaw, Poland, 7–10 September 2025.

Chapter 2

State of the art

2.1 Cortisol biological role

Cortisol is a glucocorticoid hormone produced by the adrenal cortex and is essential for maintaining biochemical and physiological homeostasis. Its secretion is controlled by the hypothalamic–pituitary–adrenal (HPA) axis, where corticotropin-releasing hormone (CRH) from the hypothalamus stimulates the release of adrenocorticotropic hormone (ACTH) from the pituitary, which in turn triggers the synthesis of cortisol in the adrenal gland and its secretion into the bloodstream [14] (Figure 2.1). Due to its lipophilic nature, cortisol readily crosses cell membranes and binds to intracellular glucocorticoid receptors (GRs). Once activated, these receptors translocate to the nucleus and regulate gene transcription, producing slow but long-lasting genomic effects that shape metabolic, immune, and homeostatic functions. In parallel, cortisol can act through rapid non-genomic mechanisms involving membrane-associated receptors and intracellular signaling cascades. Operating on timescales of seconds to minutes, these pathways enable rapid physiological adjustments, including modulation of immune cell activity, vascular tone, and short-term stress responses. Together, genomic and non-genomic mechanisms enable cortisol to coordinate responses across multiple temporal scales, supporting effective homeostatic regulation [15, 16].

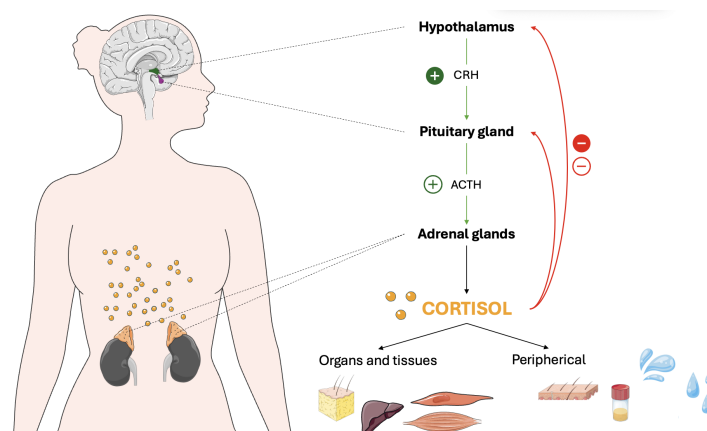


Figure 2.1. Schematics of cortisol secretion.

Given its regulatory role, cortisol is a key mediator of physiological adaptation to stress. When an individual is exposed to an acute stressor, defined as a real or perceived threat to homeostasis, whether physiological or psychological, the HPA axis transitions from its circadian basal state to an emergency response mode. This activation leads to a rapid and robust increase in cortisol secretion, promoting the mobilization of energy substrates, enhancing cardiovascular function, and reallocating metabolic and immune resources to support an effective response to the stressor. This acute cortisol surge is inherently adaptive and essential for short-term survival [17, 18]. However, the stress response system is optimized for transient activation. When stressors are persistent or repeatedly encountered, prolonged HPA axis

activation can lead to dysregulation.

2.2 Cortisol dysregulation

In healthy individuals, the physiological levels of cortisol present in sweat typically range from 8 to 140 ng/mL [19]. Persistent overactivation or dysfunction of the HPA axis can lead to sustained alterations in cortisol signaling, shifting its role from adaptive to maladaptive. Such dysregulation may result in either insufficient or excessive cortisol levels, both of which are associated with adverse physiological outcomes [13]. At the extreme ends of this spectrum, cortisol dysregulation is observed in endocrine disorders such as Addison's disease and Cushing's syndrome. Addison's disease, commonly caused by autoimmune destruction of the adrenal cortex, is characterized by chronic cortisol deficiency, leading to fatigue, hypotension, impaired stress tolerance, and, in severe cases, life-threatening adrenal crisis [20]. Cushing's syndrome results from prolonged cortisol excess due to ACTH-dependent or independent mechanisms [21, 22], and it is associated with, e.g., obesity, hypertension, mood alterations, and cognitive impairment. Although these conditions illustrate the pathological consequences of sustained cortisol imbalance, they are relatively rare and do not reflect the more subtle, yet widespread patterns of cortisol dysregulation associated with chronic psychological stress.

2.2.1 Chronic psychological stress

Chronic stress arises from sustained exposure to cumulative, persistent, and often uncontrollable stressors. In contrast to the tightly regulated and time-limited cortisol response observed during acute stress, prolonged demands on the HPA axis result in persistently elevated cortisol secretion and insufficient suppression, reflecting a state of chronic physiological alertness. This continuous activation alters cortisol signaling not only in magnitude, but critically, in its temporal organization. With continued or repeated stress exposure, cortisol dysregulation frequently progresses to a second stage characterized by loss of normal circadian regulation [18, 23]. Rather than a further linear increase in cortisol levels, the predominant alteration becomes a flattening of the diurnal cortisol rhythm, characterized by reduced day–night levels amplitude and diminished flexibility of HPA axis signaling [13]. It is commonly characterized by inappropriately elevated cortisol levels in the late afternoon or evening, when levels should typically decline, often accompanied by attenuation of the morning peak or the cortisol awakening response (CAR). This pattern indicates impaired physiological recovery and is widely regarded as a biological marker of allostatic load, reflecting the cumulative burden of prolonged stress exposure.

This disruption of cortisol rhythmicity has consequences that extend well beyond endocrine regulation, giving rise to a constellation of systemic effects that reflect the integrated nature of the stress response [24]. Altered cortisol signaling under chronic stress is consistently associated with increased cardiometabolic vulnerability, including impaired glucose regulation [25], insulin resistance, visceral adiposity, hypertension, dyslipidemia, and elevated risk of cardiovascular disease, contributing to increased all-cause and cardiovascular mortality. In parallel, chronic stress is accompanied by persistent immune imbalance and low-grade systemic inflammation [26], which further exacerbates vascular dysfunction and metabolic deterioration.

Because stress-related hormonal and inflammatory signals act on both peripheral tissues and the central nervous system, systemic and neuropsychological consequences are inherently interconnected [17, 27]. Chronic stress is associated with neuroinflammatory processes and functional and structural alterations in stress-sensitive brain regions [28, 29], including the hippocampus, amygdala, and prefrontal cortex. These neural changes manifest clinically as cognitive impairments in memory, attention, and executive function, alongside affective disturbances such as anxiety, depression, sleep disruption, chronic fatigue, and heightened pain sensitivity [7]. Together, these interrelated effects illustrate how prolonged disruption of cortisol regulation translates into progressive loss of physiological resilience and increased vulnerability to chronic non-communicable and neurodegenerative diseases [18]. Table 2.1 summarises the primary physiological functions of cortisol and the consequences of its dysregulation.

Table 2.1. Physiological roles of cortisol and their dysregulation in chronic stress.

Physiological Function	Underlying Cortisol Mechanisms	Manifestations in Chronic Stress	Primary Cause (Cortisol / HPA Axis)
Blood pressure regulation	Enhances vascular responsiveness to catecholamines and contributes to maintenance of vascular tone.	Persistent hypertension, atherosclerosis, increased cardiovascular and mortality risk.	Sustained elevated cortisol levels; flattened diurnal cortisol profile.
Immune and inflammatory modulation	Suppresses pro-inflammatory cytokine production, limits leukocyte activation, and prevents excessive immune responses.	Chronic low-grade inflammation, increased C-reactive protein (CRP).	Glucocorticoid receptor (GR) resistance; loss of normal anti-inflammatory suppression.
Energy metabolism and body weight regulation	Stimulates hepatic gluconeogenesis, reduces peripheral glucose uptake, and promotes protein catabolism and lipolysis.	Insulin resistance, type 2 diabetes mellitus, visceral obesity, muscle wasting.	Chronic hypercortisolism; increased gluconeogenesis and inhibition of insulin action.
Circadian rhythm synchronization	Follows a diurnal secretion pattern with a morning peak and nocturnal decline, supporting sleep–wake cycles.	Sleep disturbances, chronic fatigue, cognitive dysfunction, reduced stress adaptability.	Dysregulation of dynamic cortisol rhythm (CDR); flattened diurnal slope.
Stress response integration	Mobilizes metabolic reserves and modulates immune, cardiovascular, cognitive, and emotional responses during stress.	Impaired adaptive stress responses; physiological exhaustion.	Prolonged dysregulation of the HPA axis (hyper- or hypoactivity).

Cont.

Physiological Function	Underlying Cortisol Mechanisms	Manifestations in Chronic Stress	Primary Cause (Cortisol / HPA Axis)
Neurocognitive function	Acts on the hippocampus and prefrontal cortex, regulating memory, attention, and executive function.	Memory impairment, attentional deficits, executive dysfunction; hippocampal atrophy.	Neurotoxic effects of chronic hypercortisolism; loss of cortisol rhythmicity.
Psychological and emotional regulation	Modulates limbic system activity and neurotransmitter systems involved in mood regulation.	Depression, anxiety disorders, post-traumatic stress disorder (PTSD).	HPA axis dysregulation associated with neuroinflammation and chronic stress exposure.

In clinical practice, cortisol is commonly quantified using immunoassay-based methods, particularly enzyme-linked immunosorbent assay (ELISA), applied to blood, saliva, or urine samples. Although widely used, these techniques have significant limitations that make them unsuitable for stress assessment, particularly in chronic contexts [10, 30]. ELISA relies on discrete sampling and provides only a snapshot of cortisol concentration, failing to capture its pronounced circadian and ultradian variability. Given the short biological half-life of cortisol, single or infrequent measurements reflect only recent HPA axis activity and do not represent cumulative hormonal exposure associated with chronic stress. Moreover, immunoassays are highly sensitive to pre-analytical factors, including sampling time, acute stressors, and handling conditions, which can introduce substantial variability. While analytically robust, ELISA assays are not inherently low-cost when reagents, laboratory infrastructure, trained personnel, and turnaround time are considered,

limiting their feasibility for frequent or real-time monitoring [9].

In this context, the development of biosensing technologies, particularly electrochemical sensors, represents a promising approach to overcoming the limitations of conventional assays. Such sensors offer the potential for real-time monitoring, improved temporal resolution, and integration into wearable or point-of-care (POC) systems, thereby enabling a more accurate assessment of chronic stress physiology [9, 31]. These advances underpin the next chapter, which focuses on electrochemical strategies for cortisol detection and their application to long-term stress monitoring.

2.3 Electrochemical sensors for cortisol detection

Cortisol poses intrinsic challenges for direct detection due to its small molecular size, neutral charge, and limited electroactivity under mild aqueous conditions. As a result, most electrochemical sensors do not rely on the direct oxidation or reduction of cortisol. The sensor's analytical performance depends on the design of the sensing interface, i.e., the recognition element, the transduction mechanism, and the material architecture.

High selectivity and sensitivity are typically achieved by functionalizing the electrode with a recognition layer that selectively binds to cortisol and converts this interaction into an electrochemically detectable signal. Sensing strategies can be broadly divided into affinity-based approaches employing recognition elements such as antibodies, aptamers, and molecularly imprinted polymers (MIPs), and non-affinity-based approaches that rely on the interaction of cortisol with functional electrode materials.

In antibody- and aptamer-based sensors (Figure 2.2a), cortisol binding occurs through highly specific molecular interactions [32–35]. Signal transduction is generally label-free and occurs through binding-induced perturbations at the electrode–electrolyte interface, such as steric hindrance, reduced accessibility of solution-phase redox probes, changes in interfacial capacitance, or modulation of charge-transfer resistance.

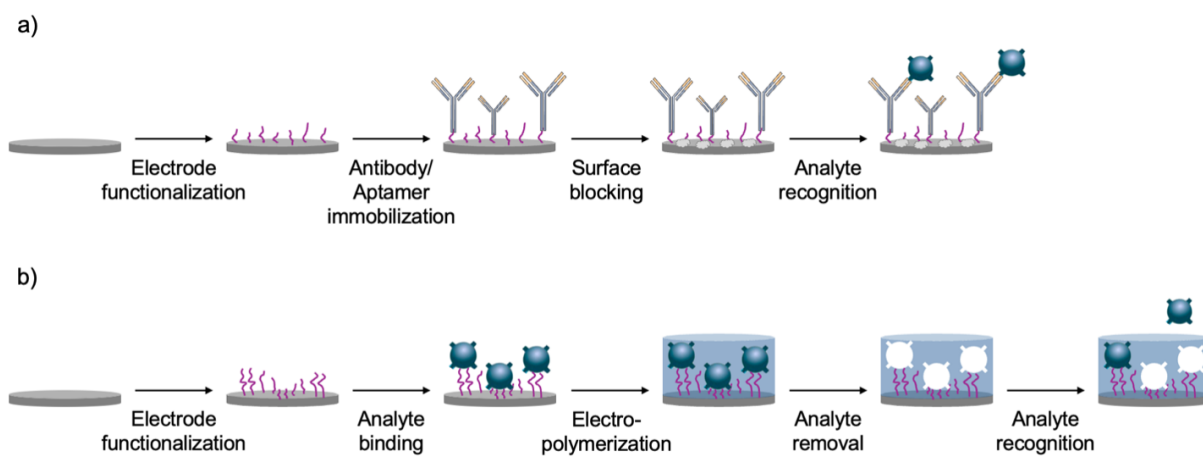


Figure 2.2. Schematics of the production of electrochemical immune (a) and MIP (b)-based sensors.

MIPs represent another class of synthetic recognition elements in which molecular selectivity is embedded within the sensing material itself [36]. Template-shaped cavities formed during polymerization in the presence of cortisol (Figure 2.2b) give rise to robust, receptor-integrated architectures, in which cortisol binding directly alters the electrochemical properties of the polymer matrix. Signal transduction is frequently mediated by probe-based signal-off mechanisms that can employ either external or built-in redox probes. In these built-in systems, the redox-active species is embedded within the imprinted polymer matrix and serves as an internal signal transducer. Cortisol binding partially blocks electron transfer between the internal redox mediator and the electrode surface, resulting in a concentration-dependent signal-off response.

Depending on how cortisol binding is transduced into a measurable signal, affinity-based electrochemical sensors can be classified into direct, competitive, or sandwich formats (Figure 2.3). In direct formats, cortisol binding at the electrode interface induces a proportional change in the electrochemical signal, yielding signal-on or signal-off responses. Competitive formats rely on competition between free cortisol and a labeled cortisol analogue for a limited number of binding sites, resulting in an inverse signal. Sandwich formats employ two recognition elements to capture cortisol, forming a complex that enhances selectivity and signal intensity. However, this strategy typically involves additional assay steps, which can increase assay complexity and analysis time. These formats often incorporate redox-active probes, either external or built-in, to facilitate electron transfer at the electrode interface and enable signal amplification, improving sensitivity and signal-to-noise ratios [35, 37, 38].

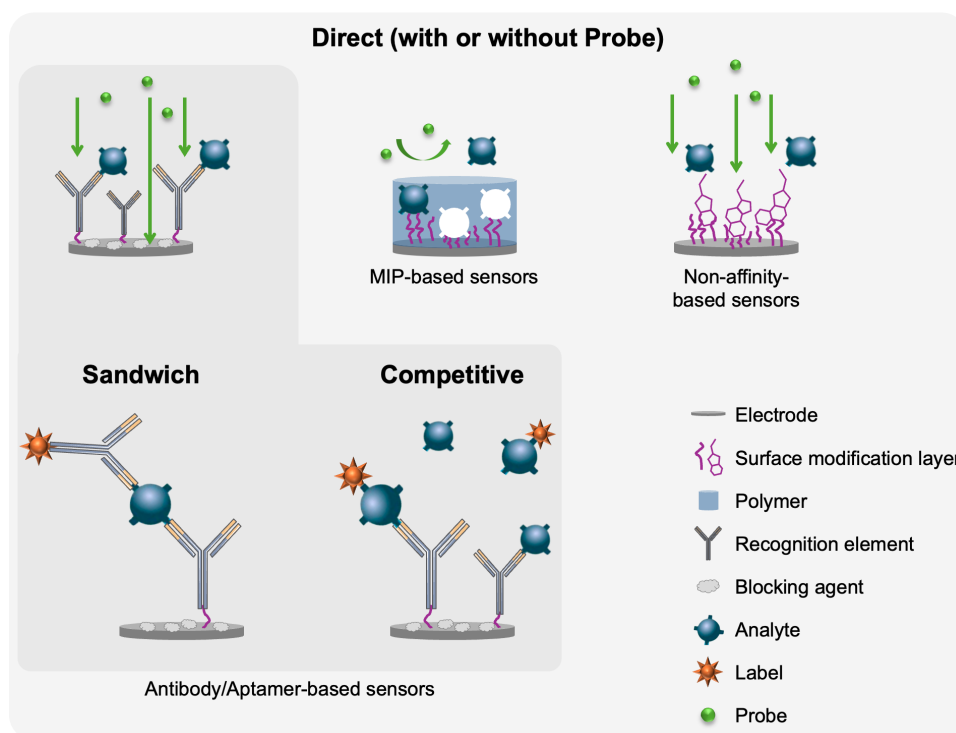


Figure 2.3. Schematics of the electrochemical detection formats: direct (with or without probe/mediator), sandwich, and competitive.

The electrochemical signal changes induced by cortisol binding at the electrode interface can be detected and quantified using a range of electroanalytical techniques. Electrochemical methods, such as chronoamperometry (CA), cyclic voltammetry (CV), differential pulse voltammetry (DPV), and electrochemical impedance spectroscopy (EIS), are commonly employed in POC cortisol biosensing applications due to their rapid response, low cost, high sensitivity, and compatibility with miniaturized systems. These techniques enable the identification and quantification of analytes in electrochemical cells by measuring current, potential, or impedance. Controlled voltage waveforms induce analyte-specific redox reactions at the sensor surface, producing characteristic electrical signals [37, 39, 40]. In CA, a constant potential is applied to the working electrode, generating a time-dependent current as the analyte concentration near the electrode's interface decreases (Figure 2.4a). Its rapid response and simple instrumentation are well-suited to low-cost POC platforms, although its lower sensitivity limits its performance relative to pulsed techniques [37]. CV involves sweeping the electrode potential between defined limits while monitoring the resulting current arising from surface-confined and diffusion-controlled redox processes. The resulting voltammogram (Figure 2.4b) provides insight into the electrochemical behavior of analytes and interfacial reaction mechanisms [41]. DPV is a pulse technique in which small potential steps are superimposed onto a slowly varying base potential, inducing analyte redox reactions at the electrode surface (Figure 2.4c). The differential current measured before and after each pulse enhances faradaic responses

while minimizing capacitive effects [42, 43], improving sensitivity, peak resolution, and detection limits. In EIS (Figure 2.4d), an alternating current (AC) potential is applied over a range of frequencies, and the resulting impedance of the electrochemical system is measured to evaluate interfacial processes [44]. Variations in charge-transfer resistance and interfacial capacitance associated with target binding at the electrode surface enable high sensitivity, although at the expense of longer acquisition times and increased instrumental complexity.

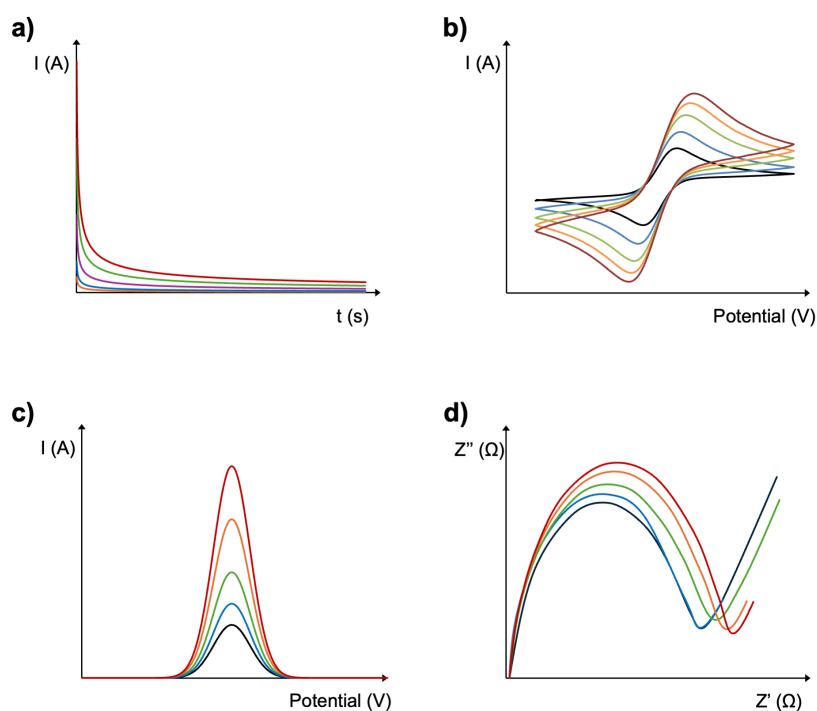


Figure 2.4. Typical responses obtained from standard electrochemical techniques used in POC: a) CV, b) DPV, c) EIS, and d) CA.

The performance of the electroanalytical response depends on the recognition strategy, transduction mechanism, and the materials employed.

Table 2.2 summarizes reported electrochemical strategies for cortisol detection, organized by recognition element type: antibody, aptamer, MIPs, and non-affinity-based. The table provides an overview of the different detection formats, signal transduction mechanisms, and material architectures used in each case, highlighting trends in sensor design and the evolving role of materials in enhancing performance.

Table 2.2. Reported non-invasive electrochemical cortisol sensors categorized by recognition element, electrode material, and surface modification, detection format, electrochemical technique, sensitivity, limit of detection (LOD), and concentration range.

Recognition element	Electrode Material	Surface modification	Electro-chemical method	Detection Format	Sensitivity	LOD	Range of concentration	Fluid	Ref
Antibody	LIG-IDE	AuNPs, 3-MPA, EDC/NHS	nF-EIS	Direct (Label-free / nF-EIS)	0.67 % ΔZ /dec (PBS) 0.82 % ΔZ /dec (Artificial sweat) [†]	0.0085 nM (PBS) 0.0108 nM (Synthetic sweat)	0.1 pM - 100 nM	PBS, Artificial sweat	[45]
	Au on porous polyamide	DSP thiol linker	nF-EIS	Direct (Label-free / nF-EIS)	8 – 28 % $\Delta Z $ [†]	8 ng/mL (range start)	8 - 140 ng/mL	Artificial sweat, Human sweat (validation)	[46]
	ITO coated glass	PEDOT:PSS with hydroxyl functional groups, ATPES linker	nF-EIS	Direct (Label-free / nF-EIS)	0.31 Ω / (ng/mL) [†]	—	50 - 200 ng/mL	Buffer (PBS)	[47]
	Nanoporous polyamide	ZnO, DSP crosslinker	nF-EIS	Direct (Label-free / nF-EIS)	19.6 – 48.6 % ΔZ (Artificial sweat) 10 – 42 % ΔZ (Human sweat)	1 pg/mL (Artificial Sweat) 1 ng/mL (Human sweat)	1 – 100 pg/mL (Artificial Sweat) 1 – 200 ng/mL (Human sweat)	Artificial sweat, Human sweat (validation)	[48]
	Pd	MoS ₂ , DSP crosslinker	nF-EIS	Direct (Label-free / nF-EIS)	10 – 42% $\Delta Z $ [†]	1 ng/mL	1 – 500 ng/mL	Human Sweat	[49]
	OECT / PEDOT:PSS	3-MPA	OECT	Direct (Label-free / OECT)	50 μ A/dec (PBS)	100 pM	1 - 1000 nM	PBS, Human sweat (validation)	[50]
	CCY	Fe ₂ O ₃ , hematite	CV, DPV	Direct (Label-free)	3.886 μ A/dec (CV) 2.133 μ A/dec (DPV) [†]	0.005 fg/mL (CV) 0.003 fg/mL (DPV)	1 fg – 1 μ g	PBS, Human Sweat (validation)	[51]
	Au	Ag/AgO NPs	CV	Direct (Label-free)	66 μ A/dec [†]	0.64 pM	1 pM – 1 μ M	PBS	[52]

Cont.

Recognition element	Electrode Material	Surface modification	Electro-chemical method	Detection Format	Sensitivity	LOD	Range of concentration	Fluid	Ref
Antibody	Au	PPAu NPs	CV, DPV	Direct (Label-free)	1.63 $\mu\text{A}/\text{dec}$ (CV) 2.39 $\mu\text{A}/\text{dec}$ (DPV) [†]	1 pM	1 pM – 100 nM	PBS	[53]
	PI	LGE, PPA	Amperometric	Competitive (HRP-label, mediated via hydroquinone)	3.04 nA/mm ² per ng/mL [†]	0.08 ng/mL	0.43 – 50.2 ng/mL	PBS, Human saliva and sweat (validation)	[54]
	AuND LEG	Protein A/G	SWV	Competitive (MB-label)	—	2.70 ng/mL	0 - 100 ng/mL	PBS, Human Sweat (validation)	[55]
	GCE	SnS ₂	DPV	Signal-Off (mediated via [Fe(CN) ₆] ^{3-/4})	0.0103 (mA/cm ²)/dec [†]	0.036 ng/mL	100 pM - 100 μM	PBS, Human saliva (validation)	[56]
	GCE	β -MnO ₂ CNs	EIS	Signal-Off (mediated via [Fe(CN) ₆] ^{3-/4})	247.5 Ω /pM (0.1 – 1 pM) 0.45 Ω /pM (100 – 1500 pM)	0.023 pM	0.1 pM – 1500 pM	PBS, Human Saliva and Sweat (validation)	[57]
	GCE	ZnO NPs, APTES cross-linker	DPV	Signal-Off (mediated via [Fe(CN) ₆] ^{3-/4})	—	5×10^{-7} nM	10^{-7} - 10^2 nM	Artificial Saliva	[58]
	GCE	GO-SiC nanocomposite	DPV	Signal-Off (mediated via [Fe(CN) ₆] ^{3-/4})	11.24 $\mu\text{A}/\text{dec}$ [†]	90 fg/mL	1 fg/mL - 1 $\mu\text{g}/\text{mL}$	PBS, Human sweat (validation)	[59]
	Au/Si	ZnO NFs	CV	Signal-Off (mediated via [Fe(CN) ₆] ^{3-/4})	7.74 $\mu\text{A}/\text{M}$	1 pM	100 pM - 100 nM	PBS, Human saliva (validation)	[60]
	IDEs	DTSP	CV	Signal-Off (mediated via [Fe(CN) ₆] ^{3-/4})	6 $\mu\text{A}/(\text{pg}/\text{mL})$	10 pg/mL	10 pg/mL - 100 ng/mL	PBS, Human saliva (validation)	[61]

Cont.

Recognition element	Electrode Material	Surface modification	Electro-chemical method	Detection Format	Sensitivity	LOD	Range of concentration	Fluid	Ref
Antibody	Conductive nylon thread	MXene, AuNPs, L-cysteine	Amperometry	Signal-Off (mediated via $[\text{Fe}(\text{CN})_6]^{3-/4}$)	$0.015 \mu\text{A}\cdot\text{cm}^{-2}/(\text{ng}\cdot\text{mL}^{-1})$ (5 - 40 ng/mL) $0.094 \mu\text{A}\cdot\text{cm}^{-2}/(\text{ng}\cdot\text{mL}^{-1})$ (40 - 180 ng/mL)	0.54 ng/mL	5 - 180 ng/mL	PBS, Artificial sweat (validation)	[62]
	Inkjet Au on paper	CMA, Ethanolamine	EIS	Signal-Off (mediated via $[\text{Fe}(\text{CN})_6]^{3-/4}$)	$25 \Omega\cdot\text{ng}/\text{mL}$ (PBS) $23 \Omega\cdot\text{ng}/\text{mL}$ (Artificial saliva) $19 \Omega\cdot\text{ng}/\text{mL}$ (Human saliva)	0.81 ng/mL (PBS) 1.09 ng/mL (Artificial saliva) 1.18 ng/mL (Human Saliva)	5 - 20 ng/mL	PBS, Artificial saliva, Human saliva (validation)	[63]
	LIG	G-PANI ink, carboxyl groups	CV CA DPV EIS	Signal-Off (mediated via $[\text{Fe}(\text{CN})_6]^{3-/4}$)	$85.72 \mu\text{A}/\text{dec}$ (CV) $6.54 \mu\text{A}/\text{dec}$ (CA) $0.35 \mu\text{A}/\text{dec}$ (DPV) $9.94 \Delta R/\text{dec}$ (EIS) [†]	$0.0813 \mu\text{g}/\text{mL}$ (CV) $0.0577 \mu\text{g}/\text{mL}$ (EIS)	100 ng/mL - 100 $\mu\text{g}/\text{mL}$	PBS	[64]
	GF	PBASE-NHS	DPV	Signal-Off (mediated via $[\text{Fe}(\text{CN})_6]^{3-/4}$)	$1.05 \mu\text{A}/\text{dec}$ (PBS) $0.84 \mu\text{A}/\text{dec}$ (Artificial saliva) [†]	0.24 fg/mL	1.0 fg/mL - 10000 pg/mL	PBS, Artificial Saliva, Human Saliva (validation)	[65]
	MWC-NTs/PDMS	AuNPs	DPV	Signal-Off (mediated via $[\text{Fe}(\text{CN})_6]^{3-/4}$)	$10.85 \mu\text{A}/\text{dec}^{\dagger}$	0.3 fg/ mL	1 fg/mL - 1 $\mu\text{g}/\text{mL}$	PBS, Human sweat (validation)	[66]
	PET coated with ITO	AuNPs, anteobind	DPV	Signal-Off (mediated via MB)	$0.543 \mu\text{A}/\text{ng}\cdot\text{mL}^{-1}$	0.58 ng/mL	0.5 - 150 ng/mL	Artificial and human sweat and saliva (validation)	[67]
	SPE	e-RGO	CV	Signal-Off (mediated via $[\text{Fe}(\text{CN})_6]^{3-/4}$)	—	0.1 ng/mL	0.1 – 200 ng	PBS, Artificial saliva and sweat (validation)	[68]

Cont.

Recognition element	Electrode Material	Surface modification	Electro-chemical method	Detection Format	Sensitivity	LOD	Range of concentration	Fluid	Ref
Antibody	Mogul-patterned substrate	3D nanostructured Au	EIS	Signal-Off (mediated via $K_3Fe(CN)_6$)	$0.25 \Omega/ng \cdot mL^{-1}$ (sensor) $0.27 \Omega/ng \cdot mL^{-1}$ (integrated patch)	1 pg/mL	1 pg/mL – 1 $\mu g/mL$	Artificial sweat, Human sweat (validation)	[69]
	SPCE on PET	AuNPs, SH-PEG-COOH	DPV	Signal-Off (mediated via Ferrocenyl methanol)	61.18 $\mu A/dec$ [†]	7.47 nM	100 nM - 500 nM	PBS, Human sweat (validation)	[70]
Aptamer	Thin-film gold	MCH (6-Mercapto-1-hexanol)	DPV	OFF/ON (MB-labeled)	19.8 %/dec [†]	4.6 nM	10 - 500 nM (3.6–181.2 ng/mL)	Buffer (PBS-based)	[71]
	Patterned graphene-modified GCE	ERGO, AuNP pre-bound TA	SWV	Signal-On (Displacement Assay)	65.387 $\mu A/cm^2$ per $\mu g/mL$ [†]	10 pg/mL	3×10^{-5} – 10 $\mu g/mL$	Citrate-phosphate buffer, Human saliva (validation)	[72]
	Nano-porous polyamide	ZnO	nF-EIS, CA	Direct (Label-free / nF-EIS)	10% – 45% $\Delta Z $ [†]	1 ng/mL	1 – 256 ng/mL	Human sweat	[73]
	Nano-porous polyamide	Porous Au thiolated-bound	nF-EIS	Direct (Label-free / nF-EIS)	50 % $\Delta Z $ [†]	1 ng/mL	1 – 256 ng/mL	Artificial and human sweat	[74]
	PDMS	CNC/CNT	CV	Direct (Label-free / Capacitive)	8.3106 $\mu F / (ng/mL)$ [†]	1.8 ng/mL	2.5 – 35 ng/mL	PBS, Human sweat (validation)	[75]
	SPGEs	Streptavidin MBs	EIS	Signal-Off (mediated via $[Fe(CN)_6]^{3-/4}$)	466.86 Ω/dec	2.14 pg/mL	1×10^2 - 1×10^5 pg/mL	Low-salt buffer, Artificial sweat (validation)	[76]
	Carbon SPE	GQDs	CV	Signal-Off (mediated via $[Fe(CN)_6]^{3-/4}$)	2.1 $\mu A/(pg/mL)$ [†]	0.1 pg/mL	0.1 pg/mL – 100 ng/mL	PBS	[77]
	Photo-paper	MWNT-Cu-PP, streptavidin-coated MNP	DPV	Direct (Label-free)	—	10 pM	100 pM – 50 nM	PBS, Human saliva (validation)	[78]

Cont.

Recognition element	Electrode Material	Surface modification	Electro-chemical method	Detection Format	Sensitivity	LOD	Range of concentration	Fluid	Ref
Aptamer	SPGE	Polyaniline (PANI), GA	CA	Signal-Off (Direct / Label-free)	0.4502 μA /dec (PBS) 0.4145 μA /dec (Artificial Saliva) [†]	27 pM	0.1 - 10 nM	PBS, Artificial saliva (validation)	[79]
	CNT/PVA/C-NTs/PU film	Ni-Co MOF, Streptavidin	DPV	Signal-Off (Direct / Label-free)	—	0.032 ng/mL	0.1 - 100 ng/mL	PBS, Artificial and human sweat (validation)	[80]
MIP	GCE	Au-doped poly-o-PD	SWV	Signal-Off (mediated via $[Fe(CN)_6]^{3-/4}$)	2.061 μA /dec	200 fM	1 pM – 500 nM	PBS, Artificial saliva (validation)	[81]
	GCE	PEDOT/SA conductive hydrogel	EIS, Chrono-coulometry (CC)	Signal-Off (mediated via $[Fe(CN)_6]^{3-/4}$)	33.14 ΔR /dec (EIS) 0.043 ΔA /dec (CC) [†]	0.314 pM (EIS) 0.131 pM (CC)	10^{-12} - 10^{-7} M	PBS, Artificial and human sweat (validation)	[82]
	Cotton textile	Au-plated poly(GMA-co-EGDMA) / PANI / CNT / CNC	CV	Signal-Off (Direct / Label-free)	0.0155 $\mu F \cdot mL$ /ng (PBS) 0.0094 $\mu F \cdot mL$ /ng (Sweat) [†]	8.00 ng/mL	9.80 - 49.5 ng/mL	Phosphate Buffer, Human sweat (validation)	[83]
	SPCEs on PET	PPy/CuPcTS	CA	Direct (Label-free)	2.44 μA /dec [†]	181 pM	100 pM - 1 μM	PBS, Artificial and human sweat (validation)	[84]
	Carbon electrodes on PET	PPy/PB	CA	Signal-Off (Built-in PB Probe)	6 $\pm$ 0.2 nA/dec (PBS) 3 $\pm$ 0.4 nA/dec (Artificial sweat)	0.16 ng/mL (PBS) 0.12 ng/mL (Artificial Sweat)	0.1 – 10000 ng/mL	PBS, Artificial sweat, Human sweat (validation)	[85]
	Au on PI film	Py-PB oxide film	CA, DPV	Signal-Off (Built-in PB Probe)	0.125 μA /dec [†]	0.1 pM	0.1 pM - 5 μM	PBS, Human sweat (validation)	[86]
	Au on PI film	PPy and PB	i-t Curve	Signal-Off (Built-in PB Probe)	0.083 mA/dec (PBS) 0.028 mA/dec (Artificial sweat)	0.1 nM	10^{-9} - 10^{-5} mol/L	PBS, Human sweat (validation)	[87]

Cont.

Recognition element	Electrode Material	Surface modification	Electro-chemical method	Detection Format	Sensitivity	LOD	Range of concentration	Fluid	Ref
MIP	SPCE	Au NPs, PPy/PB	CA	Signal-Off (Built-in PB Probe)	15.518 nA·lg(nM ⁻¹)·cm ⁻²	0.4112 fM	1 fM - 1 μM	PBS	[88]
	GCE	Cu-BTC MOF, o-PD monomer, PB	DPV	Signal-Off (Built-in PB Probe)	0.058 μA/nM [†]	0.0027 nM	0.01 - 1000 nM	PBS, Human saliva and sweat (validation)	[89]
Non-affinity based	Paper-based SPE	Sulfur-doped graphene, AuNPs, CMC binder	LSV	Direct	0.247 μA/μM [†]	15.39 ng/mL	35.24 - 18123 ng/mL	PBS, Artificial sweat (validation)	[90]
	PGE	ZnO-graphene nanocomposite	DPV	Direct	0.34 μA/nM [†]	0.15 nM	5 × 10 ⁻¹⁰ M – 115 × 10 ⁻¹⁰ M	PBS, Human saliva (validation)	[91]
	CFMEs		Fast-scan cyclic voltammetry (FSCV)	Direct	0.87 ± 0.055 nA/μM	—	10 - 40 μM	PBS, Artificial Urine (validation)	[92]
	Carbon	Graphene-PPy composite	CV	Direct	12.92 μA/cm ² per ng/mL [†]	0.5 ng/mL	0.5 - 5 ng/mL	Artificial saliva	[93]
	Graphite/Gold-leaf (86% Copper) ink	Alkyd resin varnish	CA	Direct	2.7 μA/μM [†]	10 nM (lowest detected)	10 - 250 nM	Artificial and Human sweat (on-body validation)	[94]
	SPCE	MWCNTs-CuTPP	DPV	Direct	2×10 ⁸ μA/M (50 fM–1.0 pM) 2.8×10 ⁵ μA/M (1.0 pM – 100.0 nM)	50 fM	50 fM – 100 nM	PBS, Human saliva (validation)	[95]

[†] Value derived from the reference.

Antibody-based sensors are the most established class, characterized by highly specific molecular recognition and predominantly indirect signal transduction. Some studies explored substrate-linker combinations for cortisol detection via non-Faradaic electrochemical impedance spectroscopy (nF-EIS), such as laser-induced graphene interdigitated electrode (LIG-IDE) modified with gold nanoparticles (AuNPs) and 3-mercaptopropionic acid (3-MPA) [45], Au on porous polyamide modified with dithiobis (succinimidyl propionate) (DSP) thiol linker [46], and indium tin oxide (ITO)-coated glass modified with poly(3,4-

ethylenedioxythiophene):poly(styrene sulfonate) (PEDOT:PSS) and 3-aminopropyltriethoxysilane (ATPES) linker [47]. Others report the use of flexible substrates, such as zinc oxide (ZnO)-coated nanoporous polyamide membranes [48] and molybdenum disulfide (MoS₂)-modified palladium (Pd) electrodes [49], that enable non-Faradaic cortisol sensing by amplifying and modulating the electrical double-layer (EDL) capacitance for impedance-based detection in sweat. Organic electrochemical transistors (OECTs) exploit the same principle, in which an antibody-functionalized gate modulates the EDL capacitance and amplifies the PEDOT:PSS drain current [50]. Other direct formats rely on the high electroactive surface area of nano-materials, such as ellipsoidal iron(III) oxide (Fe₂O₃, hematite) on conductive carbon yarn (CCY) [51], silver/silver oxide nanoparticles (Ag/AgO NPs) [52], and polyaniline-protected gold nanoparticles (PPAuNPs) [53] on gold electrodes, to monitor cortisol binding. More complex approaches rely on enzymatic or redox-labeled signal probes in competitive assay formats. For instance, laser-induced graphene (LIG) electrodes modified with 1H-pyrrole propionic acid (PPA) use horseradish peroxidase (HRP)-labeled cortisol, with signal generation based on the enzymatic reduction of hydrogen peroxide (H₂O₂) [54]. Methylene blue (MB)-labeled antigens immobilized on gold nanodendrite-decorated laser-engraved graphene (AuND-LEG) [55] act as a redox probe, in which analyte-induced displacement results in a decrease in the square-wave voltammetry (SWV) peak current.

The most prevalent detection strategy is the label-free signal-off (probe-mediated) format, in which cortisol binding restricts the access of an external redox probe to the electrode surface. The ferri/ferrocyanide redox couple ([Fe(CN)₆]^{3-/4-}) is widely used, and its activity is monitored by DPV, CV, CA, and both faradaic and nF-EIS. Rigid electrode materials, glassy carbon electrodes (GCEs), were modified with two-dimensional materials and nanostructures, such as tin disulfide (SnS₂) nanoflakes [56], β -manganese dioxide (β -MnO₂) cacti-like architectures [57], zinc oxide (ZnO) nanoparticles [58], or graphene oxide (GO)-silicon carbide (GO-SiC) nanocomposite [59] to provide mechanically stable, high-surface-area interfaces that support efficient probe diffusion while amplifying steric blocking effects upon cortisol binding. ZnO nanostructures [60] and dithiobis(succinimidylpropionate) (DTSP) [61] on gold substrates were also employed to enable efficient antibody immobilization. Flexible conductive nylon threads modified with MXene (Ti₃C₂T_x)/AuNP composites [62], inkjet-printed gold electrodes on paper functionalized with 4-carboxymethylaniline (CMA) [63], and graphene-polyaniline (G-PANI) ink modified with carboxyl groups on LIG Kapton electrodes [64], yield highly conductive scaffolds enabling more distinct and quantifiable signal-off current suppression. A highly porous three-dimensional commercial graphene foam (GF) functionalized with pyrene-based linkers, i.e., 1-pyrenebutyric acid N-hydroxysuccinimide ester (PBASE-NHS), combines a high electroactive surface area with efficient antibody immobilization, enabling ultralow detection lim-

its of 0.24 fg/mL [65]. Multi-walled carbon nanotubes (MWCNTs)/polydimethylsiloxane (PDMS) films decorated with AuNPs [66], and ITO/polyethylene terephthalate (PET) electrodes modified with AuNPs and biofunctional linkers [67] provided flexible conductive scaffolds, while electroreduced graphene oxide (ERGO)-modified screen-printed carbon electrode (SPCE) [68] served as signal amplification platforms. Other studies employed 3D-nanostructured Au on a mogul-patterned PDMS substrate with a potassium ferricyanide ($K_3Fe(CN)_6$) redox probe for detection via EIS [69]. Similarly, AuNPs and thiol-polyethylene glycol-carboxyl (SH-PEG-COOH)-modified SPCEs on PET used a ferrocenyl methanol mediator for cortisol detection via DPV [70].

The combination of electrode materials and label-free recognition strategies enables these sensors to achieve the sensitivity required to detect cortisol in complex biofluids, such as saliva and sweat, operating within clinically relevant concentration ranges (pg/mL to ng/mL).

Aptamer-based sensors combine the structural flexibility and tunability with the functional properties of engineered electrode materials. Structure-switching aptamers labeled with MB on gold electrodes generate a signal-on response [71], due to the proximity of the redox label to the surface upon cortisol binding. Displacement assays have also been reported in which cortisol displaces prebound analogues (e.g., triamcinolone) on gold-modified graphene electrodes [72], resulting in a detectable change in current. Label-free signal-off formats are implemented on materials such as nanoporous polyamide with ZnO [73] or porous gold functionalized with thiolated aptamers [74], enabling capacitive sensing via modulation of the EDL. Flexible composites, such as PDMS modified with cellulose nanocrystals (CNC)/MWCNT [75], were used to detect cortisol via changes in capacitance measured by CV. Screen-printed graphene electrodes (SPGEs) modified with streptavidin magnetic beads [76], or carbon screen-printed electrodes modified with graphene quantum dots (GQDs) [77], enable $[Fe(CN)_6]^{3-/4-}$ redox probe-based signal-off detection via CV and EIS, respectively. Alternatively, several direct sensing strategies avoid the use of external probes. MWCNT–copper porphyrin inks printed on paper served as a catalyst, enabling the electrochemical reduction of cortisol [78]. Polyaniline (PANI) on graphene electrodes served as an intrinsic redox material whose signal is blocked by cortisol, enabling one-step detection via CA [79]. Nickel–cobalt metal-organic framework (MOF) nanosheets [80] function as peroxidase-mimicking catalytic nanozymes to amplify electrochemical current, while cortisol binding suppresses this activity, resulting in a signal-off response via DPV. Aptamer-based sensors achieved detection limits in the pM to nM range (e.g., 27 pM, or ~ 1 ng/mL), which are higher than those of antibody-based systems. However, their design facilitates the flexible integration of electrode materials to improve performance, with dynamic ranges well-suited to measuring cortisol in sweat and saliva (0.1–100 ng/mL).

In MIP-based sensors, materials engineering plays a central role in defining both molecular recognition and signal transduction. A GCE was modified with gold-doped poly(o-phenylenediamine) (poly-o-PD) [81] to detect cortisol via SWV using an external probe, i.e., $[\text{Fe}(\text{CN})_6]^{3-/4-}$. Using the same detection format, a conductive poly(3,4-ethylenedioxythiophene) (PEDOT)/sodium alginate (SA) hydrogel [82] served as an antifouling MIP matrix, thereby minimizing nonspecific adsorption and enabling label-free EIS detection. Cotton substrates coated with MWCNT/CNC and PANI [83] exhibited electrical conductivity, enabling capacitive sensing in which cortisol binding decreases capacitance. Alternatively, the incorporation of copper tetrasulfonated phthalocyanine (CuPcTS) into polypyrrole (PPy) films on flexible carbon/PET electrodes enables electrocatalytic reduction of cortisol, with a limit of detection of approximately 181 pM [84].

Within these sensing formats, a prevalent strategy is to incorporate built-in redox probes as internal signal transducers. This approach was implemented using SPCEs [85] or gold on polyimide substrates [86] modified with conductive polymers, such as PPy, into which Prussian Blue (PB) nanoparticles were incorporated as the internal redox mediator [87]. The incorporation of AuNP into these matrices [88] (e.g., AuNP/PPy/PB) further enhances electroactive surface area and charge transport, enabling ultralow detection limits in the femtomolar range (i.e., 0.4112 fM). Hybrid architectures combining copper benzene-1,3,5-tricarboxylate (Cu-BTC) MOFs with PB nanoparticles and o-phenylenediamine (o-PD) monomers [89] yielded highly porous scaffolds that increased the density of recognition sites and improved charge-transfer kinetics. Electrochemical techniques such as DPV and CA are predominantly used in these systems to monitor electron-transfer changes, leveraging the conductive MIP layers and the embedded redox mediator. These sensors exhibit dynamic ranges spanning femtomolar to micromolar, with sensitivities in the femtomolar range, comparable to those of antibody-based immunosensors. MIP-based platforms have the potential to combine high sensitivity and selectivity with improved robustness, reusability, and lower production costs compared with traditional immunosensors, depending on the imprinting strategy and material architecture employed.

In contrast, non-affinity-based detection formats rely entirely on the electrode material to transduce cortisol into an electrochemical signal. Some studies [90] have used AuNP on paper to enhance charge transfer and interfacial conductivity at the electrode, in combination with sulfur-doped graphene (S-G). This combination reduced the overpotential required for the direct oxidation of cortisol, enabling linear-sweep voltammetry (LSV) measurements. Others used a porous graphene network modified with catalytic ZnO [91] on pencil graphite electrodes to facilitate efficient electron transport and cortisol access to the interface. Alternatively, carbon fiber microelectrodes (CFMEs) exploited their intrinsic conductivity and micrometer-scale dimensions to perform ultra-fast potential scans, capturing transient cortisol reduction

signals that are difficult to resolve with conventional voltammetry [92]. Carbon graphene-PPy composite [93] and printed graphite and gold-leaf electrodes [94] were also reported to detect cortisol via CV.

A multi-walled carbon nanotubes–copper(II) porphyrin–polypyrrole nanocomposite (MWCNT-Cu-PP) [95] was used as a biomimetic enzymatic catalyst, while the incorporation of MWCNT provided a high surface area and conductivity. This amplifies the electrochemical response, achieving an ultralow LOD of 50 fM in saliva via DPV, making it the only reported sensor of this type with performance comparable to antibody-based systems. Recognition-free sensors typically exhibit detection limits in the nanomolar to micromolar range (e.g., 10 nM or 15.39 ng/mL). Nevertheless, these systems offer important practical advantages, including simplified fabrication, mechanical and chemical robustness, long-term stability, and reduced production costs, being particularly attractive for wearable, disposable, and non-invasive applications.

A clear progression is observed in electrochemical cortisol-sensing strategies reported, from antibody-based systems dominated by interfacial modulation of external redox probes to aptamer-based platforms that enable recognition-driven signal transduction, to MIP-based sensors that couple molecular recognition with signal generation within the material, and further to fully material-driven approaches. Overall, the lowest detection limits and highest sensitivities are achieved predominantly by recognition-based sensors. However, sensor performance is an interplay between recognition element, material architecture, and transduction mechanism, which ultimately defines LOD, sensitivity, and the accessible concentration range. The overview of the reported sensors above provides the basis for a critical discussion of their limitations in the following section.

2.4 Challenges in biomarker detection

Despite the extremely high affinity constants of antibody–antigen interactions, which enable high selectivity and low LOD, antibody-based electrochemical sensors present several practical limitations. Antibody production is time-consuming, costly, and dependent on animal hosts, leading to significant batch-to-batch variability that constrains large-scale implementation. They are prone to denaturation and loss of activity under non-physiological conditions, compromising long-term operational stability [35, 96]. These systems typically rely on complex assay formats and require secondary labels, such as enzymes, nanoparticles, or redox mediators (e.g., MB, $[\text{Fe}(\text{CN})_6]^{3-/4-}$, or PB), to generate a measurable electrochemical signal. Thus, sensor fabrication and operation involve multiple modifications, incubation, and washing steps that compromise reproducible performance and limit integration into POC and wearable devices.

Aptamer-based sensors address some of the limitations of antibodies by enabling synthetic production, greater chemical versatility, and easier functional modification. However, the selection and optimization of high-affinity aptamers remain labor-intensive and expensive. Aptamers can exhibit limited stability in complex biological matrices due to nuclease degradation and matrix-induced alterations to their folded, functional structure. Like antibody-based platforms, aptamers are typically covalently immobilized on the electrode surface to achieve stable, oriented binding [96, 97]. Signal transduction often relies on redox labels or conformational-switching mechanisms, thereby increasing design complexity.

MIPs constitute an alternative as they are typically more robust, chemically stable, and cost-effective. Nevertheless, these sensors face intrinsic challenges, including incomplete template removal, potential template leakage, heterogeneous distribution of binding sites, and limited accessibility of imprinted cavities, particularly for bulky or poorly diffusing analytes. To achieve high specificity, extensive optimization of polymer composition, cross-linking density, and imprinting conditions is often required, limiting reproducibility and scalability [98, 99]. Despite closer integration of recognition and transduction, effective target binding in these systems often requires relatively long incubation times, thereby limiting their suitability for real-time and continuous monitoring.

A common limitation across recognition-based electrochemical sensors is their complex fabrication protocols, extended incubation times, and secondary signal-generation mechanisms. These factors increase assay duration, reduce operational robustness under real-world conditions, and limit seamless integration with wearable and continuous-sensing platforms [9].

Non-affinity-based electrochemical sensors eliminate the need for molecular recognition elements, yet they generally exhibit limited sensitivity and higher detection limits. Some reported platforms also rely on complex surface chemistries or costly functional materials, which can partially undermine the simplicity expected of recognition-free designs. In this context, material-driven sensing strategies offer a promising alternative, directly converting cortisol–material interactions into measurable electrochemical signals through mechanisms such as direct detection, signal suppression, or modulation. This approach has the potential to overcome several limitations of current cortisol sensors, particularly in applications demanding simple fabrication and practical implementation.

Chapter 3

Exploratory study of anhydride-functionalized
carbon nanotubes for cortisol detection

This chapter is based on the article:

Santos, A. R., Bento, F., Paiva, M. C., Silva, A. F., & Viana, J. C. "Explorative study of carbon nanotubes covalently functionalized with anhydride groups for electrochemical cortisol Sensing". (in preparation)

3 Exploratory study of anhydride-functionalized carbon nanotubes for cortisol detection

This work investigates the covalent functionalization of multi-walled carbon nanotubes (MWCNTs) with anhydride groups to tailor surface properties for cortisol sensing. Functionalization was carried out using gas-phase and solution-based methods, followed by material characterization. The materials with the highest degree of functionalization were evaluated as electrochemical sensors, with limited sensitivity toward cortisol.

3.1 Introduction

Covalent functionalization of MWCNTs with anhydride groups was selected as a rational strategy to tailor the transducer's surface chemistry while preserving the nanotube network's intrinsic electrical conductivity. Anhydride moieties provide highly reactive, polar surface sites that engage in hydrogen bonding and dipole–dipole interactions with oxygenated molecules such as cortisol. In addition, the introduction of anhydride functionalities enhances nanotube wettability and dispersion on the electrode surface, potentially improving analyte accessibility and interfacial electron transfer. MWCNT-anhydride groups (ANI) materials have the potential to promote detectable changes in the electrochemical response upon cortisol adsorption through surface blocking and interfacial modulation effects.

In the present work, the covalent modification of the MWCNTs surface was investigated using both gas-phase and solution-based approaches, followed by thorough characterization of the resulting materials. Finally, the MWCNTs exhibiting the highest degree of functionalization were selected and evaluated for the electrochemical sensing of cortisol.

3.2 Experimental section

MWCNTs were obtained from Nanocyl (NC 7000). Diethylene glycol dimethyl ether (diglyme), and 3-sulfolene were purchased from Sigma-Aldrich. Screen-printed carbon electrodes (SPCE 110D, Figure 3.1) were purchased from DropSense (Metrohm, Portugal). These consist of a carbon counter electrode (CE), a working electrode (WE) with a diameter of 4 mm, and a silver (Ag) reference electrode (RE).



Figure 3.1. Screen printed carbon electrode (SPCE) 110D.

MWCNTs were functionalized in the gas phase, avoiding the use of a solvent. In this case, 50 mg of MWCNTs were placed on a Pyrex shallow dish above 500 mg of 3-sulfolene, and the system was heated to induce decomposition of 3-sulfolene into 1,3-butadiene and SO_2 . The reaction was carried out for 4 days under two different temperature conditions: at 135 °C ($\text{MWCNT-ANI}_{\text{vp135}}$) and 150 °C ($\text{MWCNT-ANI}_{\text{vp150}}$). MWCNTs were also functionalized in the liquid phase ($\text{MWCNT-ANI}_{\text{lp}}$), using diglyme as solvent. Briefly, 200 mg of MWCNTs were mixed with 400 mg of 3-sulfolene, and the reaction was carried out in 60 mL of diglyme at 135 °C for 5 days under stirring, as described in a previous study [100]. The solution was filtered through a polytetrafluoroethylene (PTFE) membrane with 200 μm porosity, washed with ethanol, and then dried under vacuum overnight at 80 °C.

Thermogravimetric analysis (TGA) of MWCNTs and MWCNT-ANI was conducted on a TA Instruments Q500 (DE, USA), at a heating rate of 10 °C $\cdot$ min⁻¹ from 40 °C to 780 °C under an N_2 atmosphere. X-ray photoelectron spectroscopy (XPS) of MWCNT-ANI was performed using an ESCALAB 250Xi (Thermo Fisher Scientific) equipped with a hemispherical analyzer and a monochromated Al $K\alpha$ source ($(h\nu) = 1486.68$ eV, 220 W, 14.6 kV, spot size 650 μm). Survey and high-resolution spectra were collected at pass energies of 100 eV and 40 eV, respectively. Spectra were processed using Advantage with a Shirley background, and binding energies were referenced to the adventitious C1s peak at 284.8 eV.

A suspension of 1 mg/mL MWCNT-ANI in water/methanol (1:1) was prepared and subjected to 30 min of ultrasonic tip (UP100H, 100% A, 0.9 cycle). The screen-printed carbon electrodes (SPCEs) preparation consisted of casting 7 μL of the MWCNT-ANI suspension on the SPCE WE. Phosphate-buffered saline (PBS) and cortisol solutions were prepared as previously reported [101]. Differential pulse voltammetry (DPV) measurements were performed at room temperature with a pulse amplitude of 25 mV, a potential step of 5 mV, a modulation time of 0.05 s, and an interval of 0.5 s, using an Autolab PGSTAT30 potentiostat

controlled by the GPES 4.9 software (EcoChemie). A drop of solution (70 μL) covered all three electrodes of the modified SPCE/MWCNT-ANI.

To evaluate the effect of incubation on the analytical response, the modified SPCE/MWCNT-ANI electrodes were incubated with varying cortisol concentrations for 15 minutes at room temperature prior to DPV measurements.

3.3 Results

3.3.1 Materials characterization

The pristine and functionalized MWCNT-ANI were characterized by TGA and XPS. The pristine MWCNTs exhibited high thermal stability in N_2 (Figure 3.2), showing negligible weight loss over the analyzed temperature range, consistent with their graphitic structure. The MWCNT-ANI_{vp135} and MWCNT-ANI_{vp150} exhibit weight losses of approximately 1.6% and 6.7%, respectively, at 750 °C. These results indicate that increasing the functionalization temperature leads to a higher degree of surface modification. At higher temperatures (150 °C), the decomposition of 3-sulfolene is enhanced, increasing the concentration of reactive species. This promotes the extent of functionalization of the MWCNT surface, resulting in higher weight loss during TGA analysis. MWCNT-ANI_{lp} exhibited a significantly higher weight loss (28.7%), attributed to the thermal decomposition of covalently attached organic functional groups on the nanotube surface. This result indicates a much higher degree of functionalization. The difference highlights the greater efficiency of the liquid-phase process, likely due to improved contact between the reactants and the nanotube surface, enabling more extensive covalent modification than the gas-phase approach.

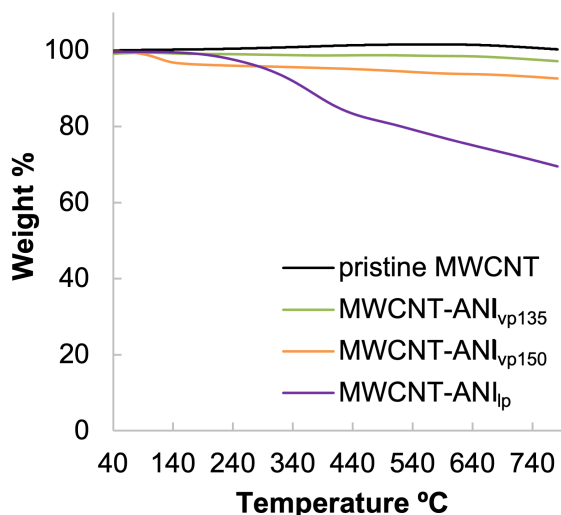


Figure 3.2. TGA results of pristine MWCNTs, MWCNTs functionalized with anydride groups in the liquid (MWCNT-ANI_{lp}) and vapor phase at 135 °C (MWCNT-ANI_{vp135}) and 150 °C (MWCNT-ANI_{vp150}).

The XPS survey spectra of of pristine MWCNT, MWCNT-ANI_{lp}, MWCNT-ANI_{vp135}, and MWCNT-ANI_{vp150} are presented in Figure 3.3, evidencing the increase in concentration of oxygen-containing groups with functionalization, observed at a binding energy (BE) near 530 eV. The overall composition of all MWCNTs is mostly carbon and oxygen. The MWCNTs functionalized in solution and in the gas phase at 150 °C also showed a residual sulfur content. The atomic content (at.%) obtained from the analysis of the high-resolution spectra of C1s, O1s, and S2p is summarized in Table 3.1, and the corresponding high-resolution spectra are presented Figures SS3.1–SS3.5 (Appendix). The overall oxygen atomic % measured for MWCNT-ANI_{lp}, MWCNT-ANI_{vp135}, and MWCNT-ANI_{vp150} is approximately 18.71%, 2.09%, and 3.06%, respectively, confirming the lower degree of functionalization achieved in the gas-phase functionalization. The higher oxygen content observed for the MWCNT functionalized in solution, and the increase in oxygen content with gas phase functionalization temperature, are consistent with the residual weight measured by TGA.

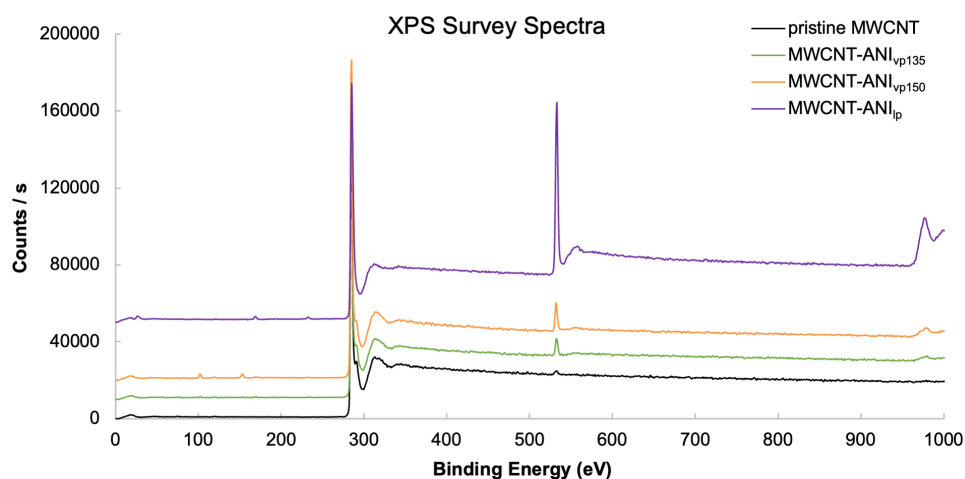


Figure 3.3. XPS survey spectra of pristine MWCNTs, MWCNTs functionalized with anydride groups in the liquid (MWCNT-ANI_{lp}) and vapor phase at 135 °C (MWCNT-ANI_{vp135}) and 150 °C (MWCNT-ANI_{vp150}).

The O1s high-resolution spectra can be deconvoluted into two component peaks centered at BE $\sim$ 532 eV (corresponding to double-bonded oxygen in O=C=O) and $\sim$ 534 eV (corresponding to single-bonded oxygen in O-C=O), and the corresponding at.% obtained are presented in Table 3.1. The ratio of the at.% associated with these component peaks, O1s (532)/O1s (534) indicates the relative contribution of oxygen bonded to a double bond to carbon (O=C) versus a single bond to carbon (O-C). For the pristine MWCNTs, the low surface oxidation is reflected in a low O1s (532)/O1s (534) ratio, indicating a larger contribution from single-bonded functions, such as phenol groups. The gas phase functionalized MWCNTs present, in both cases, a O1s(532)/O1s(534) ratio near 1, indicating a similar contribution of doubly bonded and single bonded oxygen, consistent with the formation of functional groups such as carboxylic acid and lactone (Figure 3.4). For MWCNT-ANI_{lp}, a C:O ratio of 81:19 was observed, indicating a significantly higher degree of functionalization compared to the gas-phase samples. In this case, the deconvolution of the O1s peak shows a O1s(532)/O1s(534) ratio greater than 3, showing a predominance of the $\sim$ 532 eV component. This predominance of doubly bonded oxygen suggests the formation of anhydride groups, consistent with results from a previous study [100]).

Additionally, a residual amount of sulfur was observed at the surface of the MWCNTs functionalized in the liquid and gas phases at 150 °C. Although the high-resolution S2p peak presents a low signal-to-noise ratio, the BE positions associated with S ($\sim$ 168.5 – 169.0 eV) suggest the presence of sulfonic acids.

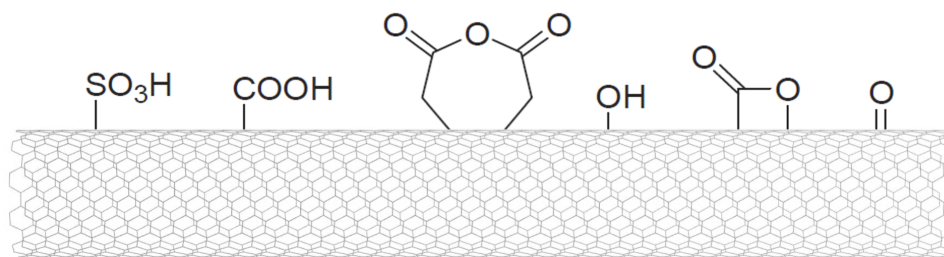


Figure 3.4. Possible functional groups functionalized at the MWCNT surface. Adapted from [100].

Table 3.1. Elemental and functional group analysis from XPS.

	Atom % and atomic ratio			
	Pristine MWCNT	MWCNT-ANI _{lp}	MWCNT-ANI _{vp135}	MWCNT-ANI _{vp150}
C1s(total)	88.71	76.89	89.05	85.87
O1s (~532 eV)	0.17	14.26	1.06	1.39
O1s (~534 eV)	0.55	4.45	1.03	1.67
S2p (~169 eV)	–	0.5	–	0.1
O/C (total)	0.008	0.24	0.023	0.036
O (532)/O (534)	0.31	3.2	1.03	0.83
S/C	–	0.0065	–	0.001

In conclusion, a lower degree of functionalization was observed for the gas-phase samples, whereas the liquid-phase approach led to significantly higher surface modification. The results demonstrate the influence of both reaction temperature and phase on the extent of functionalization, with higher temperatures and liquid-phase conditions promoting more effective modification. Considering the significantly higher degree of functionalization observed for MWCNT-ANI_{lp} and the presence of a high concentration of C=O, including anhydride groups, this functionalization was selected for further modification of SPCE and subsequently tested in cortisol sensing experiments.

3.3.2 Voltammetric characterization of the sensor response

Figure 3.5 presents the electrochemical response of SPCEs modified with MWCNT-ANI_{lp} as a function of cortisol concentration (1 to 100 μ M; 10 to 500 nM) via DPV. A peak around -0.45 V is observed in the absence of cortisol (Figure 3.5a), suggesting that this peak is associated with the electrode material itself (MWCNT-ANI_{lp}). This peak indicates that the MWCNT-ANI_{lp} electrode exhibits a characteristic background signal, likely due to redox reactions involving surface functional groups.

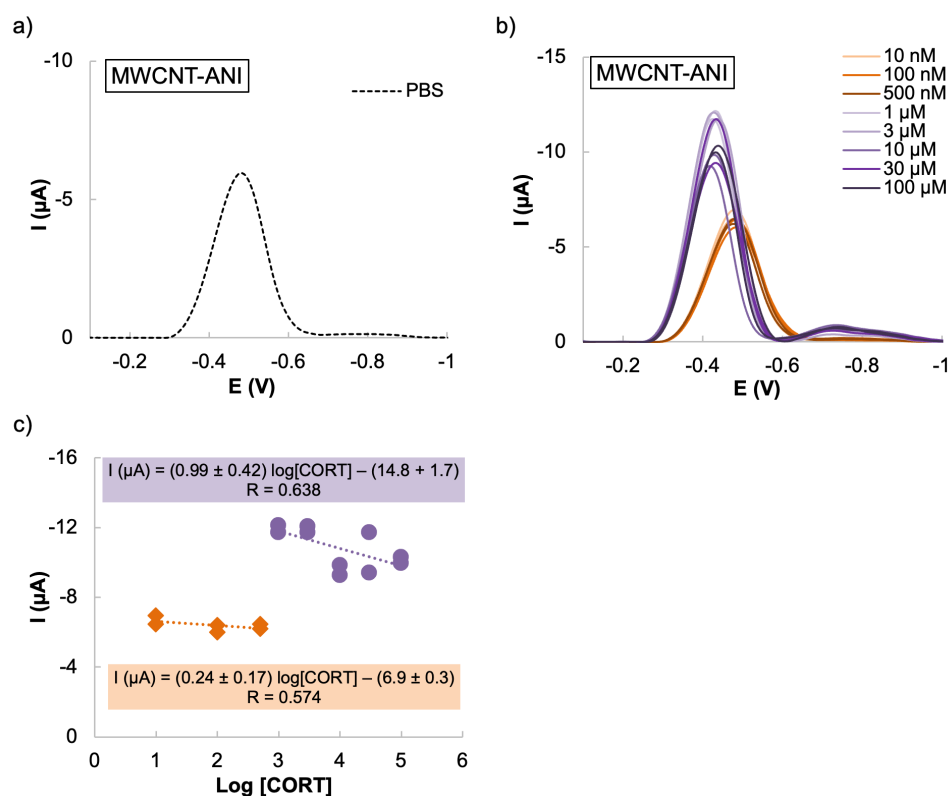


Figure 3.5. DPV responses recorded using SPCE/MWCNT-ANI_{ip} electrodes in the absence of cortisol (a) and at varying cortisol concentrations (b). (c) Corresponding variation in current intensity as a function of increasing cortisol concentration across different concentration ranges. Two replicates are shown for each measurement.

In Figure 3.5b, the electrodes modified with MWCNT-ANI_{ip} and tested with μM cortisol showed a decrease of the characteristic peak near -0.45 V with increasing cortisol concentration, indicating that interactions between the anhydride groups on the MWCNT and cortisol, possibly via hydrogen bonding or electrostatic interactions, partially block the electrode's active sites. For MWCNT-ANI_{ip} tested at the nM cortisol concentration range, a slight positional shift and a decrease in peak intensity were observed, attributed to inherent variability in electrode baseline responses. No significant change in peak height was observed with increasing cortisol concentration (Figure 3.5c). Also, incubation with cortisol did not improve the electrochemical response of MWCNT-ANI_{ip} (Figure SS3.6, Appendix).

The results suggest that the interaction between cortisol and the anhydride-functionalized surface is relatively weak and likely dominated by nonspecific physicochemical effects. These findings indicate that further improvements are needed, such as optimizing nanotube dispersion or adjusting measurement conditions to enhance adsorption and surface interactions.

3.4 Conclusions

This exploratory study confirmed the covalent functionalization of MWCNTs with anhydride groups, highlighting the influence of both reaction phase and temperature. While gas-phase modification showed a measurable degree of functionalization that increased with temperature, the liquid-phase approach was more effective, achieving a 28.7% weight loss and a C:O ratio of 81:19. Despite these results, the sensing performance remained limited, particularly at low cortisol concentrations. These findings underline the constraints of anhydride-based surfaces in this context and motivated the exploration of alternative functionalization strategies in subsequent work.

3.5 Appendix

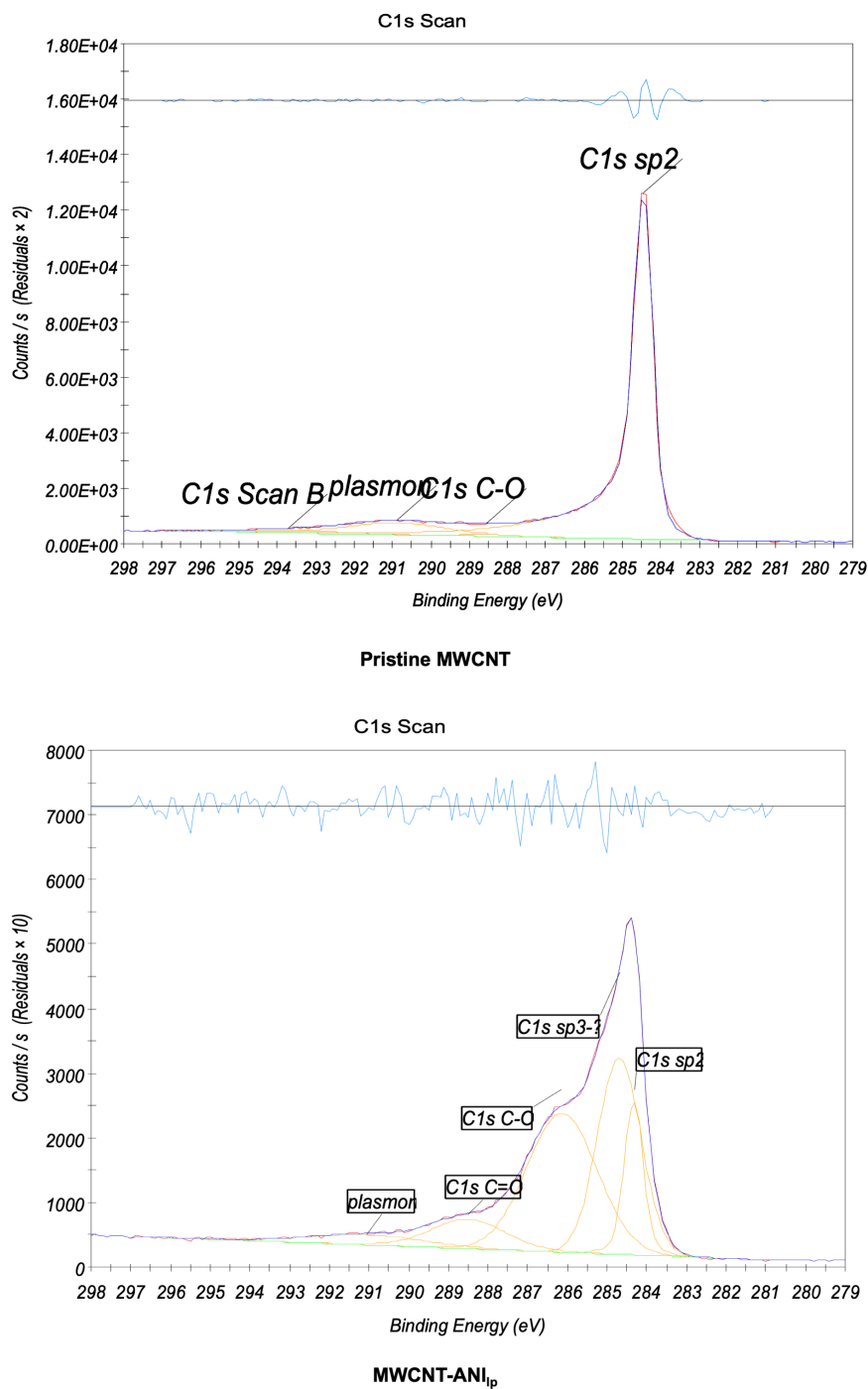
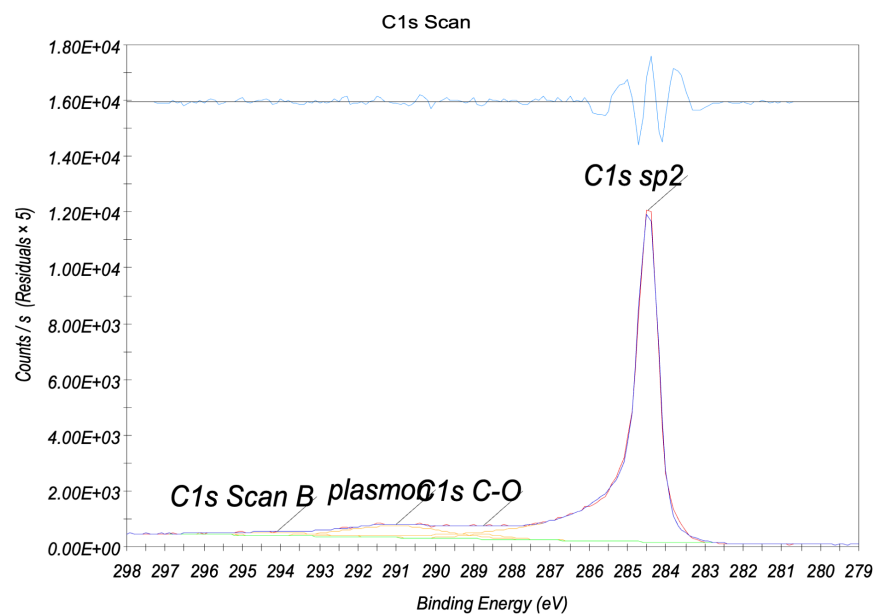
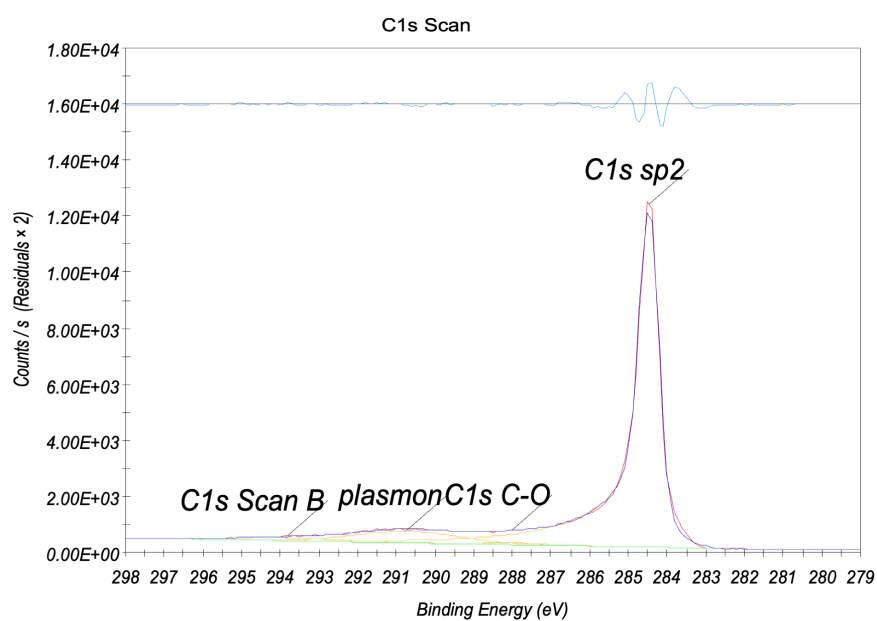


Figure S3.1. High-resolution XPS spectra (C1s) of the functionalized MWCNTs samples (part 1).

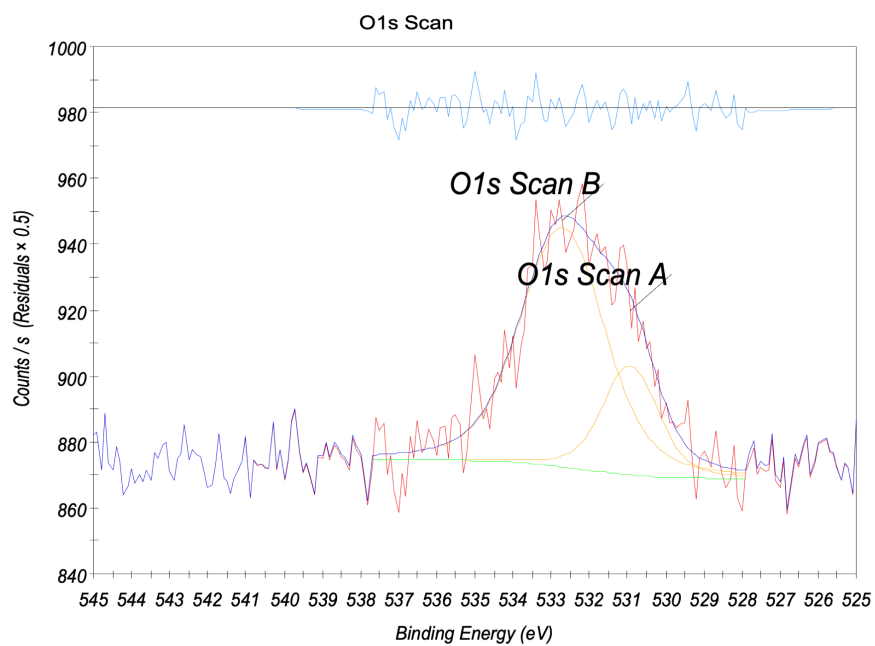


MWCNT-ANI_{vp135}

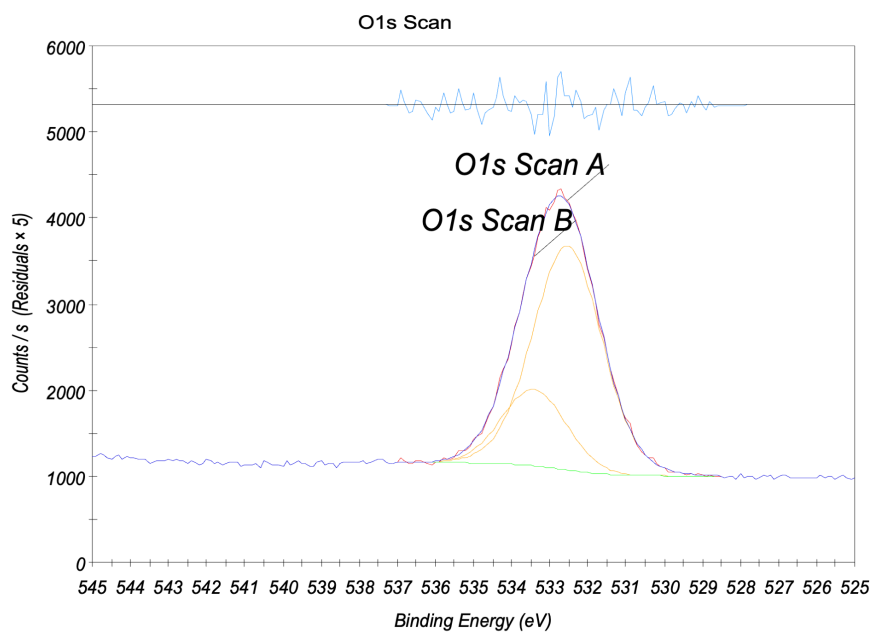


MWCNT-ANI_{vp150}

Figure S3.2. High-resolution XPS spectra (C1s) of the functionalized MWCNTs samples (cont.).

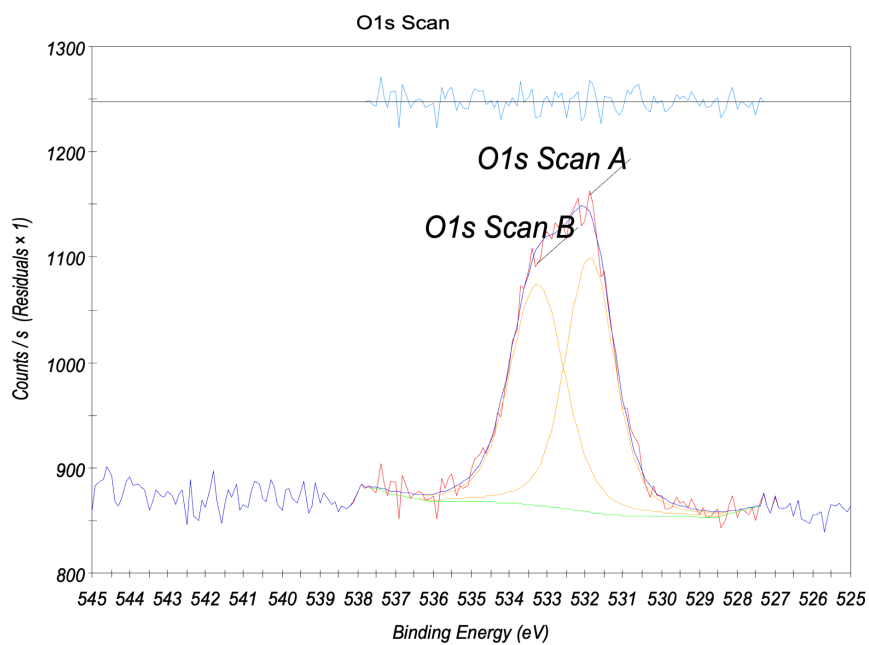


Pristine MWCNT

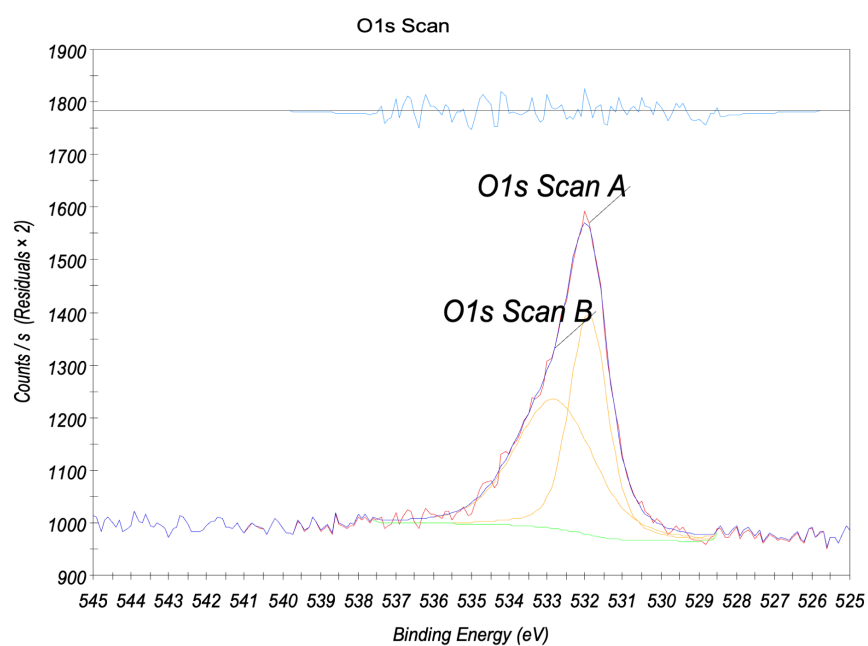


MWCNT-ANI_p

Figure S3.3. High-resolution XPS spectra (O1s) of the functionalized MWCNTs samples (part 1).



MWCNT-ANI_{vp135}



MWCNT-ANI_{vp150}

Figure S3.4. High-resolution XPS spectra (O1s) of the functionalized MWCNTs samples (cont.).

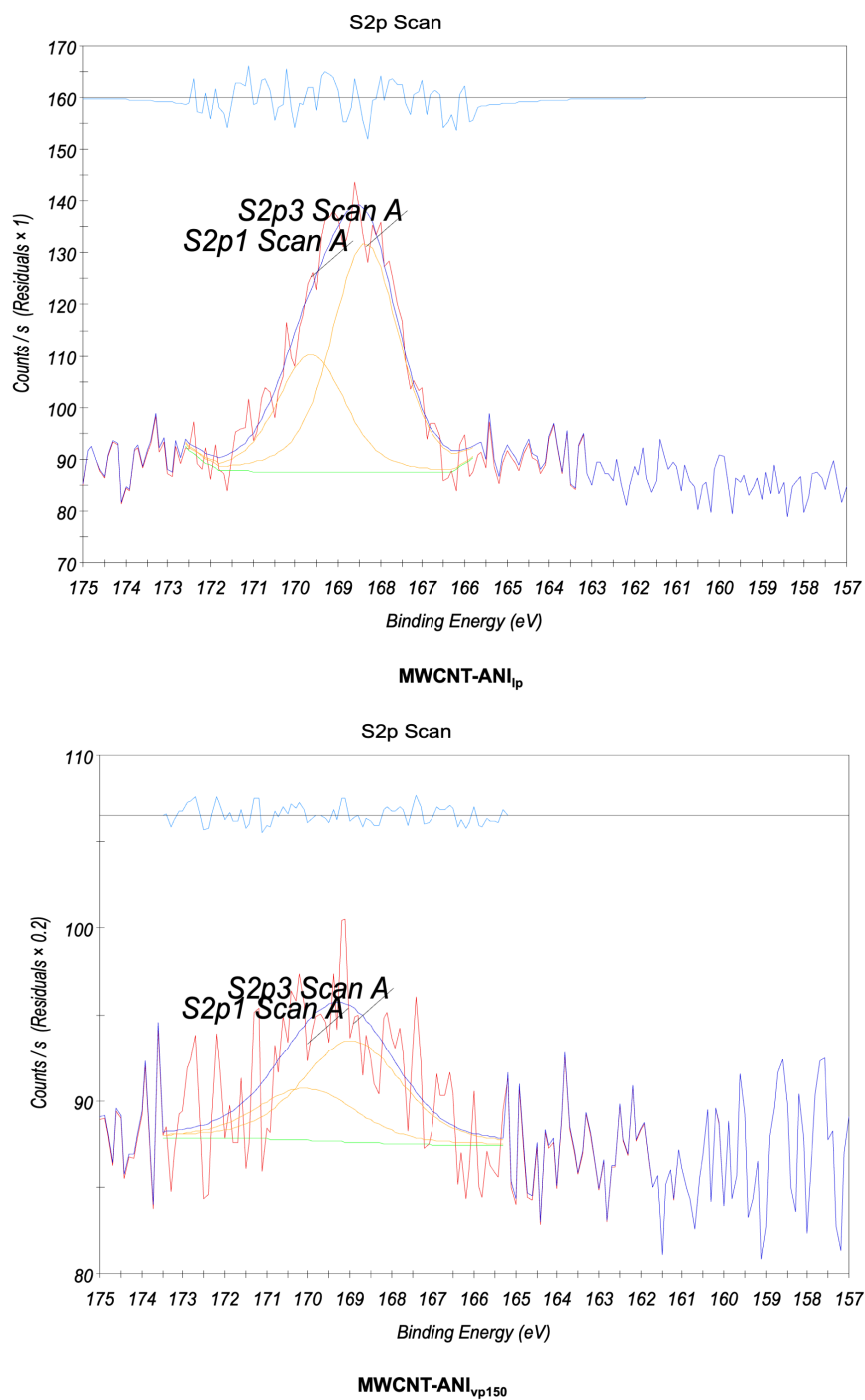


Figure S3.5. High-resolution XPS spectra (S2p) of the functionalized MWCNTs samples.

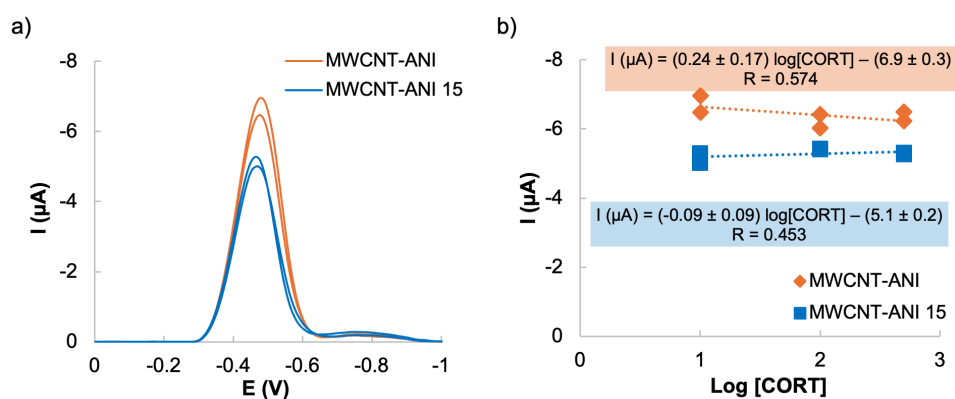


Figure S3.6. a) Comparison of the DPV response and b) current intensity with cortisol increment (nM) of SPCE/MWCNT-ANI_p without and with 15 minutes of incubation time. Two replicates are shown for each measurement.

Chapter 4

Pyrene derivative based sensing platforms

This chapter is based on the articles:

Santos, A. R., Bento, F., Faria, B., Paiva, M. C., Silva, A. F., Viana, A. S., & Viana, J. C. "Pyrene derivative-based electrode for biomarker detection". (Submitted to Small Methods - under review)

Santos, A. R., Bento, F., Faria, B., Paiva, M. C., Silva, A. F., & Viana, J. C. "High-Sensitivity Non-Affinity Cortisol Sensor Based on a Novel Pyrene Derivative: Electrochemical Performance and DFT-Driven Mechanistic Insights". (in preparation)

Santos, A. R., Bento, F., Paiva, M. C., Silva, A. F., & Viana, J. C. "Aqueous pyrene modification of carbon electrodes for cortisol sensing". (in preparation)

4.1 Pyrene derivative-based electrode for biomarker detection

In this work, screen-printed carbon electrodes were modified with a pyrene derivative (PY) derivative to enhance their electrochemical sensing performance. Their applicability was demonstrated in cortisol detection, used here as a model biomarker. This derivative consists of a pyrene functionalized with a pendant group containing a carboxyl function bonded to an aromatic moiety through an amide linkage. The electrode's performance in relation to cortisol was assessed using differential pulse voltammetry (DPV). The results demonstrate the potential of this PY-based electrode for cortisol monitoring without the need for biological recognition elements or molecular probes. Molecular dynamics (MD) simulations provided molecular-level insights into the interactions between the pyrene derivative and cortisol. Overall, the study supports the use of this pyrene derivative as a versatile platform for developing low-cost sensors and simplified detection protocols for cortisol and other biomarkers.

4.1.1 Introduction

Electrochemical sensors have excellent potential for the rapid, non-invasive, and accessible assessment of biomarkers in physiological fluids such as saliva, sweat, and urine, providing user-friendly alternatives for real-time, point-of-care (POC) monitoring of human health, rather than traditional blood laboratory testing (e.g., enzyme-linked immunosorbent assay, enzyme-linked immunosorbent assay (ELISA)). To efficiently detect these analytes at sub-millimolar concentrations, sensors must exhibit both high selectivity and sensitivity. To achieve this, standard sensor configurations rely on recognition elements such as antibodies (Abs), aptamers (Apts), and molecularly imprinted polymers (MIPs) [38, 102]. These biorecognition elements are coupled with signal transduction strategies adapted to the detection mechanism and the specific analyte, commonly assessed by electrochemical techniques such as cyclic voltammetry (CV), DPV, electrochemical impedance spectroscopy (EIS), or square-wave voltammetry (SWV).

Despite antibodies exhibiting extremely high antibody-antigen complex formation constants, which enable low detection limits and high selectivity, the associated measurement assays are usually quite complex. They often involve sandwich or competitive formats and require labeling agents, such as enzymes, nanoparticles, or redox probes (like methylene blue (MB), ferrocene, or Prussian Blue (PB)), to generate a measurable electrochemical signal. Various sensors using antibodies as recognition elements for non-invasive biomarker detection have been reported in the literature, as summarized in Table 4.1.

Table 4.1. Examples of reported electrochemical biosensors for non-invasive biomarker detection, categorized by recognition element, electrode platform, surface modification, detection format, and target analyte.

Recognition element	Electrode Material	Surface modification	Electrochemical method	Detection Format	Analyte	Ref.
Antibody	Gold (Au)	Phenylboronic acid	EIS	Direct and sandwich	C-reactive protein in phosphate buffer and serum	[103]
	Indium tin oxide (ITO) with hydroxyl groups	Poly(3-thiophene acetic acid)	CV and EIS	Direct	TNF α antigen in saliva	[104]
	Graphene oxide (GO)	GO chemically functionalized	CV	Direct	SARS-CoV-2 spike protein in saliva	[105]
	ITO	Pt nanoparticles (NP); hydrazine	Chronocoulometric	Sandwich; Competitive; MB label	Malaria biomarker in saliva	[106]
	Au chip	Alkaline phosphatase (ALP)	Amperometry	Competitive; ALP label	Cortisol in phosphate buffer and serum	[107]
	Carbon yarn	ellipsoidal iron (III) oxide Fe ₂ O ₃	CV and DPV	Fe ₂ O ₃ mediator	Cortisol in sweat	[51]
Aptamer	Fluorine-doped Tin Oxide (FTO)	ZnO nanorods; Au layer	EIS	Direct	Carcino embryonic antigen in saliva	[108]
	Au thin film	Thiolated guanine-rich DNA	SWV	Ferrocene label	K ⁺ in sweat	[109]
	Au NP-MXene	MB-tagged ssDNA	SWV	Competitive; MB redox probe	Estradiol in sweat	[110]
	Graphene quantum dots (GQDs)	[Fe(CN ₆)] ^{3-/4-}	CV	[Fe(CN ₆)] ^{3-/4-} redox probe	Cortisol in saliva	[77]
	AuNP	Triamcinolone pre-bound	SWV	Competitive; Triamcinolone redox probe	Cortisol in phosphate buffer and serum	[72]
	Nitrogen-doped porous reduced graphene oxide (N-prGO)	Pyrene ligands (py-COOH/py-polyethylene glycol)	DPV	[Fe(CN ₆)] ^{3-/4-} redox probe	Cardiac protein biomarker (cTnl) in saliva	[111]
MIPs	Au screen printed electrode (SPE)	Electro-polymerized with acrylamide Micro-patterned MIP	DPV and EIS	[Fe(CN ₆)] ^{3-/4-} redox probe	Glucose in saliva	[112]
	Boron-doped bio-inert nano-crystalline diamond (NCD)	by N,O-bismethacryloyl ethanolamine photopolymerization	EIS	Direct	Testosterone in urine and saliva	[113]
	Au SPE	Cysteamine; Pyrrole electropolymerization Copper	SWV	[Fe(CN ₆)] ^{3-/4-} redox probe	α -amylase in saliva	[114]
	Polypyrrole (PPy) SPE	phthalocyanine tetrasulfonate (CuPcTS)	Amperometry	Direct	Cortisol in artificial sweat	[84]

Likewise, aptamers were used in labeled and label-free detection formats, frequently incorporating

redox-active probes such as MB, ferrocene, or PB. Aptamer-based sensors incorporating various nanomaterials as transducers, including metal nanoparticles, carbon-based nanostructures, metal oxide nanostructures, and 2D materials, have been developed to enhance electron transfer and immobilization efficiency. Representative examples from the literature are listed in Table 4.1.

MIPs, which are polymeric structures tailored to mimic the natural receptors of biomarkers, can provide more robust and cost-effective platforms by using direct detection methods, in which the binding of the target analyte causes measurable changes in the imprinted polymer properties (e.g., impedance, capacitance) [115, 116]. Examples of this methodology are also summarized in Table 4.1.

Each recognition strategy offers distinct advantages and limitations. Although highly selective, Ab production is time-consuming (up to 9 months), expensive, dependent on animal hosts, and subject to batch-to-batch variability. Additionally, they are susceptible to denaturation under non-physiological conditions. Electrochemical biosensors based on Ab often need lengthy incubation times and complex detection methods, such as sandwich or competitive formats. Aptamers are synthetically produced and offer high versatility, though their design and selection remain costly and labor-intensive. They can also be unstable in complex biological environments due to nuclease degradation. Both Ab and Apt must be covalently immobilized on the electrode surface to ensure stable and oriented binding. MIPs tend to be more robust and have lower production costs. However, they can exhibit lower specificity, limited binding site accessibility, template leakage, and often require extensive optimization of synthesis conditions. Regardless of the recognition strategy employed, these biosensors typically require long incubation times to ensure effective target interaction, which limits their suitability for some wearable applications. Signal generation in these systems frequently depends on secondary labels, such as enzymes, redox probes, or metallic nanoparticles, to produce a detectable electrochemical response.

The use of catalytic or electrochemically active materials can present a more straightforward detection method than traditional recognition elements. Materials such as metal oxides, nanozymes, or redox-active nanostructures can interact selectively with target analytes through redox reactions, surface adsorption, or modulation of material activity. This simplifies the sensor design by removing the need for complex biorecognition or labeling steps. Although generally less specific, these materials still provide selective responses through intrinsic chemical affinity or by tailoring surface functionalities. They are ideal for wearable and non-invasive applications that demand durability, low cost, and simpler production. Recently, metalloporphyrin-MWCNT [95] and flexible alginate/copper(II) oxide (CuO) [117] electrodes demonstrated potential for detecting cortisol and ascorbic acid, respectively.

Building on recent advances in biomarker electrochemical sensing, materials that enable straightfor-

ward electrode modification and provide the potential for selective molecular recognition are gaining more attention. Screen-printed carbon electrodes (SPCEs) offer a versatile, low-cost platform, making them ideal for developing sensors for POC applications [118]. In this context, pyrene derivatives have been explored as functional components in electrochemical sensing systems, particularly for modifying carbon-based electrodes. Their immobilization is straightforward and effective because they can form stable $\pi-\pi$ interactions with graphitic surfaces. Gold nanoparticle (AuNP)/multi-walled carbon nanotube (MWCNT) nanocomposite was modified with 1-pyrene carboxylic acid (PCA) for higher electrocatalytic activity, allowing dopamine and uric acid detection via DPV in human serum [119]. Graphene foam was functionalized with pyrene-boronic acid to serve as a molecular recognition site for lactate detection via changes in capacitance in artificial sweat [120]. MWCNT electrodes modified with pyrene derivatives act as mediators for the electrochemical detection of H_2O_2 using horseradish peroxidase (HRP) via CV and amperometric techniques [121]. MWCNT functionalized with a ruthenium complex bearing pyrene chains (RuPEG), which served as an anchor and mediator, was used for glucose detection via chronoamperometry (CA) [122]. MXene nanosheets functionalized with pyrene-tetrasulfonic acid sodium salt (PyTS), an electrocatalytic agent, were used for uric acid detection via DPV and CA measurements in sweat [123]. Although pyrene derivatives have been previously employed as anchors, mediators, and/or electrocatalytic agents in biosensing, their intrinsic electrochemical activity as a direct analytical signal has not been explored. Its molecular structure allows functionalization with various chemical groups, which can be selected based on the target analyte's properties. In particular, the pyrene derivative [124] functionalized with amide and carboxylic acid groups can form hydrogen bonds with various biomarkers of interest and is therefore explored in this study.

In this work, a PY- modified electrode was developed and experimentally tested for cortisol detection as a proof-of-concept. Electrochemical measurements were performed to evaluate the electrode's response to low concentrations of cortisol. MD simulations, widely used in sensor design studies [125–130], were performed to gain molecular-level insights into the interactions between PY and cortisol that underlie the electrochemical response. The results elucidated the binding behavior and showed the potential of the selected pyrene derivative for cortisol detection. This approach eliminates the need for costly biorecognition molecules and complex chemical modifications, offering a potential solution for real-time monitoring in wearable devices.

4.1.2 Experimental section

Screen-printed carbon electrodes (SPCE 110D) were purchased from DropSense (Metrohm, Portugal). Di-Sodium hydrogen phosphate dihydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$), sodium dihydrogen phosphate dihydrate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$), Sodium chloride (NaCl), Hydrocortisone (H0888), and 1-aminopyrene (A77903) were acquired from Merck KGaA, Darmstadt, Germany. The functionalized pyrene (PY) represented in Figure 4.1 was synthesized according to the method described elsewhere [124].

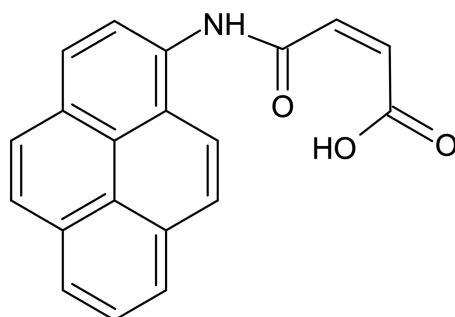


Figure 4.1. Schematics of the functionalized PY.

The SPCE 110D working electrode was modified by drop-casting 5 μL of hydroalcoholic PY solutions of different concentrations. The solutions were prepared from a PY/ethanol stock solution with a concentration of 0.88 mg/mL. This stock solution was diluted with water. Specifically, 1.5 mL, 2.5 mL, and 3.5 mL of the stock solution were diluted to a final volume of 5 mL with water, producing the target concentrations of 0.30 mg/mL (PY3), 0.44 mg/mL (PY44), and 0.62 mg/mL (PY62), respectively. Although the solutions had different ethanol:water ratios, namely 1:2.33 (PY3), 1:1 (PY44), and 2.33:1 (PY62), complete solubilization of PY was ensured in all cases. Finally, the solvent was fully evaporated at room temperature.

Phosphate-buffered saline (PBS) solution (18.8 mM, pH 7) was prepared by dissolving a mixture: 2.759 g of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.52 g of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, and 8.5 g of NaCl in 1 L of water (18.2 M Ω .cm of electrical resistivity obtained from a Milli-Q Gradient A10 system). A hydrochloric acid solution (0.12 mM) was used to adjust the pH.

Cortisol stock solution (20 mM) was prepared by dissolving 724.9 mg of cortisol in 100 mL of ethanol and stored at -22°C . Cortisol working solutions were prepared before each experiment by diluting the stock solution with PBS (supporting electrolyte).

Electrochemical measurements were conducted using an Autolab PGSTAT30 potentiostat controlled by the GPES 4.9 software (EcoChemie). A drop of solution (70 μL) was placed onto the modified SPCE, covering all three electrodes. CV measurements were performed at room temperature with a scan rate

of $100 \text{ mV} \cdot \text{s}^{-1}$. DPV measurements were performed at room temperature with a pulse amplitude of 25 mV, a potential step of 5 mV, a modulation time of 0.05 s, and an interval of 0.5 s.

The working electrode (WE) surface was observed using microscope images obtained with a Leica DM4 P microscope (Wetzlar, Germany), equipped with incident light and bright-field illumination. Images were acquired using 2.5 $\times$ and 50 $\times$ objective lenses. The samples were coated with 9 nm of gold (Au)/palladium (80/20 %W) to enable scanning electron microscopy (SEM) analysis using a NanoSEM-200 apparatus from FEI Nova. The sample's morphology was further characterized by atomic force microscopy (AFM) analysis using a Multimode 8 HR microscope (Bruker) coupled to a Nanoscope V controller, operated in Peak Force Tapping and ScanAsyst modes, with silicon nitride ScanAsyst-Air probes (nominal spring constant of 0.4 N/m, tip radius $\sim$ 2% nm) and a scan rate of ca. 1 Hz.

4.1.3 Computational approach

MD simulations were performed using LAMMPS software [131] on a local Supermicro server equipped with AMD EPYC multi-core processors. The modeled systems contained 4000 ethanol molecules, 20 PY molecules, and 10 cortisol molecules. Although the number of molecules does not reflect experimentally realistic concentrations, this composition was chosen to ensure enough interaction events within a feasible simulation timescale. The simulations were performed in an ethanol solvent model, a deliberate simplification to isolate interactions between PY and cortisol. In mixed ethanol–water systems, strong hydrogen-bonding interactions involving water introduce significant competition effects, which can obscure direct PY–cortisol association. Ethanol was therefore selected as a simpler protic solvent that allows hydrogen bonding while reducing competitive solvent effects, enabling clearer observation of interactions within accessible simulation timescales. Accordingly, the simulations were intended to provide qualitative mechanistic insight into PY–cortisol interactions, rather than a quantitative representation of the experimental hydroalcoholic or aqueous electrolyte systems. Ethanol was modeled using the OPLS-AA force field [132], while PY and cortisol molecules were described using parameters from the GROMOS54A7 force field [133]. The use of these two distinct force fields, which follow different parametrization strategies, was motivated by the availability of suitable parameters for each component. These force fields are widely used for organic systems and provide a reasonable qualitative description of intermolecular interactions. However, no specific reparameterization or validation was performed for the pyrene derivative, and the simulations are therefore presented qualitatively. Given the aim to qualitatively explore intermolecular interactions, and since no quantitative free energy estimations were intended, the combination of these force fields was considered acceptable.

Intermolecular non-bonded interactions were handled using standard Lennard-Jones potentials, with care taken to ensure consistent combination rules and unit conventions.

A cutoff distance of 12 Å was employed for non-bonded and coulombic interactions. The standard particle-particle particle-mesh (PPPM) was used to solve long-range Coulombic interactions. Periodic boundary conditions were applied in all directions.

The system was first equilibrated under canonical (NVT) conditions using the Nosé–Hoover thermostat for 200 ps to randomize molecular positions and relieve steric overlaps. Subsequently, the simulation box was gradually compressed under NVT conditions to a volume that corresponds to a density slightly above the theoretical value (0.813 g/cm³), calculated as a mass-weighted average of the pure-component densities (Equation 4.1):

$$\rho_{mix} = \sum_i \omega_i \rho_i \quad (4.1)$$

Where ρ_i and ω_i are the density and mass fraction of component *i* (ethanol, PY, cortisol), respectively. ω_i is calculated by Equation 4.2.

$$\omega_i = \frac{N_i m_i}{\sum_j N_j m_j} \quad (4.2)$$

Where N_i is the number of molecules, and m_i is the mass per *i* molecule ($m_i = \frac{M_i}{N_A}$, M_i is the molar mass, and N_A is Avogadro's number).

After reaching the approximate target volume, the system was further equilibrated for 1 ns under constant volume and temperature (NVT). This was followed by a 10 ns equilibration in an isothermal-isobaric (NPT) ensemble at 300 K and 1 atm, using the Nosé–Hoover thermostat and barostat. A 20 ns production run was then performed under NPT conditions, with only the last 10 ns analyzed to ensure complete equilibration. A timestep of 1 fs was used throughout the simulation, with covalent bonds involving hydrogen atoms constrained using the SHAKE algorithm, applying a relative tolerance of 10^{−4} to ensure numerical stability. The interaction energy (eV) between PY and cortisol molecules was recorded during the production run. This energy between pyrene and cortisol was computed using the compute group/group command in LAMMPS, which calculates the total pairwise non-bonded energy (Lennard-Jones and Coulomb) between two defined atom groups at each timestep.

A Python program was used to quantify the number of PY molecules per cortisol molecule within a 4 Å radius. The visual molecular dynamics (VMD) software [134] was used to analyze hydrogen bonds (HBonds) between specific PY and cortisol functional groups.

4.1.4 Results

4.1.4.1 Morphological characterization

Morphological characterization of both bare and modified WE surfaces was performed using optical microscopy, SEM, and AFM to investigate the effect of PY modification and its influence on the surface area. At low magnification (Figure 4.2a), optical images of the bare WE showed a relatively uniform surface. At higher magnification, a carbonaceous structure consistent with the typical morphology of SPCE was evident, characterized by various-sized cavities and a rough texture (Figure 4.2b). In contrast, the PY-modified WE showed light reflective zones scattered across the surface at lower magnification (Figure 4.2c), indicating a heterogeneous deposition or aggregation of the PY. At higher magnification (Figure 4.2d), the presence of needle-like structures became clear. These formations are likely due to the self-assembly of PY molecules, which can form extended supramolecular architectures under certain conditions.

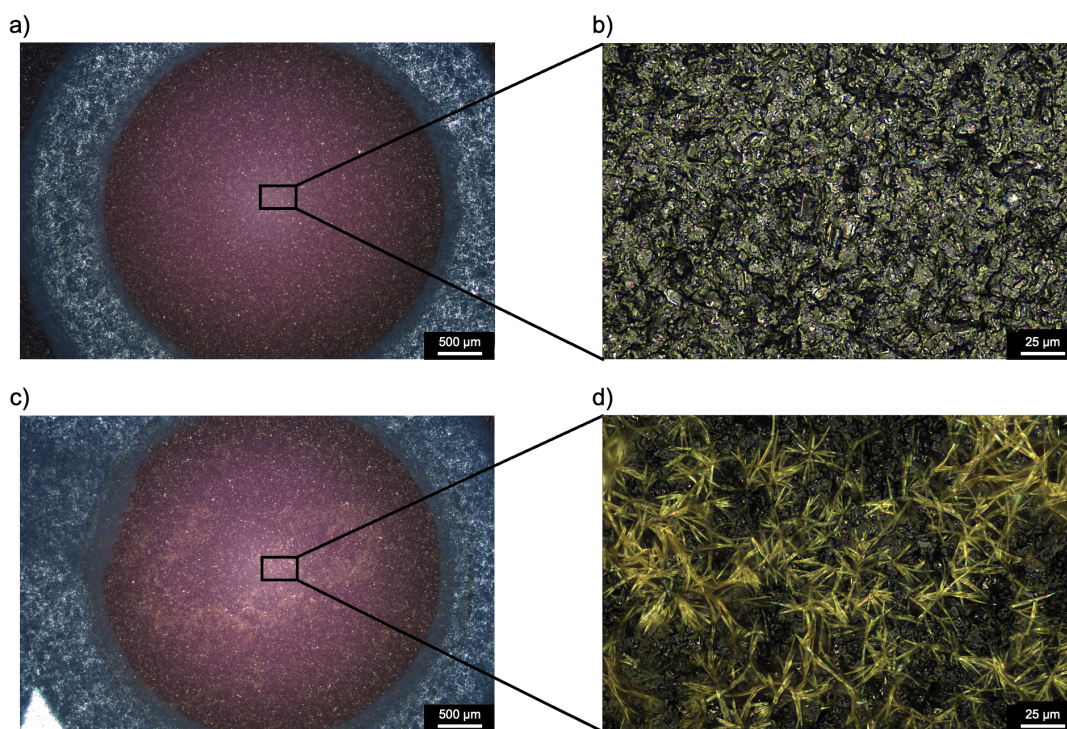


Figure 4.2. Optical microscope images of the WE surface of SPCE, showing bare (a, b) and PY-modified (c, d) electrodes at low and high magnification, respectively.

SEM images provided additional insight into the morphology of the WE. The bare WE (Figure 4.3a,b) exhibited a surface with carbon nanoparticles interspersed among randomly distributed graphite flakes, resulting in visible pores and surface roughness. The PY-modified WE presented a random distribution of elongated ribbon-like structures [135–138] across the entire surface (Figure 4.3c). These PY-based

structures were partially elevated from the carbon substrate, indicating a non-collapsed architecture. These ribbon structures varied in length and morphology, with measured thicknesses ranging from approximately $1.5\ \mu\text{m}$ to $4.0\ \mu\text{m}$ (Figure 4.3d).

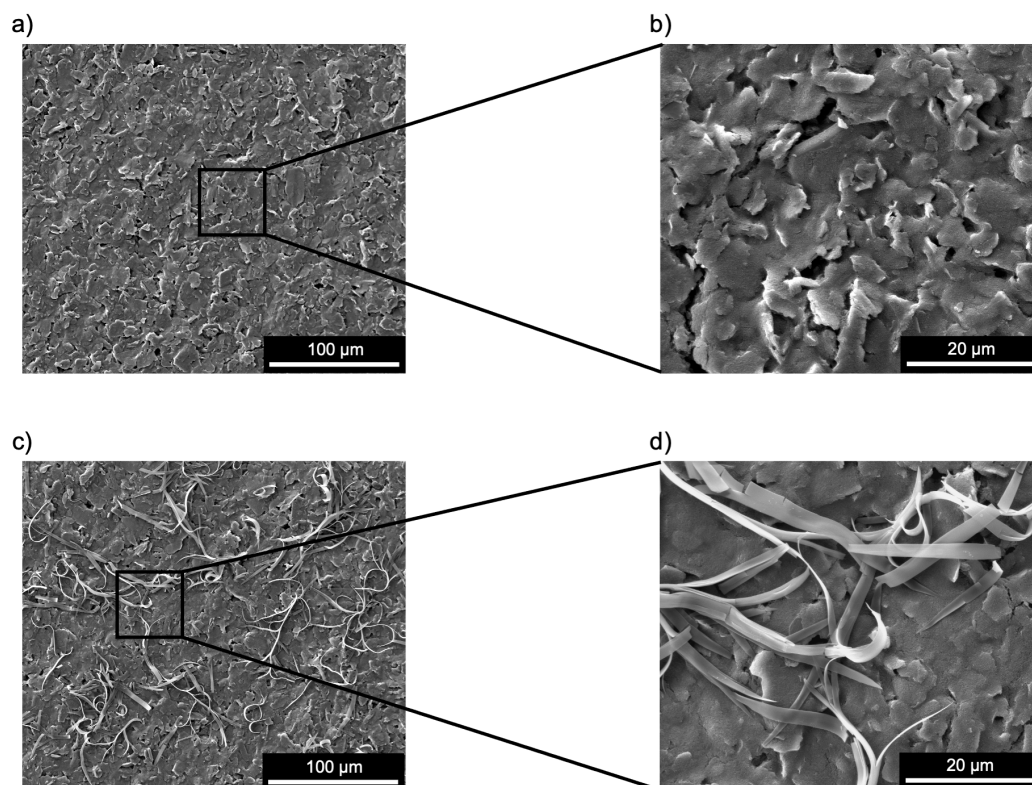


Figure 4.3. SEM images of the WE surface of SPCE, showing bare (a, b) and PY-modified (c, d) electrodes at different magnifications.

AFM 3D images provided high-resolution information on the surface topography of the bare SPCE (Figure 4.4a) and PY-modified SPCE (Figure 4.4b). The bare SPCE shows an uneven surface typical of a printed carbon layer. In contrast, the PY-modified electrode clearly exhibits a distinctive tubular structure, which can be associated with the ribbon-like PY already observed in optical and SEM images. The relatively large size of the pyrene crystals and the resulting high surface roughness of the modified electrodes limited the achievable AFM resolution. Nevertheless, these observations confirm the presence of PY on the WE surface and highlight the morphological differences introduced by the modification.

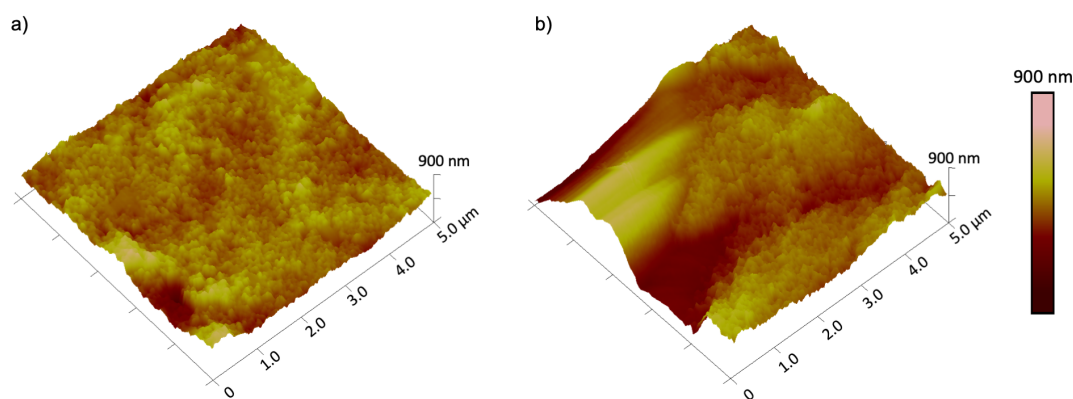


Figure 4.4. AFM topography images of the WE surface of a) bare and b) PY modified SPCE.

The increased surface roughness and three-dimensionality brought by the ribbon-like PY structures are expected to significantly enhance the electroactive surface area and provide additional active sites for interaction with cortisol molecules.

4.1.4.2 Voltammetric characterization of the sensor response

The SPCE modified with the pyrene-containing amide and carboxylic groups (PY) was characterized using CV over a wide potential range. Figure 4.5a shows the voltammetric response of SPCE/PY in PBS in the absence of cortisol over three cycles. In the first cycle, a single oxidation peak (I) appears at 0.67 V followed by three reduction peaks (II, III, and IV) at 0.12, -0.1, and -1.2 V, respectively. In the second and third cycles, two partially overlapping oxidation peaks (II' and III') appear at around 0.2 V while the peak already visible in the first cycle at about -0.87 V which may be associated with O_2 reduction, decreased significantly. The intensities of reduction peaks II and III, and oxidation peaks II' and III', increase over successive cycles, while the intensity of peak I decreases progressively. This behavior suggests electroactive processes associated with PY, possibly involving the formation of intermediate species, which alter the surface or lead to partially irreversible reactions. Peak IV, observed in the reduction scan at approximately -1.22 V, does not show a counter-peak. Unlike peaks II and III, it considerably decreases from cycle 1 to cycle 2. This behavior indicates that the reduction of PY at the electrode surface during the first cycle was extensive and irreversible.

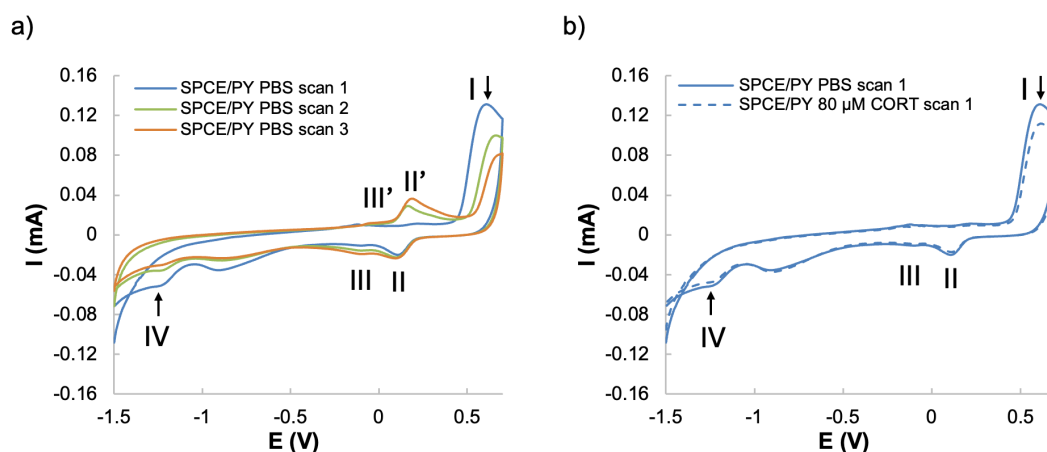


Figure 4.5. CV of a) SPCE/PY in PBS, pH 7.0, over three cycles in the potential range of -1.5 to 0.7 V, and b) comparison of the 1st cycle of SPCE/PY in PBS and 80 μ M cortisol solution in the same potential range.

Figure 4.5b shows the comparison of the voltammetric response (1st cycle) of SPCE/PY in PBS, in the absence (solid line) and in the presence (dotted line) of cortisol ([CORT] = 80 μ M). The voltammetric response of SPCE/PY in the presence of cortisol is similar to that in its absence, showing no process directly attributable to this molecule. This indicates that cortisol is not electroactive despite the SPCE surface modification, and there is also no evidence of its participation in any catalytic reaction. However, the voltammogram recorded in the presence of cortisol tends to show lower peak currents, especially for peak I in the anodic scan and for peak IV in the cathodic scan. These differences can be attributed to the stabilization of PY by cortisol, which promotes cortisol adsorption at the electrode surface via intermolecular interactions. This shift was further analyzed within restricted potential ranges using the more sensitive electrochemical technique, DPV. The most significant changes in current were observed in the potential regions corresponding to Peaks I and IV. Although cortisol also affects the oxidation peak (Peak I), it exhibited higher variability, likely due to overlap with background oxidation processes. For this reason, the reduction peak (Peak IV) at approximately -1.1 V was selected for quantitative analysis due to its greater stability and reproducibility. For additional experimental data, see Figure S4.1 (Appendix).

Figure 4.6 compares the DPV voltammograms obtained using SPCE modified by drop-casting 5 μ L of the different PY solutions: 0.30 mg/mL (PY3), 0.44 mg/mL (PY44), and 0.62 mg/mL (PY62). Figure 4.6a shows the responses of the three modified electrodes without cortisol, while Figure 4.6b shows the responses in the presence of cortisol at micromolar concentrations (namely 80, 190, and 380 μ M). The displayed voltammetric curves correspond to the first scan, as a substantial decrease in the peak height is observed in subsequent scans, as was described above for the CV results.

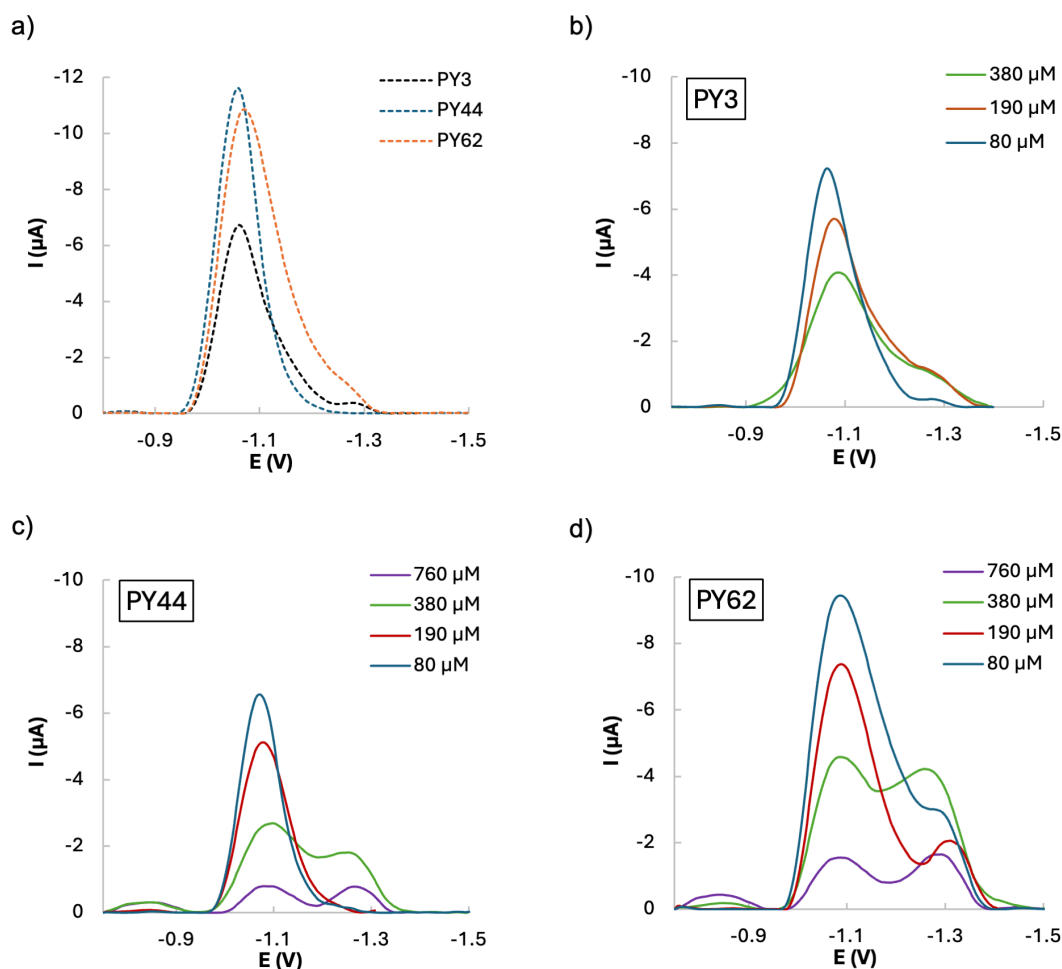


Figure 4.6. Comparison of DPV responses obtained using SPCE modified with 5 μ L of PY solutions, 0.30 mg/mL (PY3), 0.44 mg/mL (PY44), and 0.62 mg/mL (PY62) in PBS in the absence (a) and presence of cortisol at different concentrations (b, c, and d).

In the absence of cortisol, the reduction process gives rise to a main peak around -1.1 V followed by a smaller one at approximately -1.22 V which is not entirely resolved from the first peak (Figure 4.6a). The lowest current intensity was observed for the electrodes modified with the smallest PY amount, PY3. At the same time, no significant difference was found between the peak heights of PY44 and PY62. This result indicates that the WE surface may be saturated for PY44, so additional PY deposited can form a second layer or form aggregates that are not electroactive.

In the presence of cortisol (Figure 4.6b, 4.6c, and 4.6d), no additional peaks are observed. However, the main peak current intensity (I_p) decreases progressively with increasing cortisol concentration, indicating interference with the electrochemical reduction of PY at the electrode surface. This effect is consistent with cortisol adsorption onto the PY layer, stabilizing its oxidized form and thereby limiting its reduction. This interaction is further investigated through MD simulations, presented in the following section. A slight shift of the main peak potential towards more negative values is observed, more clearly for the PY3

electrode (Figure 4.6b), which may be due to partial overlap of electrochemical contributions within the overall signal. While these structures were already present in the SPCE/PY, their importance increases substantially in the presence of cortisol, suggesting that their formation becomes more pronounced. The effect of cortisol concentration is not straightforward because the peak partially overlaps the main peak, which tends to decrease with cortisol concentration. Comparable DPV responses were obtained from two independent SPCE replicas for each PY concentration at low and high cortisol concentrations (Figure S4.2).

The concentration effect was further analyzed using a semi-log plot (Figure 4.7) over the cortisol concentration range of 80-760 μM . The three SPCE/PY displayed linear trends within the cortisol concentration range studied, with high correlation coefficients, suggesting that adsorption processes control variation in the electrical current intensity. From the slope values, it can be observed that SPCE/PY62 leads to the highest sensitivity for cortisol detection (slope: 8.1 $\mu\text{A}/\text{dec}$). The SPCE/PY44 showed a slightly lower sensitivity (slope: 6.3 $\mu\text{A}/\text{dec}$), while SPCE/PY3 exhibited the lowest sensitivity (slope: 4.6 $\mu\text{A}/\text{dec}$). This configuration has lower PY loading, which decreases the number of active sites and thus lowers detection sensitivity. These results demonstrate the suitability of PY-modified SPCEs as electrochemical sensing platforms, effectively enabling cortisol detection under the tested conditions.

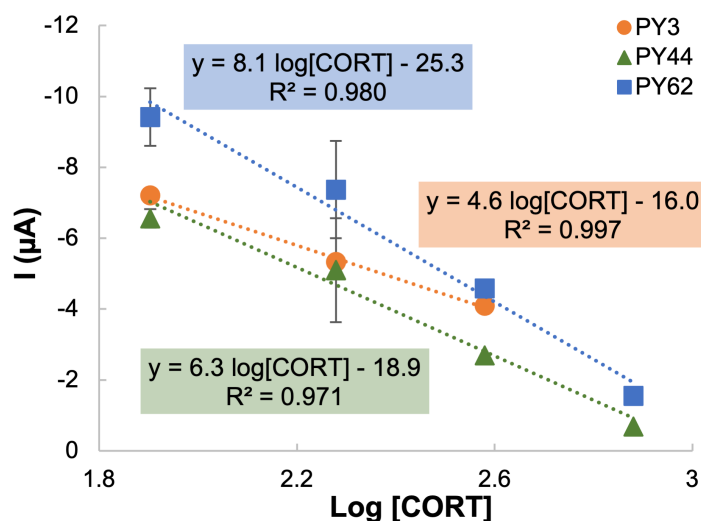


Figure 4.7. Comparison of the response of SPCE modified with 5 μL of each PY solution, 0.30 mg/mL (PY3), 0.44 mg/mL (PY44), and 0.62 mg/mL (PY62), to different cortisol concentrations (μM).

4.1.4.3 Molecular dynamics simulations

MD simulations were used to study the interaction mechanism between PY and cortisol. The simulated equilibrium density was $\rho_{\text{sim}} = 0.808 \pm 0.002 \text{ g/cm}^3$ (average $\pm$ standard deviation over the last 10 ns of the production run). Compared with the theoretical mixture density ($\rho_{\text{mix}} = 0.813 \text{ g/cm}^3$), the small

deviation of 0.59% indicates that the system reached a stable equilibrated state with a physically reasonable density. However, this comparison should not be interpreted as a full validation of the solvent model, and the simulation results are therefore discussed qualitatively.

Figure 4.8a,b) compares the interaction energy (eV) between PY and CORT registered over 10 ns of simulation with the calculated number of PY per CORT ($N^{\circ}\text{PY}/\text{CORT}$). The interaction energy is negative, indicating an attractive force between the two molecule types. Its value fluctuates between -0.1 and -3.1 eV throughout the simulation due to the dynamics of the molecules, reaching a maximum around 5.3 ns. This behavior is consistent with the oscillations in the number of PY per CORT. The spatial proximity between PY and CORT molecules directly influences the interaction energy. When a larger number of PY molecules are located within 4 Å of a CORT molecule, the attractive interaction increases, resulting in a more negative energy. The maximum $N^{\circ}\text{PY}/\text{CORT}$ value of 12, recorded at around 5.3 ns, coincides with the lowest interaction energy, confirming this relationship. Conversely, when fewer PY molecules are found within this range, the interaction weakens, and the energy becomes less negative.

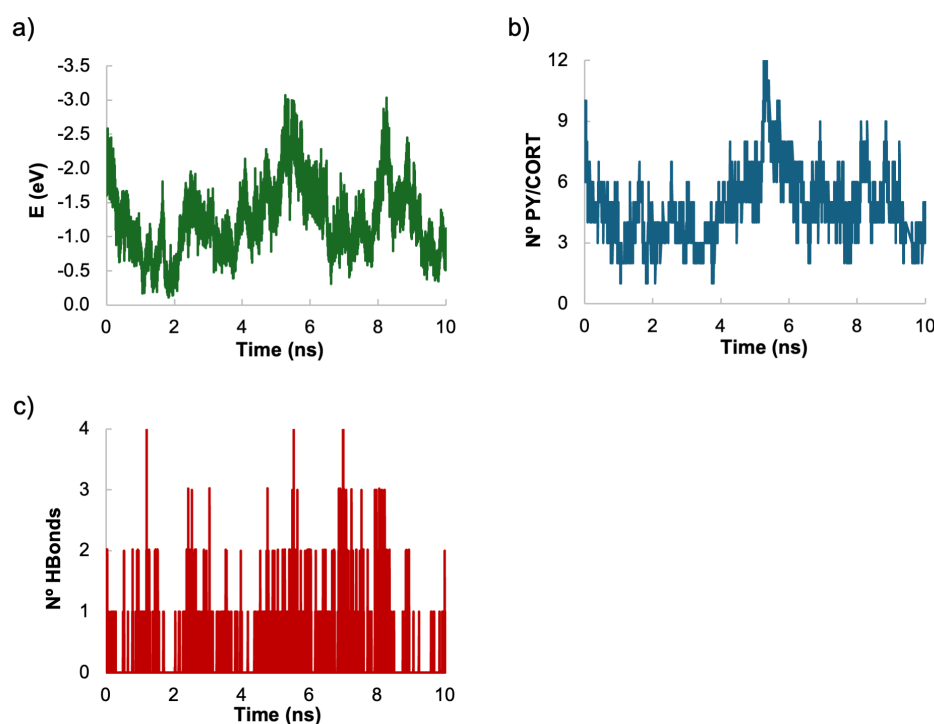


Figure 4.8. Comparison of the a) interaction energy (E (eV)), b) number of PY per cortisol molecules ($N^{\circ}\text{PY}/\text{CORT}$), and c) number of hydrogen bonds ($N^{\circ}\text{HBonds}$) throughout 10 ns of MD simulation.

VMD software was used to perform hydrogen-bond (HBond) analysis between PY and cortisol, using cutoffs of 110° and $3.5 \text{ \AA}$. The minimum and maximum values of the HBond coincide with the oscillations in interaction energy and $N^{\circ}\text{PY}/\text{CORT}$ (Figure 4.8b,c) since much of the interaction between the two molecules is due precisely to H bonds. The interactions between specific PY atoms (amide nitrogen

– N, carbonyl oxygen – Oid10 and Oid4, and hydroxyl group – OHid2) and CORT hydroxyl functional groups (Ohi39, Ohi42, and Ohi55), which are the most likely sites for hydrogen bond formation, were analyzed and are summarized in Figure 4.9. The global hydrogen bond analysis per PY atom shows that the carbonyl group Oid10 forms the highest overall percentage of hydrogen bonds (32.6%) with CORT hydroxyl groups. Among these, the Oid10–Ohi55 pair is the most frequent, suggesting a slightly preferred interaction site. Additionally, the remaining interaction sites, N, OHid2, and Oid4 – CORT hydroxyl groups also show considerable percentages of 18.4%, 19.6%, and 25.8%, respectively. These results indicate strong interactions between the two molecule types at multiple sites, which help explain the observed electrochemical behavior. Detailed HBond results for each PY/CORT atom pair are available in Table S4.1 (Appendix).

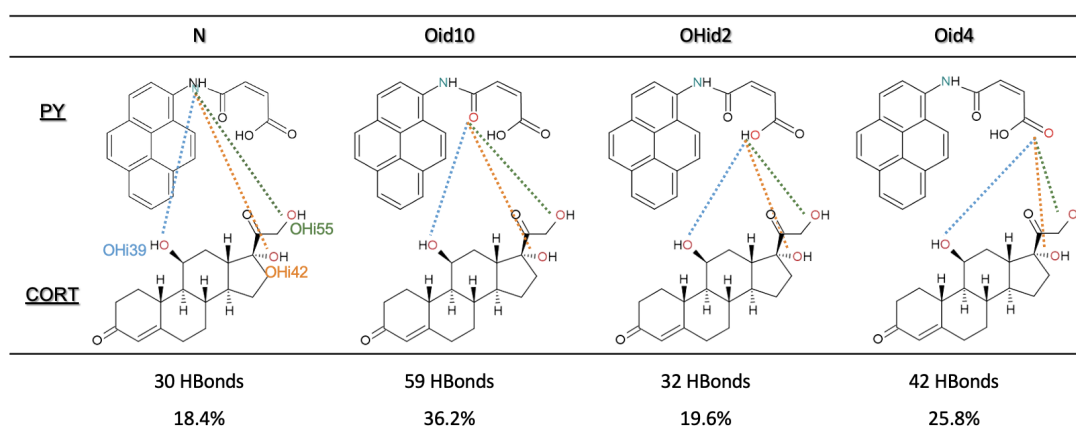


Figure 4.9. Schematics of PY and cortisol interactions and respective hydrogen bond results.

These comprehensive analyses, which combine interaction energy, intramolecular distance ($N^{\circ}PY/-CORT$), and HBond, support the existence of stable and specific interactions between PY and CORT. These findings highlight a complex interaction profile involving multiple binding modes, predominantly governed by hydrogen bonding.

4.1.5 Conclusions

The PY-modified SPCE exhibited characteristic electrochemical behavior, showing a distinct reduction peak at -1.1 V in cortisol-free solution. As cortisol concentration increased, the peak's intensity gradually decreased. This behavior indicates that the interaction between PY and cortisol stabilizes the PY molecule, leading to a less extensive reduction process or a shift toward more negative potentials. The sensitivity toward cortisol was found to depend on the PY loading on the working electrode, reaching a maximum of $8.1 \mu A/dec$ (correlation coefficient 0.980) for 0.62 mg/mL PY. A positive linear correlation between sensitivity and PY concentration on the electrode was observed (within the range of variation considered).

MD simulations provided further insight into the mechanism of PY and cortisol interactions. Hydrogen bond analysis revealed that the hydroxyl groups of cortisol can interact with PY's multiple functional groups. These results corroborate the hypothesis that hydrogen bonding with cortisol contributes to PY stabilization, thereby decreasing its electrochemical reduction response.

Overall, this straightforward approach demonstrates the potential of PY as an electrochemical sensing material, enabling analyte detection without the need for complex recognition elements, labels, or mediators. To further advance this platform toward practical POC diagnostics, an assessment of the cross-reactivity with other glucocorticoids and the evaluation of matrix effects in complex biological fluids remain essential. Such investigations are vital for establishing the platform's analytical reliability and confirming its effectiveness in practical POC applications.

4.1.6 Appendix

Figure S4.1 shows the voltammetric response (3 cycles) of SPCE/PY in PBS, in the absence and presence of cortisol ([CORT] = 80 μ M). The dotted line represents the electrochemical response of the SPCE/PY in the presence of cortisol ([CORT] = 80 μ M).

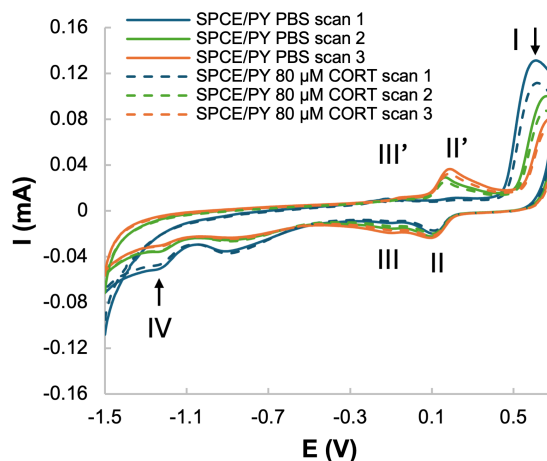


Figure S4.1. CV of SPCE/PY in PBS, pH 7.0, and 80 μ M cortisol solution over 3 three cycles in the potential range from -1.5 to 0.7 V.

Figure S4.2 compares the DPV response of SPCE modified with PY3, PY44, and PY62 to high and low limit cortisol concentrations (μ M). For each cortisol concentration, the sensor replicas showed a consistent behavior, reflecting the precision and reproducibility of the PY-based sensors.

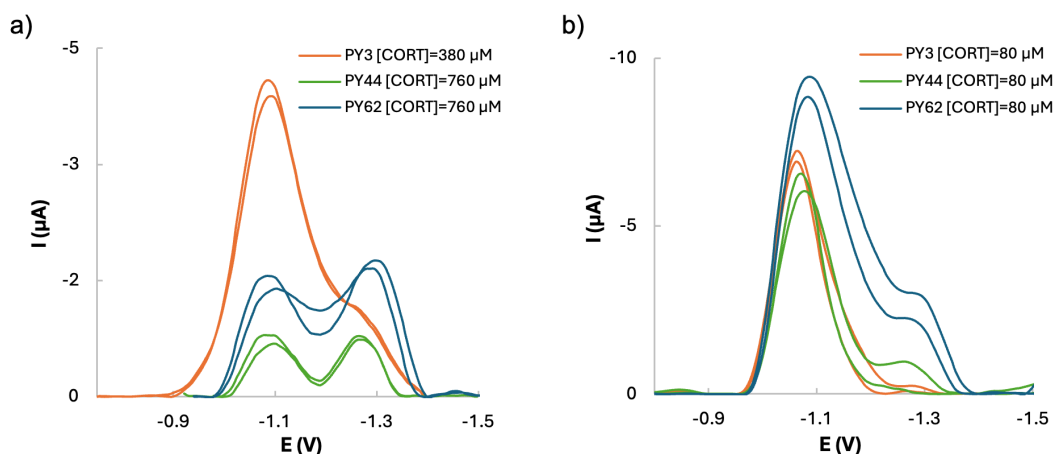


Figure S4.2. Comparison of the DPV response of SPCE modified with three PY concentrations: PY3 (0.30 mg/mL PY), PY44 (0.44 mg/mL PY), and PY62 (0.62 mg/mL PY) to high (a) and low (b) cortisol concentrations (μ M). Two replicates are shown for each measurement.

Table S1 presents the detailed hydrogen bond analysis over 10 ns of MD simulation. The interactions between each PY atom (N, Oid10, OHid2, and Oid4) and CORT hydroxyl groups (OHi39, OHi42, and OHi55) were analyzed in terms of the number of unique hydrogen bonds (N° HBond), hydrogen bonds per PY (HBond/PY), hydrogen bonds per CORT (HBond/CORT), and the percentage of PY and CORT molecules participating in the interaction. The Oid10–Ohi55 pair showed the highest values for most parameters. However, other pairs, such as Oid4–Ohi55, also exhibited high hydrogen bonds per PY and a significant percentage of CORT molecules involved.

Table S4.1. Hydrogen bond analysis results of MD simulations.

PY Atom	CORT Atom	N° HBond	HBond/PY	HBond/CORT	% PY	% CORT
N	OHi39	6	0.3	0.6	25	40
	OHi42	10	0.5	1	30	50
	OHi55	14	0.7	1.4	40	60
Oid10	OHi39	15	0.75	1.5	60	60
	OHi42	20	1	2	60	80
	OHi55	24	1.2	2.4	75	90
OHid2	OHi39	10	0.5	1	30	50
	OHi42	9	0.45	0.9	30	60
	OHi55	13	0.65	1.3	40	70
Oid4	OHi39	14	0.7	1.4	55	50
	OHi42	16	0.8	1.6	50	80
	OHi55	22	1.1	2.2	65	90

4.2 High-Sensitivity Non-Affinity Cortisol Sensor Based on a Novel Pyrene Derivative: Electrochemical Performance and DFT-Driven Mechanistic Insights

Efficient real-time cortisol monitoring necessitates low-cost, stable, and scalable sensing platforms. Herein, we report a non-affinity electrochemical platform based on a novel PY-modified electrode for the direct detection of cortisol at nanomolar levels. The sensor exhibits a robust logarithmic response across a 3–500 nM range, yielding a sensitivity of 2.21 $\mu\text{A}/\text{decade}$ and a high correlation coefficient ($R = 0.987$). An exceptional limit of detection (LOD) of 3 nM was attained, positioning this platform among the most competitive non-affinity cortisol sensors. Density functional theory (DFT) calculations provide quantitative insight into multisite PY–cortisol interactions, consistent with the observed electrochemical response. This synergy between analytical performance and mechanistic clarity supports the development of next-generation wearable stress monitoring devices.

4.2.1 Introduction

In a modern, highly competitive society, chronic workplace stress has emerged as the epidemic of the 21st century [1, 2], leading to burnout syndrome, affecting millions of people worldwide, according to the World Health Organization (WHO). Cortisol, a primary glucocorticoid, serves as a critical physiological biomarker. Persistent dysregulation of its diurnal rhythms and elevated baseline levels are clinical hallmarks of chronic HPA axis activation [18, 23].

As discussed in the state-of-the-art (Chapter 2), electrochemical sensors based on complex biorecognition molecules (e.g., antibodies and aptamers) or MIPs, integrated with diverse electrode materials, have been extensively investigated for POC cortisol sensing, alongside emerging non-affinity-based approaches. Chapter 2 further provides a comprehensive overview of sensing architectures, summarized in Table 2.2. These recognition-based systems achieve high selectivity through specific molecular interactions. However, they often require complex fabrication protocols and involve high production costs. Their operational stability may also be compromised in non-physiological environments. Current non-affinity-based approaches still rely on complex surface chemistries or expensive functional materials, which limit their practical applicability. Nevertheless, these material-based sensors offer important advantages by directly transducing cortisol–material interactions into electrochemical signals, enabling simpler fabrication and improved suitability for real-world applications.

At the molecular level, these interactions have also been investigated using theoretical approaches. DFT calculations have been employed to investigate cortisol–material interactions at the molecular level. Reports demonstrate the use of DFT to select functional monomers for MIPs by calculating binding energies between cortisol and monomers such as pyrrole or o-phenylenediamine [89, 139–142], enabling the identification of stable pre-polymerization configurations and associated hydrogen-bonding interactions. This method has also been applied to study the electronic structure and equilibrium geometry of cortisol and related steroid molecules [143, 144]. Others investigate the formation of supramolecular complexes through non-covalent interactions in novel sensing probes [145], as well as to study the electrochemical reduction of cortisol catalyzed by metalloporphyrins [95, 146]. These studies demonstrate the utility of theoretical calculation for the design of sensing materials and the mechanistic interpretation of experimentally observed electrochemical responses.

In our previous study, a pyrene-derivative-modified electrode was introduced as an electrochemically active sensing interface for cortisol detection, in which cortisol interaction was shown to partially block and stabilize one of the pyrene reduction peaks. This effect was supported by MD simulations that provided initial molecular-level insight into PY–cortisol interactions.

In the present work, the same material platform is developed and optimized to enable cortisol detection at nanomolar concentrations. In addition, DFT calculations are employed to complement the previous MD analysis by enabling a quantitative assessment of interaction energies between specific PY–cortisol atomic pairs and providing deeper insight into the interaction-driven sensing mechanism. By avoiding costly biorecognition elements and complex functionalization, this platform provides a proof-of-concept for real-time wearable stress monitoring.

4.2.2 Experimental section

Screen-printed carbon electrodes (SPCE 110D, DropSens, Metrohm, Portugal) were used as received. The functionalized pyrene (PY) was synthesized following a previously reported procedure [124].

Artificial sweat was prepared following a modified EN 1811:2011 European Standard to simulate a normal hydration state [147]. A 500 mL solution containing NaCl, KCl, urea, and lactic acid was prepared with final concentrations of 8.5 mmol/L, 1.3 mmol/L, 1.6 mmol/L, and 1.7 mmol/L, respectively. The solution corresponded to a 1:10 dilution of a dehydrated sweat profile, and the pH was adjusted to 6.5 using sodium bicarbonate.

The SPCE working electrode was modified by drop-casting 5 μ L of a hydroalcoholic PY solution (0.44 mg/mL in ethanol:water, 1:1 v/v), followed by drying at room temperature. PBS and cortisol solu-

tions were prepared as described previously [101]. Electrochemical measurements were also performed using the same protocol. The SPCE/PY-based electrodes were incubated with the cortisol solutions for 15 min prior to DPV measurements.

4.2.3 Computational approach

To investigate molecular interactions between modified pyrene and cortisol, the ground-state geometries of the isolated molecules and their complexes were fully optimized at the DFT level. Theoretical calculations were carried out using the ORCA software package (version 6.0.0). The Becke, 3-parameter, Lee-Yang-Parr (B3LYP) hybrid functional was employed due to its well-established reliability in describing organic molecules and steroid structures [78]. Long-range van der Waals interactions and weak non-covalent forces were accounted for using Grimme's D3 dispersion with Grimme's D3 dispersion with Becke-Johnson damping (D3BJ). The def2-TZVP basis set was selected to provide an accurate description of the electronic structure while maintaining a reasonable computational cost [142, 148]. Geometry optimizations were performed using tight convergence criteria for the self-consistent field cycle (TightSCF) to ensure the stability of the stationary points. Twenty initial pair configurations of cortisol-pyrene were generated using Avogadro. For each configuration, a different pair of atoms (one from cortisol and one from pyrene) was selected and positioned at an initial separation of approximately 2.4 Å. This procedure ensured systematic sampling of different relative orientations and potential interaction sites prior to geometry optimization. The interaction energies (ΔE) were calculated as the difference between the total energy of the optimized pair configuration and the sum of the energies of the individual isolated molecules (Equation 4.3).

$$\Delta E = E_{pairconfiguration} - (E_{cortisol} + E_{PY}) \quad (4.3)$$

The ΔE values represent the interaction energies for each optimized geometry, enabling comparisons of both the relative stabilities of pair configurations and the predicted strength of the cortisol-pyrene interaction (within the chosen level of theory). Atomic partial charges were obtained using the CHELPG scheme, based on electrostatic potentials calculated at the same level of theory for the optimized geometries.

4.2.4 Results

4.2.4.1 Voltammetric characterization of the sensor response

The electrochemical performance of the optimized PY-modified electrode was evaluated across a physiologically relevant range (3–500 nM). In Figure 4.12a, the DPV response of SPCE/PY44 over a cortisol concentration range of 3–500 nM is shown. A characteristic PY reduction peak at approximately -1.05 V (peak I) partially overlaps with a smaller feature around -1.2 V. The intensity of peak I decreases with increasing cortisol concentration, consistent with our previous observations [101]. This behavior may be attributed to cortisol adsorption onto PY, reducing its electrochemical reduction extent.

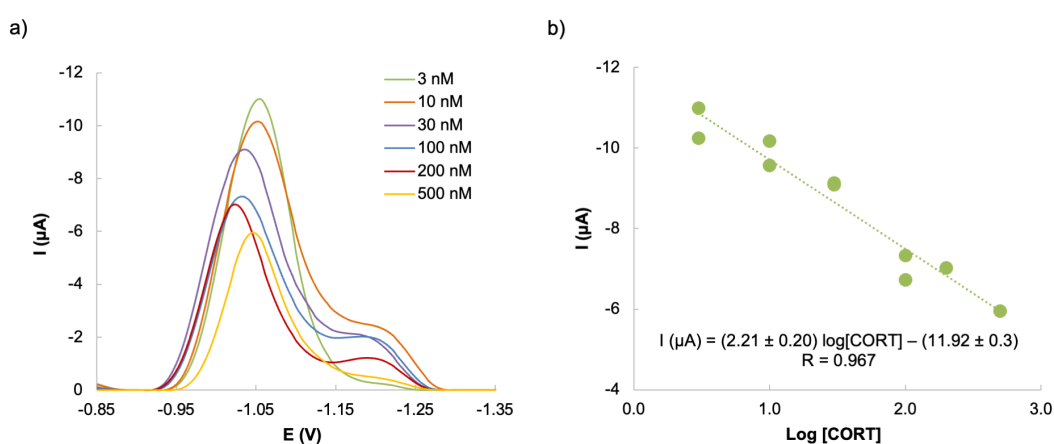


Figure 4.12. Electrochemical response of PY-modified SPCE (0.44 mg/mL, PY44) across nM cortisol concentrations (a) and its calibration curve (b).

The sensor exhibits a logarithmic response (Figure 4.12b), with the calibration curve described by $I (\mu A) = 2.21 \log[CORT] + 11.92$. This corresponds to a sensitivity of $2.21 \mu A/\text{dec}$ with a correlation coefficient (R) of 0.967, indicating good linearity and indicating a linear response of the sensing interface across independently fabricated electrodes. The LOD of 3 nM places this platform within the lower range reported for non-affinity-based cortisol sensors (see Table 2.2). This represents an improvement over fast-scan voltammetric approaches based on carbon fiber microelectrodes, which typically operate in the micromolar range. It also compares favorably with several printed electrode systems, such as sulfur-doped graphene/gold nanoparticle platforms (~ 42 nM), and is within the range targeted by wearable epidermal sensors (~ 10 nM). Although lower LODs have been reported for more complex material systems, these often rely on multi-step fabrication or nanostructured composites.

A preliminary DPV study was conducted to evaluate the electrochemical response of the PY-modified electrode (SPCE/PY44) in artificial sweat at approximately pH 7, over a cortisol concentration range of 3–200 nM. In the absence of cortisol, the typical electrochemical behavior of PY observed in PBS was

significantly altered (Figure 4.13). In PBS, the characteristic PY reduction peak appears at approximately -1.05 V (peak I), with a peak current of around $12\text{ }\mu\text{A}$. Transitioning from controlled PBS environments to artificial sweat (pH ~ 7) induces a profound attenuation of the PY reduction peak (Peak I). The signal intensity drops from $12\text{ }\mu\text{A}$ to a residual $\sim 2\text{ }\mu\text{A}$, accompanied by the emergence of parasitic features at -0.7 V and -1.4 V. This suppression indicates a significant chemical perturbation of the PY interfacial layer, likely driven by the competitive adsorption of sweat constituents such as urea or lactic acid. No discernible response to varying cortisol concentrations (incubation time 15 min) was observed under these conditions.

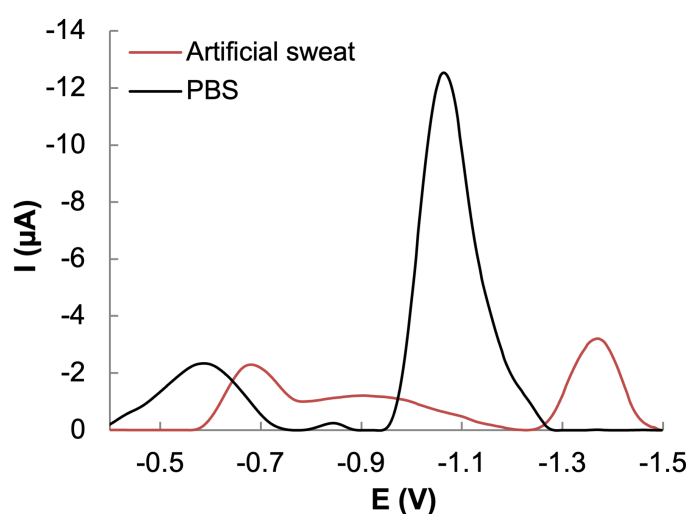


Figure 4.13. DPV response of the SPCE/PY44 in PBS and in artificial sweat (pH ~ 7) in the absence of cortisol.

The loss of cortisol sensitivity in the 3–200 nM range suggests that the high ionic strength and organic content of the sweat matrix act as a charge-transfer barrier or competitive ligand. The displacement of the characteristic PY response underscores the need for selective membranes or localized pH stabilization to recover the sensor's analytical performance in real biofluids. To restore cortisol responsiveness, a systematic deconvolution of the matrix effect is mandatory. Future investigations will evaluate the individual impact of lactic acid and urea on the PY double-layer capacitance and explore anti-fouling surface coatings (e.g., Nafion or chitosan) to shield the electroactive sites from non-specific matrix interactions.

Overall, the PY-modified electrode enables nanomolar cortisol detection with a comparatively simple fabrication strategy, supporting its potential for scalable, wearable sensing applications.

4.2.4.2 DFT calculations

DFT calculations were performed to investigate key intermolecular interactions in the PY–cortisol pair configurations, with particular focus on the polar sites of PY (N, OHid2, Oid4, and Oid10) and of cortisol (OHi39, OHi42, OHi55, Oi1, and Oi51) (Figure 4.14). Geometry optimizations from 20 initial pair config-

urations converged to several stable minima, confirming the presence of multiple possible binding sites between the two molecules.

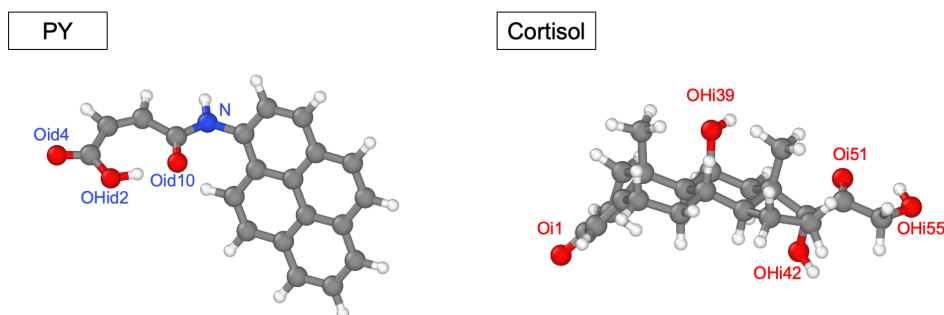


Figure 4.14. Schematics of PY and cortisol interaction sites.

Table 4.3 summarizes the initial (l_{initial}) and optimized (l) interatomic distances and the computed interaction energies (ΔE) for each pair configuration. All pair configurations converged to stabilized structures with negative interaction energies ranging from approximately -0.60 to -1.05 eV, indicating a favorable intermolecular association. These values represent gas-phase electronic interaction energies associated with the optimized local minima and are therefore intended for relative comparison, not as absolute binding free energies.

Table 4.3. Initial (l_{initial}) and optimized (l) distances ($\text{\AA}$) of each atom pair, and corresponding interaction energies (ΔE) for the 20 PY-cortisol configurations.

PY-cortisol configuration	Atom pair	l_{initial} ($\text{\AA}$)	l ($\text{\AA}$)	ΔE (eV)
1	OHid2_OHi39	2.37	2.95	-0.68
2	OHid2_OHi42	2.39	3.04	-0.61
3	OHid2_OHi55	2.40	3.02	-0.63
4	OHid2_Oi1	2.41	8.25	-0.67
5	OHid2_Oi51	2.41	3.13	-0.65
6	Oid4_OHi39	2.38	2.87	-0.6
7	Oid4_OHi42	2.41	2.83	-1.05
8	Oid4_OHi55	2.41	4.78	-0.87
9	Oid4_Oi1	2.42	5.36	-0.76
10	Oid4_Oi51	2.41	6.79	-1.04
11	Oid10_OHi39	2.38	3.12	-0.97
12	Oid10_OHi42	2.38	3.15	-0.68
13	Oid10_OHi55	2.40	3.03	-0.71
14	Oid10_Oi1	2.39	4.61	-0.87
15	Oid10_Oi51	2.41	3.31	-0.69
16	N_OHi39	2.40	3.15	-0.62
17	N_OHi42	2.38	3.08	-1.04
18	N_OHi55	2.40	2.99	-0.97
19	N_Oi1	2.43	2.94	-0.89
20	N_Oi51	2.42	2.94	-0.91

No clear correlation was observed between the shortest selected intermolecular distance and the magnitude of ΔE . This behavior reflects the fact that the overall stability of the pair configurations arises from the combined contributions of electrostatics, dispersion (included via D3BJ), polarization, and steric effects, rather than from a single pairwise contact. In several cases, geometry optimization led to the formation of additional intermolecular contacts (<3.5 Å), evidencing a multisite binding mechanism (Figures 4.15–4.18 and Figures S4.3–S4.18).

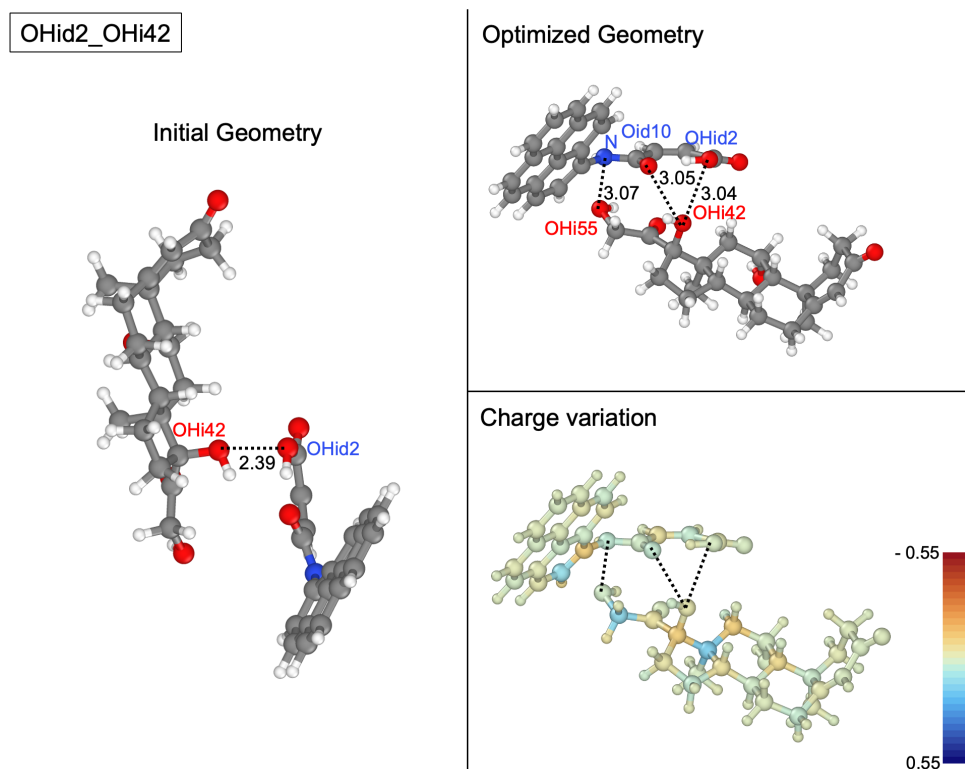


Figure 4.15. Comparison of initial and optimized geometries and CHELPG charge variation for the PY–cortisol pair configuration 2.

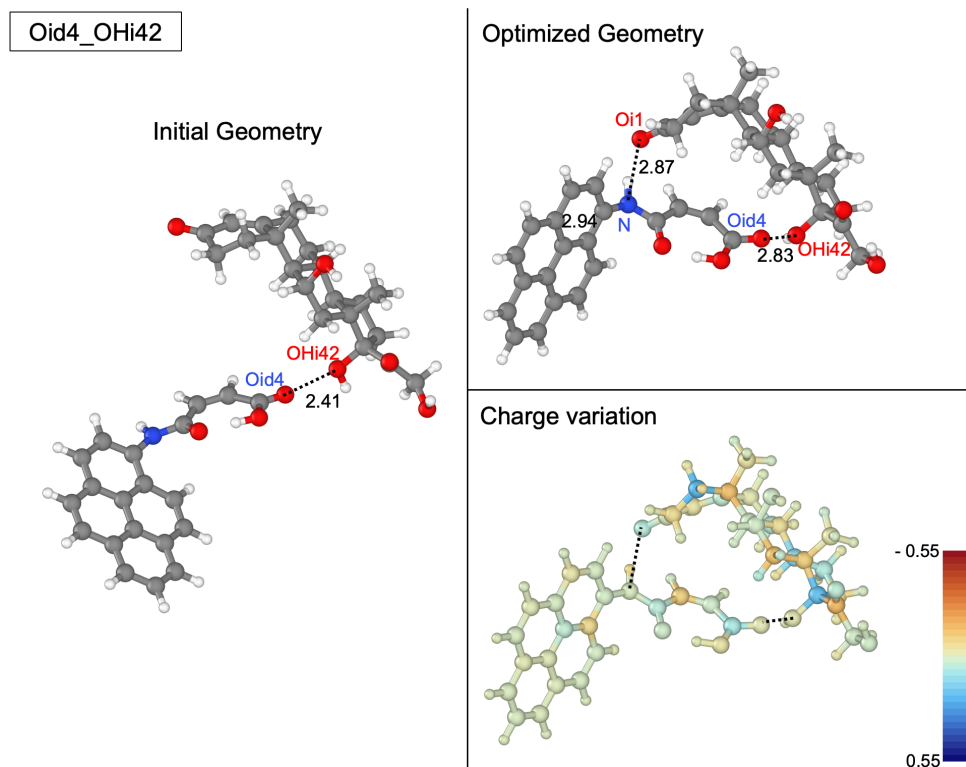


Figure 4.16. Comparison of initial and optimized geometries and CHELPG charge variation for the PY–cortisol pair configuration 7.

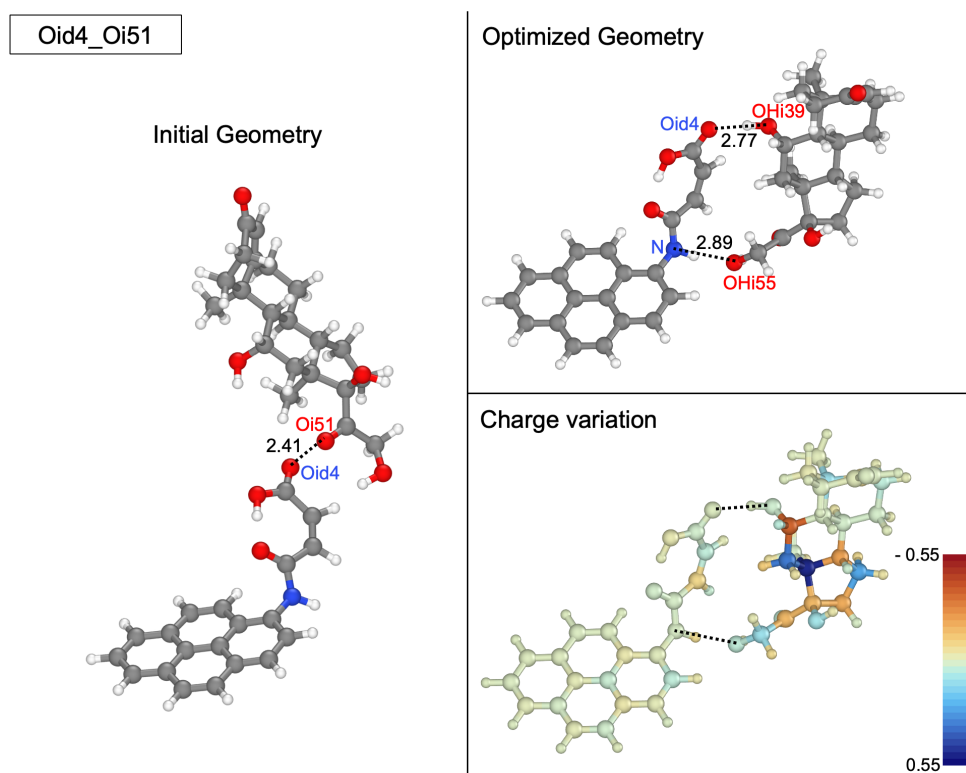


Figure 4.17. Comparison of initial and optimized geometries and CHELPG charge variation for the PY–cortisol pair configuration 10.

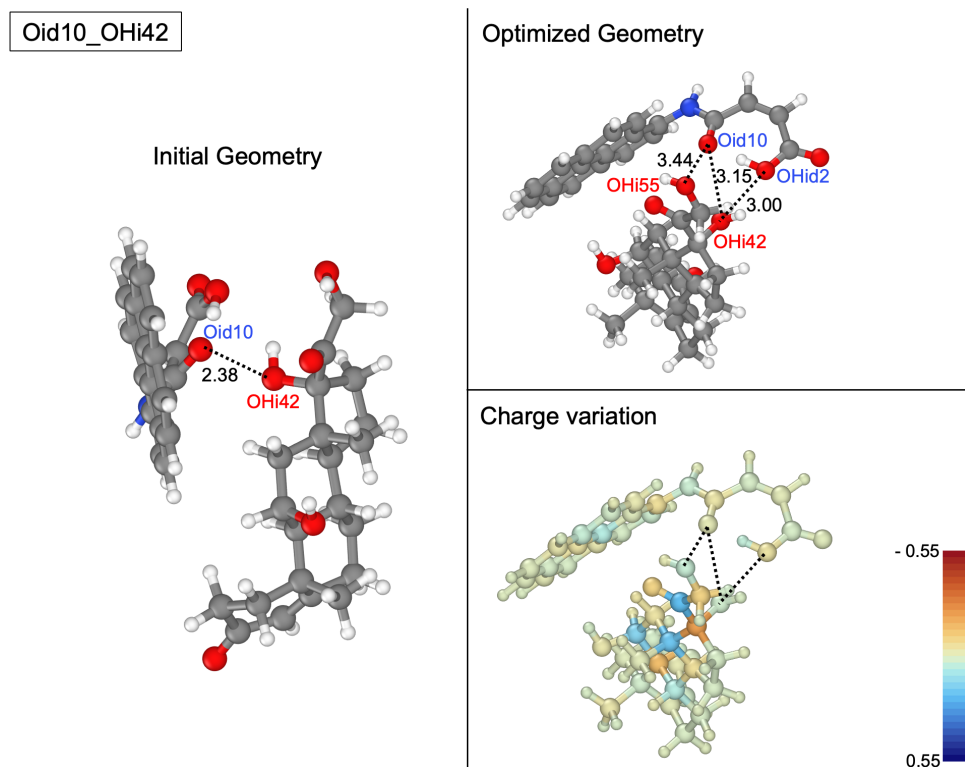


Figure 4.18. Comparison of initial and optimized geometries and CHELPG charge variation for the PY–cortisol pair configuration 12.

Among the PY functional groups, carbonyl oxygens Oid4 and Oid10 generally form the most stable pair configurations, suggesting stronger hydrogen-bond acceptor character and a more favorable local electronic environment. Configurations involving the OHid2 group tend to exhibit lower negative interaction energies, possibly due to suboptimal orientation and/or local steric constraints. For the N-based initial pair configurations, the interaction energies span from weak (N–OHi39) to medium and moderately strong interactions (e.g., N–OHi42, N–OHi55, N–Oi1, and N–Oi51), even though the optimized structures present a single-site binding. This suggests that the amide nitrogen can establish a reasonably strong localized hydrogen bond. However, steric hindrance and lower involvement in cooperative contacts likely restrict the formation of fully stabilized multisite arrangements. This interpretation is consistent with the lower dynamical prevalence of nitrogen-centered interactions reported in our previous MD study [101].

The CHELPG charge analysis (Figures 4.15–4.18) reveals a measurable redistribution of electrostatic potential-derived atomic charges upon optimized pair configuration formation. In most configurations, the largest atomic charge variations are observed on the cortisol molecule, whereas the PY framework shows comparatively smaller, more localized changes. This behavior reflects the more localized electronic structure of the functional groups in cortisol and the more delocalized electronic response of the extended aromatic PY system. No straightforward correlation is observed between the magnitude or location of the charge variations and the geometrically identified hydrogen bonds, indicating that intermolecular stabi-

lization cannot be attributed to a single localized electrostatic contribution but arises from the combined action of electrostatic, dispersive, and polarization effects.

Taken together, the MD and DFT results consistently support a geometry-dependent multisite PY–cortisol binding mechanism, with the Oid4 and Oid10 carbonyl groups of PY most frequently providing the strongest stabilizing interactions. The resulting electronic redistribution and intermolecular polarization effects are likely to perturb the local electronic environment of the modified pyrene. Although no direct redox calculations were performed, these results may suggest modulation of the electroactive PY sites, providing a plausible molecular-level explanation for the experimentally observed decrease in the PY reduction peak intensity.

4.2.5 Conclusions

This study presents a direct, non-affinity electrochemical sensor for cortisol detection based on a PY-modified electrode. The sensor exhibited a logarithmic response over a physiologically relevant range (3–500 nM), with a sensitivity of 2.21 $\mu\text{A}/\text{decade}$ and a high correlation coefficient ($R = 0.967$). The achieved limit of detection of 3 nM falls within the range relevant for monitoring cortisol in human biofluids such as sweat and saliva.

DFT calculations provided quantitative insight into the molecular interactions between PY and cortisol, supporting a geometry-dependent multisite binding mechanism. The resulting charge redistribution and polarization effects are consistent with the observed modulation of the PY reduction peak.

A key advantage of this device is its straightforward fabrication. By avoiding costly biorecognition elements and complex nanomaterial synthesis, the PY-modified electrode provides a stable, cost-effective interface for cortisol sensing. These results lay a practical foundation for developing low-cost, wearable devices for real-time stress monitoring.

4.2.6 Appendix

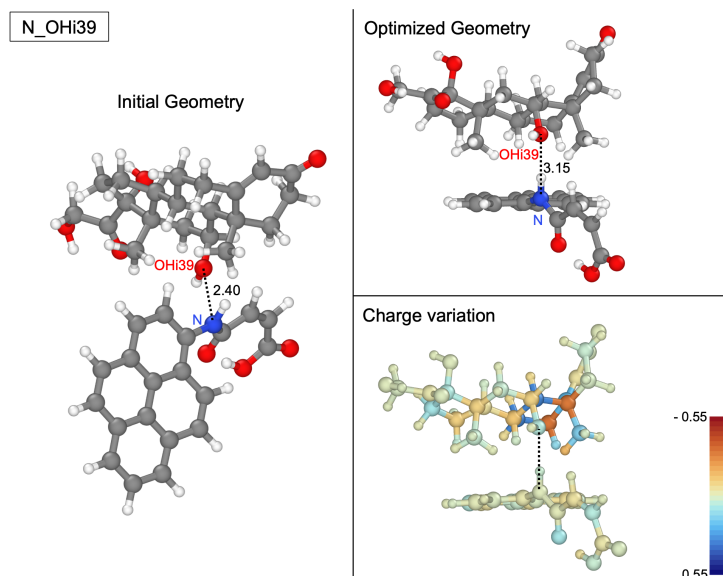


Figure S4.3. Comparison of initial and optimized geometries and CHELPG charge variation for the PY–cortisol pair configuration 1.

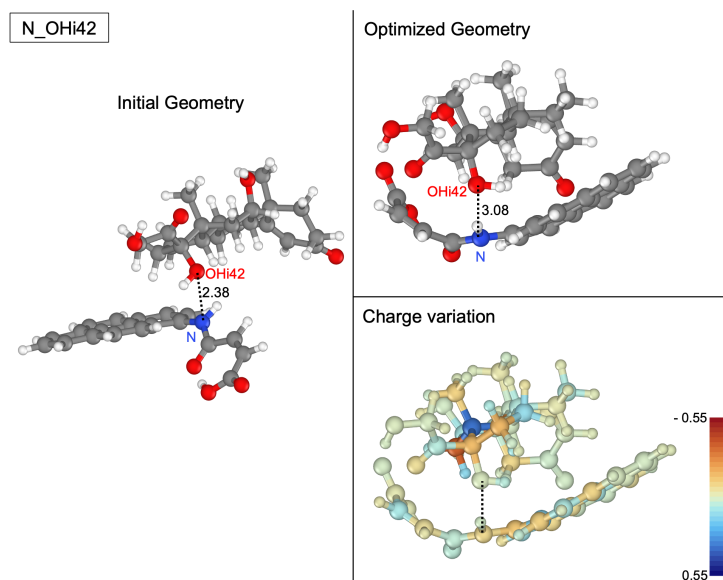


Figure S4.4. Comparison of initial and optimized geometries and CHELPG charge variation for the PY–cortisol pair configuration 3.

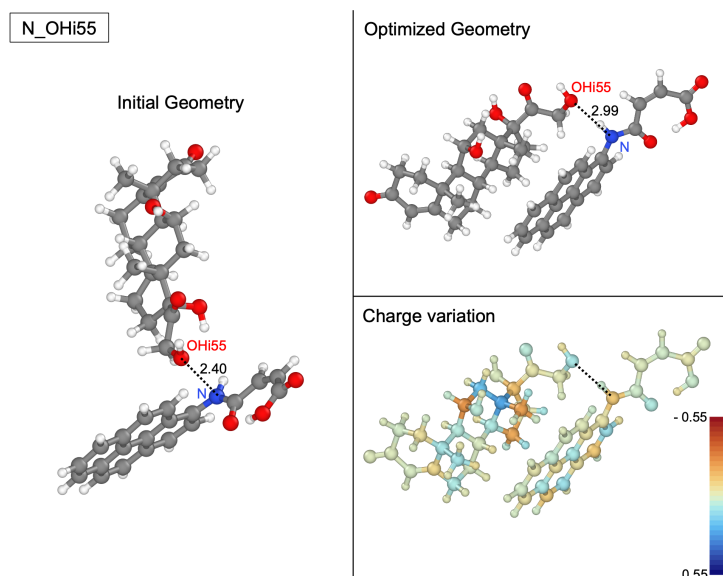


Figure S4.5. Comparison of initial and optimized geometries and CHELPG charge variation for the PY–cortisol pair configuration 4.

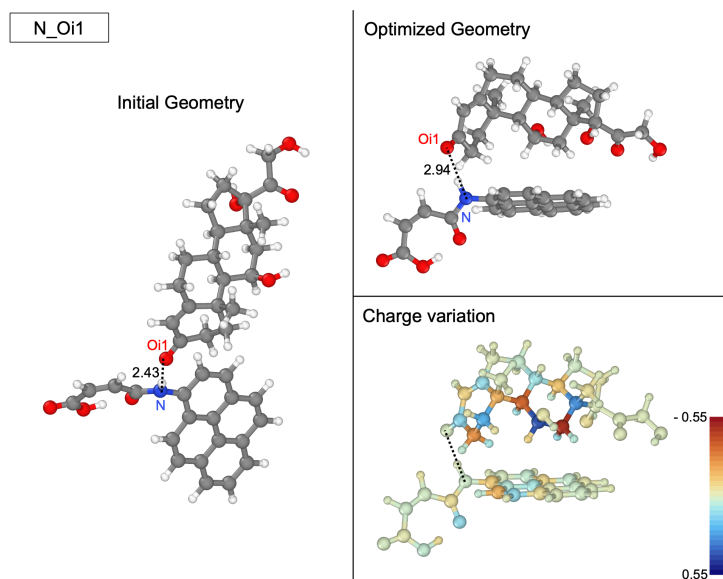


Figure S4.6. Comparison of initial and optimized geometries and CHELPG charge variation for the PY–cortisol pair configuration 5.

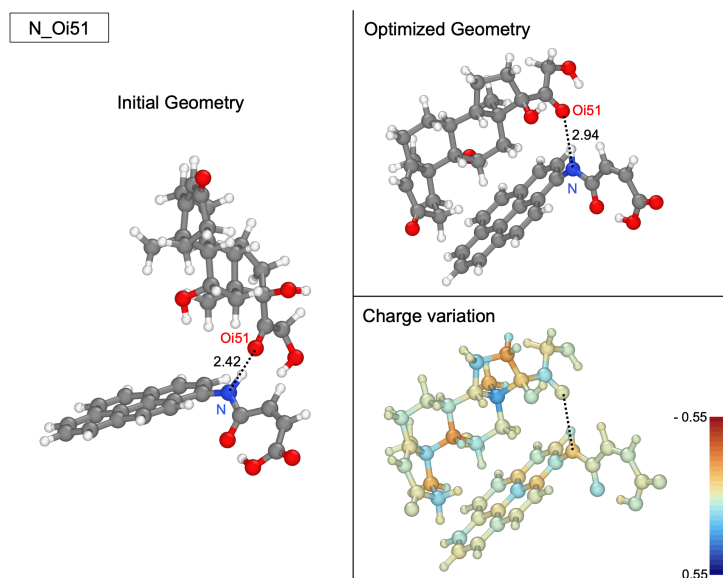


Figure S4.7. Comparison of initial and optimized geometries and CHELPG charge variation for the PY–cortisol pair configuration 6.

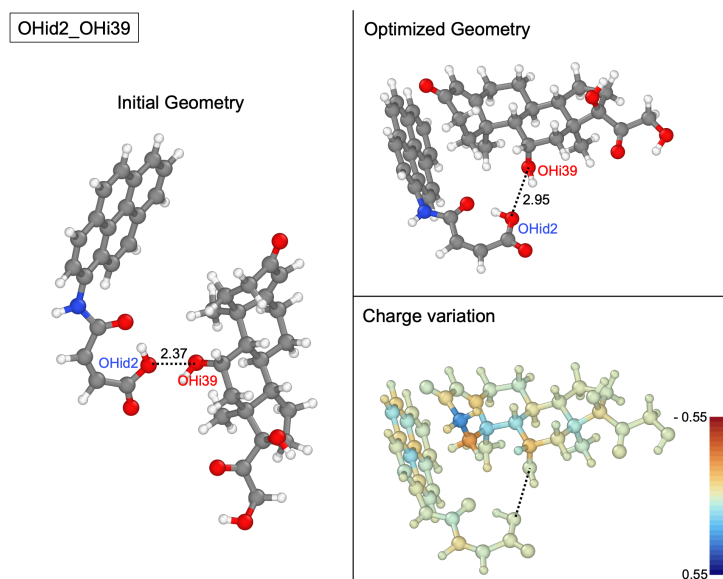


Figure S4.8. Comparison of initial and optimized geometries and CHELPG charge variation for the PY–cortisol pair configuration 8.

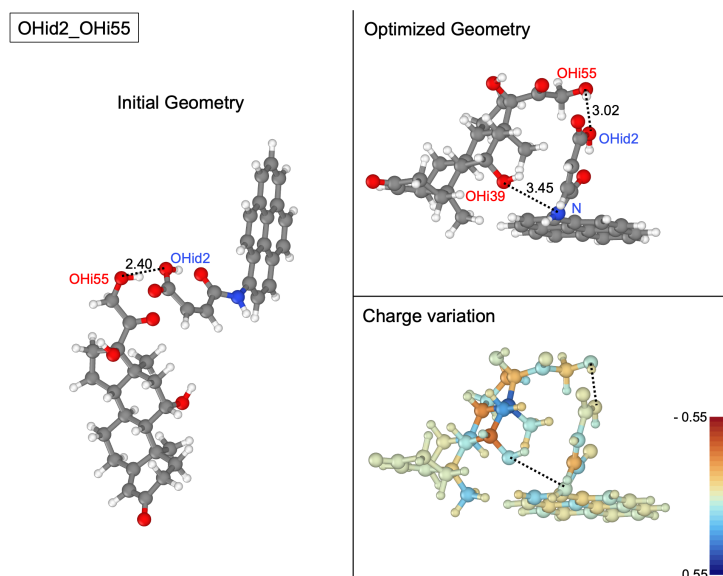


Figure S4.9. Comparison of initial and optimized geometries and CHELPG charge variation for the PY–cortisol pair configuration 9.

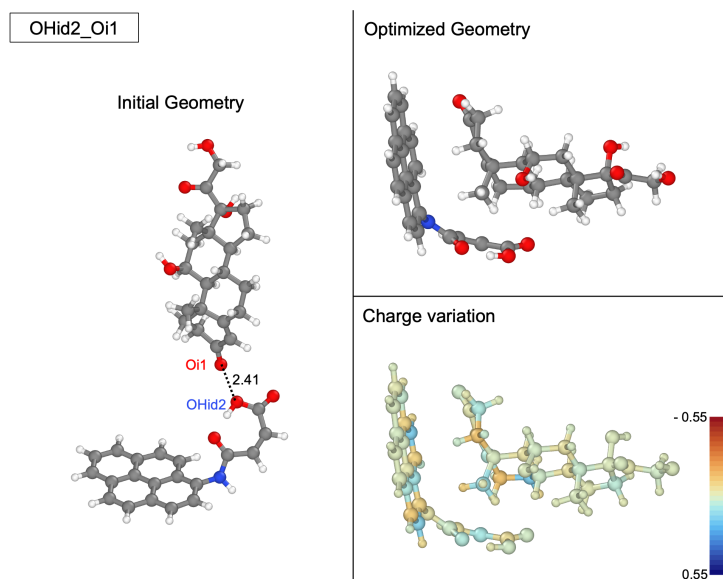


Figure S4.10. Comparison of initial and optimized geometries and CHELPG charge variation for the PY–cortisol pair configuration 11.

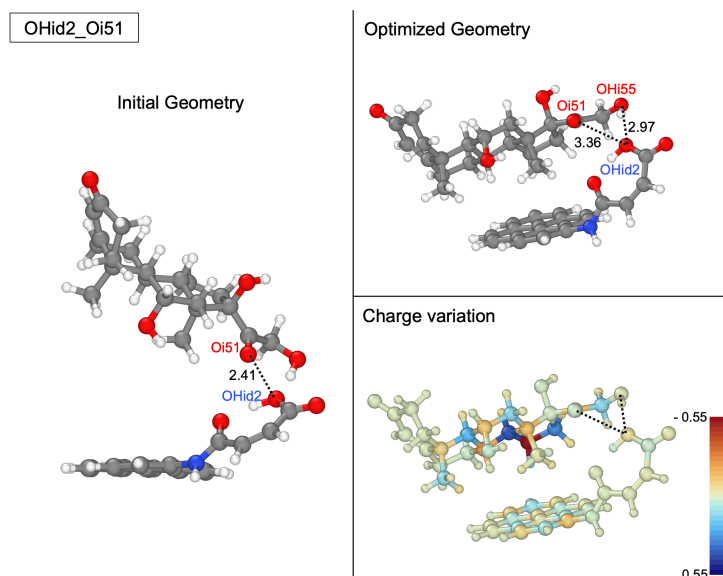


Figure S4.11. Comparison of initial and optimized geometries and CHELPG charge variation for the PY-cortisol pair configuration 13.

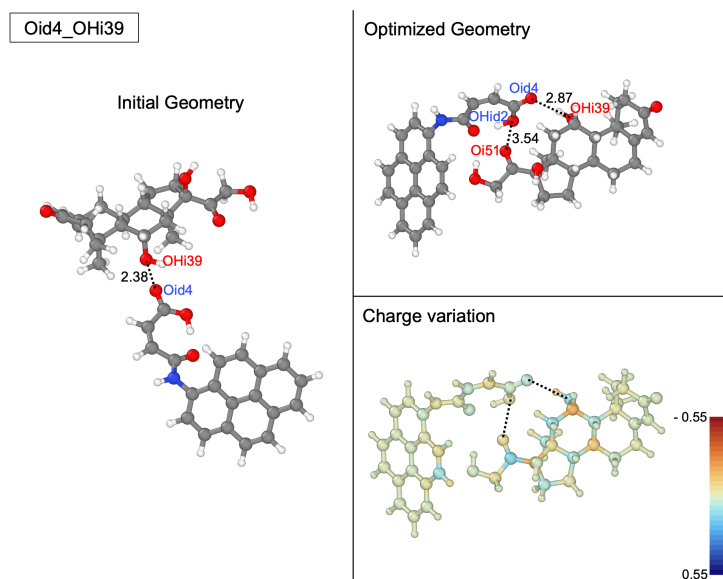


Figure S4.12. Comparison of initial and optimized geometries and CHELPG charge variation for the PY-cortisol pair configuration 14.

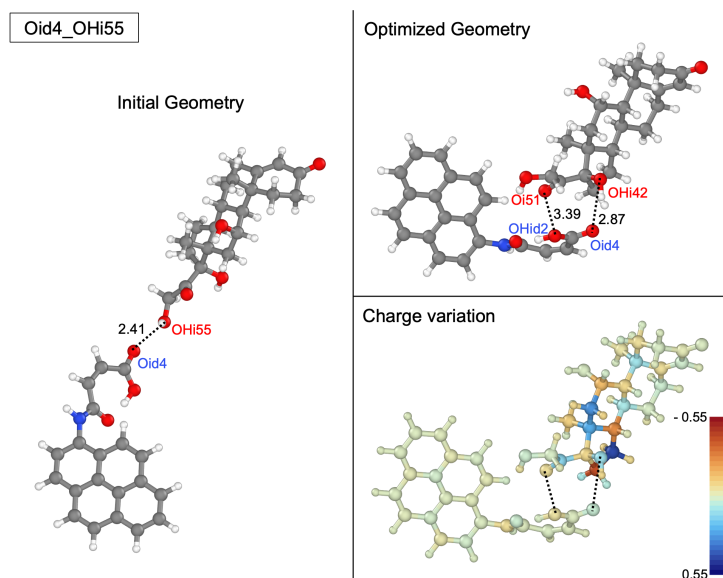


Figure S4.13. Comparison of initial and optimized geometries and CHELPG charge variation for the PY–cortisol pair configuration 15.

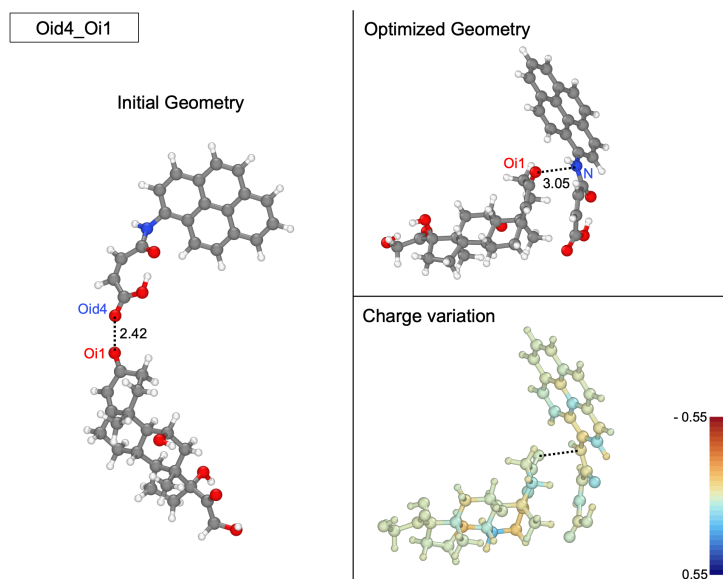


Figure S4.14. Comparison of initial and optimized geometries and CHELPG charge variation for the PY–cortisol pair configuration 16.

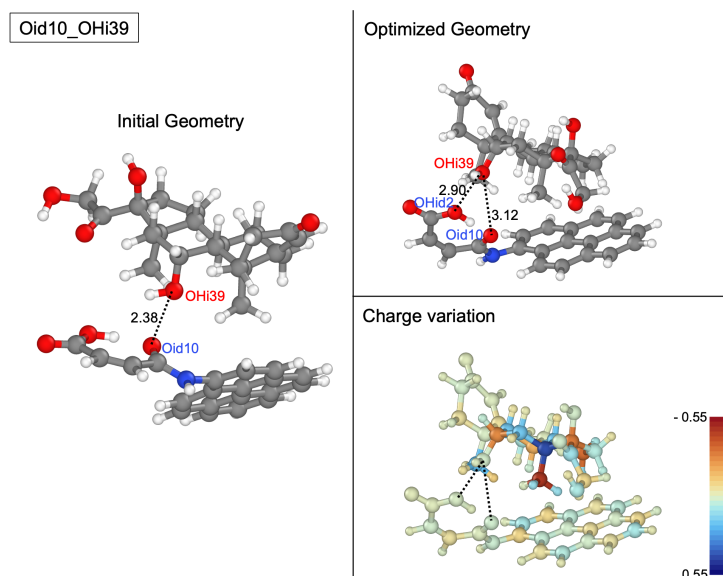


Figure S4.15. Comparison of initial and optimized geometries and CHELPG charge variation for the PY–cortisol pair configuration 17.

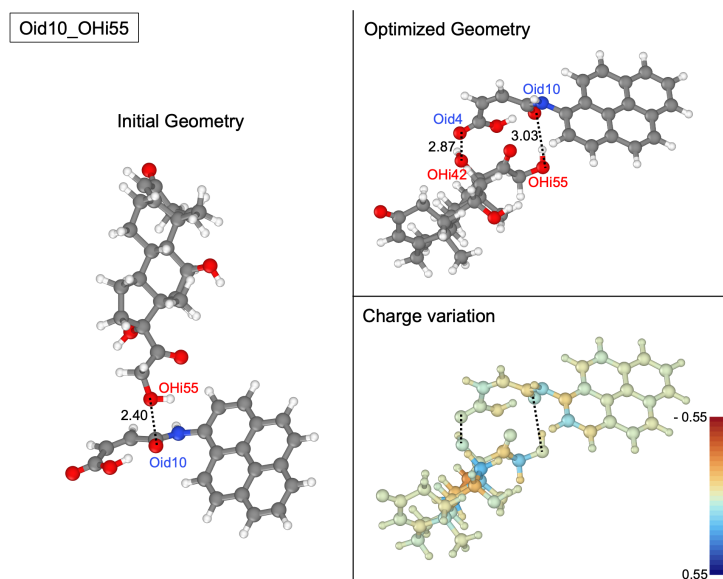


Figure S4.16. Comparison of initial and optimized geometries and CHELPG charge variation for the PY–cortisol pair configuration 18.

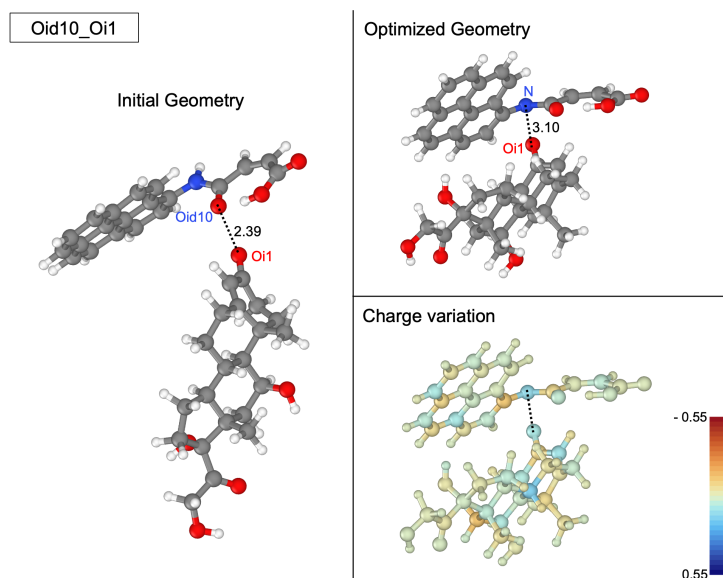


Figure S4.17. Comparison of initial and optimized geometries and CHELPG charge variation for the PY–cortisol pair configuration 19.

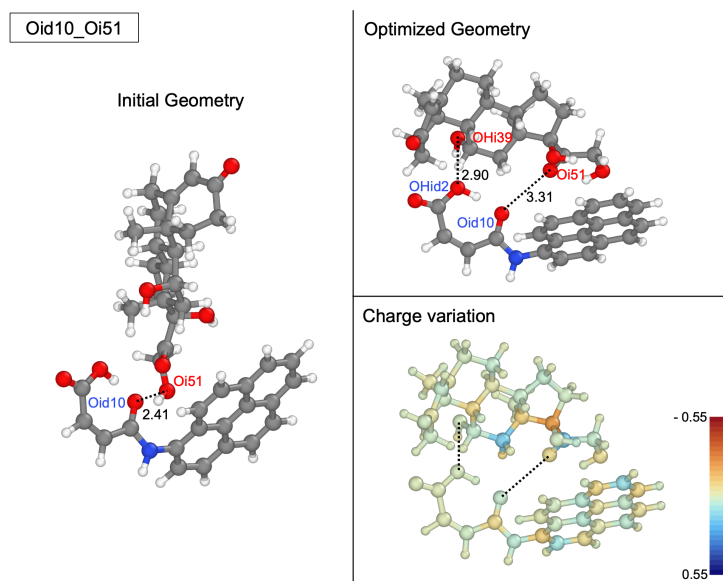


Figure S4.18. Comparison of initial and optimized geometries and CHELPG charge variation for the PY–cortisol pair configuration 20.

4.3 Aqueous pyrene modification of carbon electrodes for cortisol sensing

This study proposes an alkaline aqueous deposition method ($\text{pH} > 7$) for modifying SPCEs with a PY for cortisol sensing. This approach improves droplet confinement and surface uniformity compared to traditional organic-aqueous solvents. Scanning electron microscopy (SEM) revealed that the modified surfaces consist of ribbon-like structures (50–300 nm). Electrochemical analysis showed that cortisol detection is driven by interfacial interactions that modify the intrinsic PY redox peaks rather than direct analyte electroactivity. Optimal analytical performance was achieved with a $4.4 \mu\text{g}$ PY loading and a 15-minute incubation time, yielding a sensitivity of $4.36 \mu\text{A}/\text{dec}$. In contrast, higher PY loadings and electrode pre-treatment were found to hinder the sensor's response. These results demonstrate the importance of controlled surface modification in developing reproducible, sensitive electrochemical devices for cortisol monitoring.

4.3.1 Introduction

Electrochemical detection of cortisol is a promising approach for monitoring stress-related physiological changes [12, 149]. SPCEs are widely used for this purpose due to their low cost, portability, disposability, and versatility in surface functionalization. To improve their performance, these electrodes are often modified with functional materials via drop-casting [150–152]. However, this step can be difficult to control, leading to nonuniformity in the sensing layer and variability in the electrochemical response. Several factors can influence the final morphology of the drop-cast deposited film. For example, the coffee-ring effect, in which particles migrate toward the edges of a drying droplet, can result in uneven material distribution [153, 154]. In addition, the surface tension of the casting solution plays an important role in droplet spreading and confinement on the electrode surface. Low-surface-tension liquids, such as organic–aqueous mixtures, may spread beyond the conductive area or overflow onto the insulating substrate, making the modification process harder to control and reducing electrode-to-electrode consistency [155, 156].

In our previous work, a cortisol-sensing platform based on SPCEs modified with a PY was deposited from an ethanol/water solvent mixture. Although this system enabled cortisol detection in the micromolar range, the solution's relatively low surface tension made the modification step difficult to control.

To circumvent the limitations of low-surface-tension organic mixtures, we report a novel alkaline aque-

ous deposition protocol. This strategy leverages water's high surface tension to mitigate the 'coffee-ring effect' and ensure precise droplet confinement, promoting the formation of a homogeneous, high-density sensing interface. Since PY is insoluble in neutral water, an alkaline medium enables its dissolution and promotes a more homogeneous dispersion in the casting solution, improving the uniformity of the deposited layer. The effect of potential limits on the electrochemical response of SPCE/PY electrodes was evaluated, along with the influence of electrode pretreatment prior to modification. A multi-peak analysis was performed in both the oxidation and reduction regions. Oxidation processes were investigated by CV, in the absence and presence of a Fe(III) redox probe, at different scan rates and incubation times. The reduction region was analyzed by DPV as the surface concentration of pyrene and incubation time varied. In addition, SEM was used to characterize the morphology of the modified electrodes. This combined approach enables the identification of suitable electrochemical conditions and potential windows for the detection of cortisol at nanomolar levels.

4.3.2 Experimental section

Functionalized pyrene (PY) was synthesized according to a procedure previously reported [124]. Screen-printed carbon electrodes (SPCE 110D, DropSens, Metrohm, Portugal) were modified by drop-casting with aqueous PY solutions with pH adjustment ($pH > 7$) to allow PY dispersion in water. Three PY loadings were used (Table 4.4): PYw4 – 4.4 μg (5 μL of 0.88 mg/mL solution), PYw6PT – 6.4 μg (7.3 μL of 0.88 mg/mL solution) with pre-treatment (PT) of the SPCE, and PYw13 – 13 μg (5 μL of 2.64 mg/mL solution).

Table 4.4. Summary of PY loadings, drop-casting volumes, and concentrations of the PY solutions.

SPCE/PYw	m_{PY} (μg)	$V_{\text{drop—casting}}$ (μL)	[PY solution] (mg/mL)
PYw4	4.4	5	0.88
PYw6PT	6.4	7.3	0.88
PYw13	13	5	2.64

SPCE pre-treatment prior to PY deposition was performed by potential cycling from -0.6 to 0.6 V at $50 \text{ mV} \cdot \text{s}^{-1}$ for 20 cycles in PBS. Electrodes PYw4PT and PYw6PT underwent this pre-treatment.

SEM analysis was conducted using a NanoSEM-200 (FEI Nova). Samples were coated with a 9 nM Au/Pd (80/20 % W) prior to analysis. PBS and cortisol solutions were prepared as described previously [101]. Electrochemical measurements were performed at room temperature using CV and DPV according to the reported protocols. A 70 μL drop of solution was applied onto the modified SPCE, covering all three electrodes. Measurements were conducted using an Autolab PGSTAT30 potentiostat controlled by GPES

4.9 software (EcoChemie).

4.3.3 Results

4.3.3.1 Morphological characterization

SEM analysis was performed to evaluate the morphology of the modified electrodes. Three electrodes prepared with increasing PY concentrations (SPCE/PYw4, SPCE/PYw6, and SPCE/PYw13) were examined to assess the effect of PY content and solvent system on film structure and surface coverage (Figure 4.35).

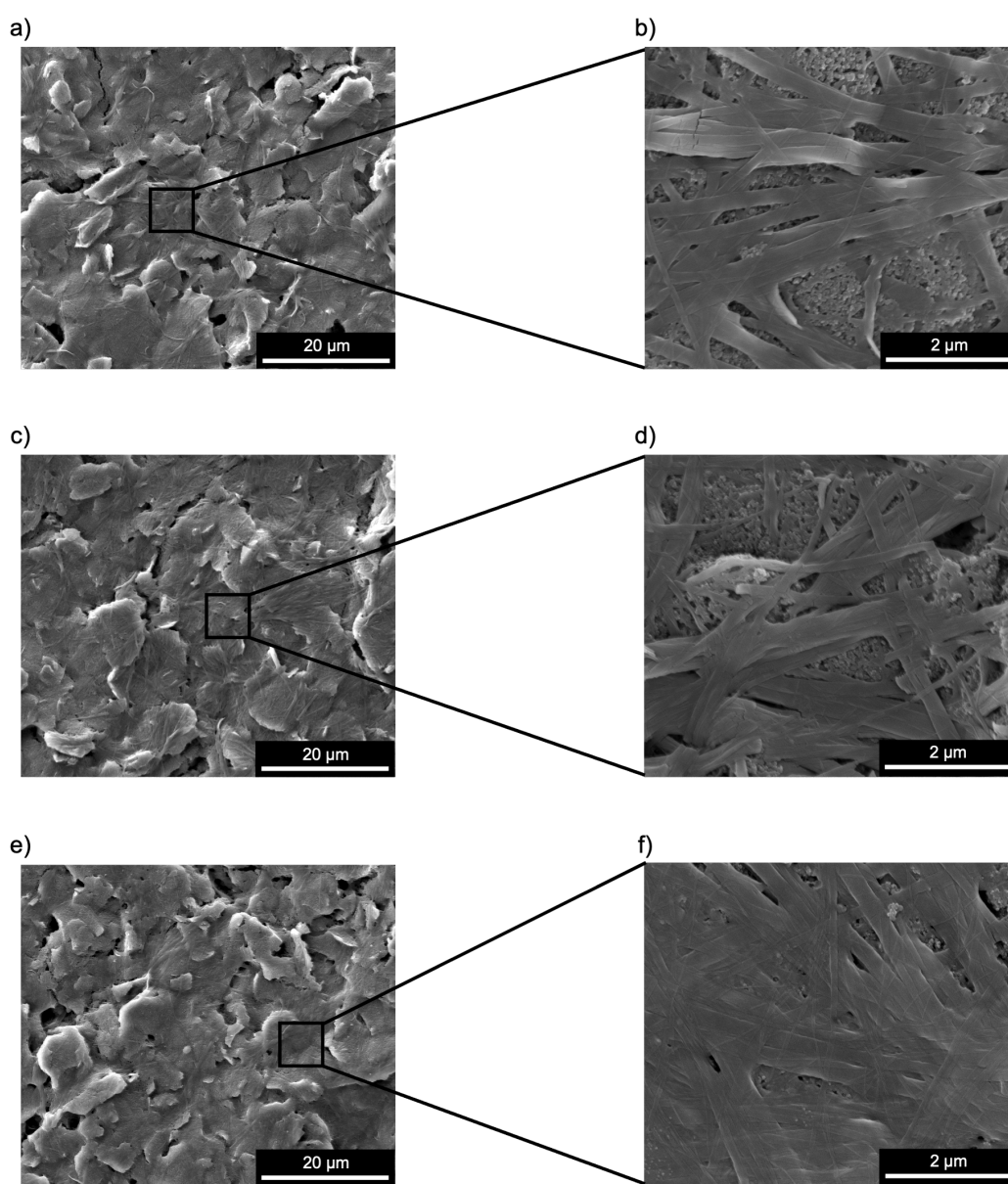


Figure 4.35. SEM images of SPCE/PYw electrodes at different PY concentrations, SPCE/PYw4, SPCE/PYw6, and SPCE/PYw13.

The deposited material exhibits ribbon-like collapsed structures with widths ranging approximately from 50 to 300 nm, observed across all three electrodes. Increasing PY concentration results in greater electrode surface coverage, while the ribbon width distribution remains similar. Higher PY content also appears to enhance ribbon stacking and overlap, producing a more densely packed surface. These differences compared to the previously reported ethanol/water system, where more defined three-dimensional ribbons with micrometer-scale dimensions were obtained, may be attributed to the distinct physicochemical properties of the solvents. In particular, the aqueous-alkaline environment likely affects solute–solvent interactions and the kinetics of aggregation, favoring the formation of smaller, collapsed ribbons rather than larger 3D structures.

4.3.3.2 Potential limits effect on the SPCE/PY electrochemical response

Electrochemical characterization of SPCE/PYw4 electrodes was performed by CV over different potential windows to assess the effect of the applied limits on the oxidation–reduction response.

Figure 4.36a shows the SPCE/PYw4 response recorded in PBS over the potential range from -1.5 to 0.25 V. The first and second cycles are very similar, with a pair of redox peaks centered near 0 V. In the third cycle, a slight shift of this redox couple toward more positive potentials is observed, suggesting minor changes at the electrode surface upon cycling. At more negative potentials (around -1.2 V, an additional non-well-defined cathodic process appears, whose intensity and position vary slightly from cycle to cycle. This behavior suggests that the cathodic process observed at more negative potentials is associated with changes within the surface-confined PY layer during cycling, such as the formation of new electroactive species or their reorganization.

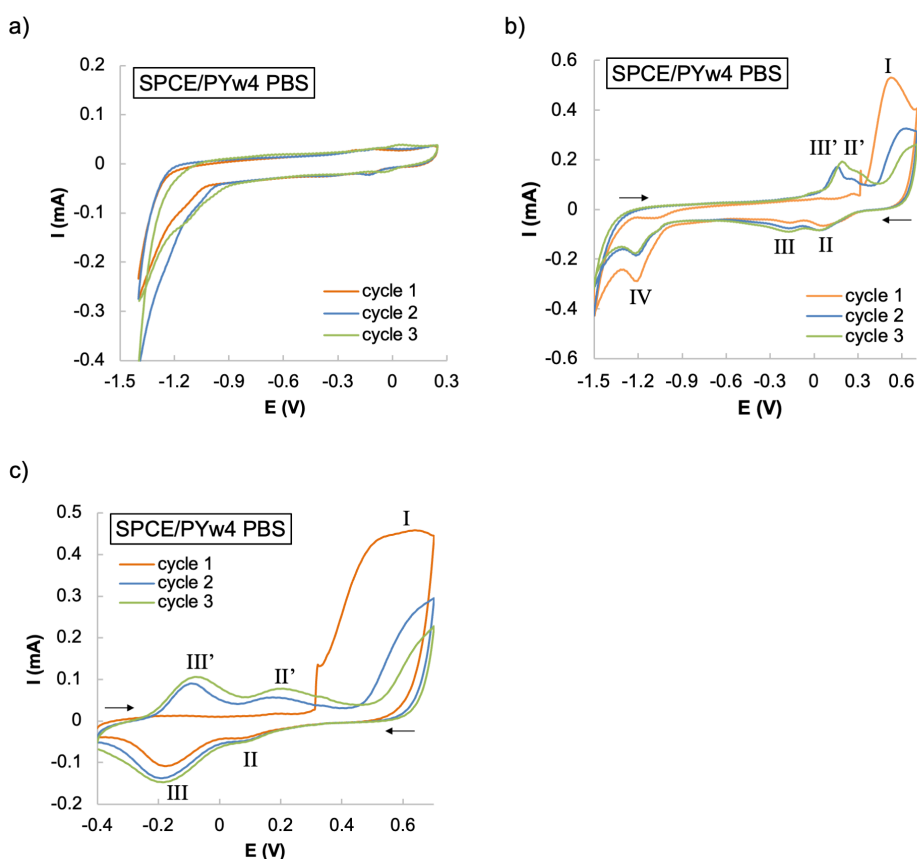


Figure 4.36. CV of SPCE/PYw4 in PBS over three cycles at a scan rate of 100 mV · s⁻¹ within the reduction potential ranges of: a) -1.5 to 0.25 V and b) -1.5 to 0.7 V and c) oxidation potential range of -0.4 to 0.7 V

When the anodic limit is extended to 0.7 V (Figure 4.36b), clear changes in the response are observed across the whole potential range. In the first forward scan, a well-defined anodic peak at 0.5 V (peak I) corresponding to the oxidation of PY immobilized on the electrode surface is observed. During the reverse scan, two smaller cathodic peaks, II and III, are observed at 0.06 and -0.15 V respectively, along with a more pronounced peak IV at -1.2 V. In the second forward scan, two additional anodic peaks (II' and III') appear at 0.17 and 0.27 V accompanied by a decrease in the intensity of peak I. In the corresponding reverse scan, peaks II and III show a slight increase in current, whereas peak IV remains essentially unchanged. The third cycle closely resembles the second, with a slight increase in the intensity of peaks II/II' and III/III' and a decrease in peak I. The emergence of the II/II' and III/III' redox couples upon expanding the anodic limit (0.7 V) signifies a potential-driven chemical transformation of the PY layer. This reorganization triggers the formation of secondary electroactive species, effectively redistributing the charge transfer sites and stabilizing the surface-confined response.

The voltammograms recorded within the narrower potential window (-0.4 to 0.7 V Figure 4.36c) show a similar trend across the three cycles. When comparing with Figure 4.36b, peaks II' and III' become clearly defined and shift slightly toward more negative potentials, while peak III increases in intensity and

peak II decreases. These changes further support the idea that repeated cycling promotes stabilization of surface-confined redox processes, likely associated with modified or partially oxidized PY species.

A comparison of the voltammetric responses obtained across the different potential intervals for the first and second cycles (Figures 4.37a and 4.37b) highlights the strong influence of the applied potential window. Extending the anodic limit to 0.7 V results in a significantly higher oxidation current for peak I, indicating that PY oxidation is more extensive under these conditions. Although the overall process is not electrochemically reversible, partial reduction of the oxidized PY species is still observed at negative potentials. The redox peaks associated with surface-confined processes become better defined and slightly shifted when less negative cathodic limits are applied, suggesting improved electrochemical stability of the modified layer within a limited potential range.

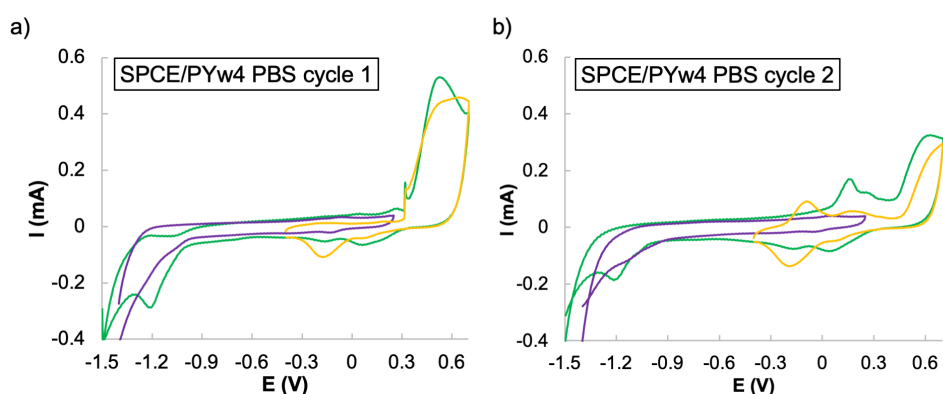


Figure 4.37. Effect of cathodic and anodic limits on the SPCE/PYw4 signal of a) cycle 1 and b) 2 in PBS.

The same analysis was performed in the presence of the $K_3Fe(CN)_6$ redox probe. Figure 4.38a shows the voltammetric response of SPCE/PYw4 recorded between -1.5 and 0.25 V over three consecutive cycles. The responses over the three cycles are nearly identical, showing the characteristic Fe(III)/Fe(II) redox couple (peaks X and X') overlapped with the PY-related peaks (II/II') previously observed in PBS. For comparison, the response of the bare SPCE toward the $[Fe(CN)_6]^{3-/4-}$ probe is also shown (dashed line).

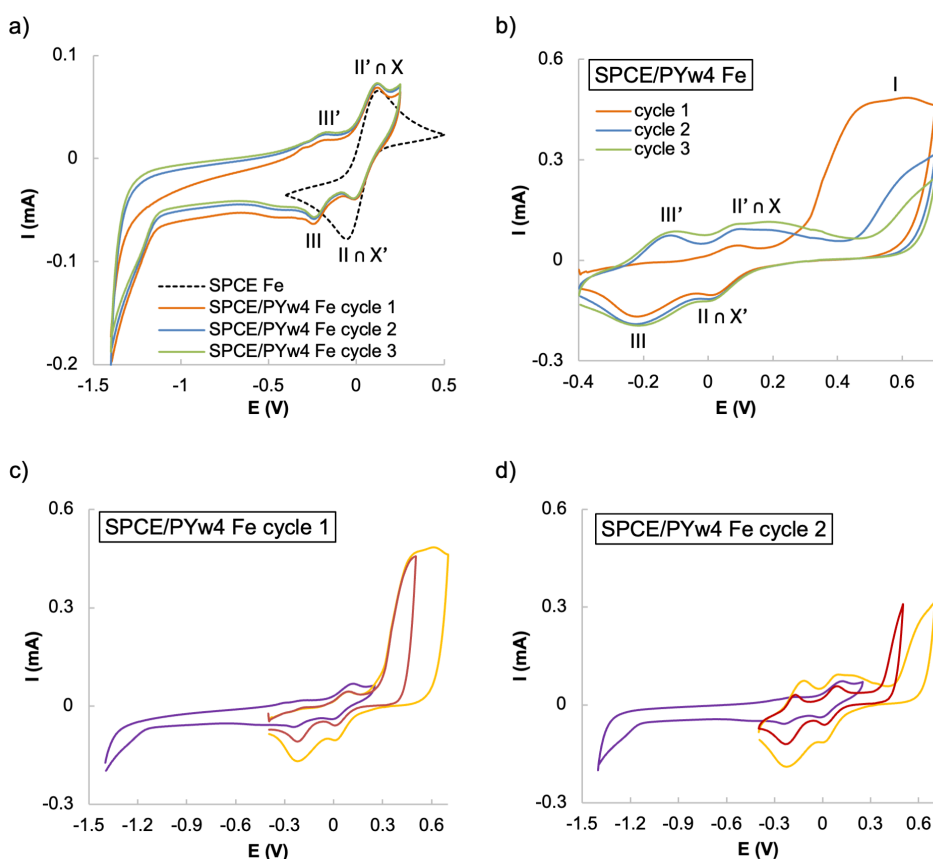


Figure 4.38. CV response of SPCE/PY in 5 mM $K_3Fe(CN)_6$ over three cycles at a scan rate of $100 \text{ mV} \cdot \text{s}^{-1}$ within the: a) reduction potential range of -1.5 to 0.25 V and b) oxidation potential range of -0.4 to 0.7 V Effect of cathodic and anodic limits on the signal of the cycles c) 1 and d) 2.

Within the -0.4 to 0.7 V range (Figure 4.38b), a clear difference is observed between the first and subsequent cycles. In the first cycle, the forward scan shows the oxidation of both $[Fe(CN)_6]^{4-}$ (peak X) and PY (peak I), while the reverse scan reveals the corresponding reduction processes of $[Fe(CN)_6]^{3-}$ (peak X') and PY (peaks II and III). The PY-related peaks II' and III' become evident only in the second and third cycles. This behavior is consistent with that observed in the absence of the probe, indicating that the PY electrochemical response is not significantly altered by the redox mediator.

The definition of the $[Fe(CN)_6]^{3-/4-}$ response (peaks X/X') is partially hindered by overlap with the PY-related peaks (II' and II), whose intensities increase upon cycling at higher anodic potentials and restricting the cathodic potential limit (Figures 4.38c and 4.38d).

Both in the absence and presence of the redox probe, a similar trend is observed: peak I decreases in intensity with successive cycles, while the remaining peaks show a slight increase. This further supports the interpretation that the electrochemical behavior is dominated by progressive modification of the PY layer during potential cycling.

Figure S4.19 (Appendix) compares the CV responses obtained in the presence and absence of the

Fe(III) probe. The intensities of peaks II, II', III, and III' increase in the presence of the probe, suggesting that it facilitates charge transfer and enhances these processes. A well-defined probe response, with minimal contribution from residual current, is observed only when the electrode surface is not extensively oxidized. This occurs either within the -1.5 to 0.25 V range (Figures S4.19a and S4.19b) or during the first cycle between -0.4 and 0.7 V (Figure S4.19c). In the second cycle (Figure S4.19d), the response becomes less defined due to an increase in background current, indicating significant surface modification after the initial oxidation.

4.3.3.3 Characterization of the probe's electron transfer process on SPCE/PY

The electron transfer behavior of the Fe(III) probe on SPCE and SPCE/PYw4 was investigated by CV. Figure 4.39 shows the corresponding voltammograms recorded in a 5 mM Fe(III) solution at scan rates (ν) ranging from 20 to 200 $\text{mV} \cdot \text{s}^{-1}$. In both cases, the peak current increased with increasing scan rate. Measurements with SPCE were performed using a single electrode, whereas each SPCE/PYw4 measurement was carried out using a single-use electrode.

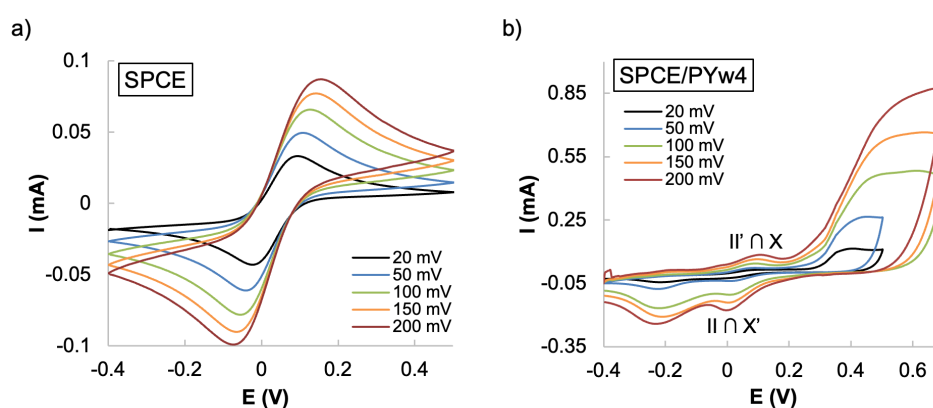


Figure 4.39. CV of a) SPCE and b) SPCE/PY in 5 mM Fe (III) solution over different scan rates ($\text{mV} \cdot \text{s}^{-1}$).

Figure 4.40 presents the relationship between peak current and scan rate. For the bare SPCE (Figure 4.40a), a linear dependence of peak current on the square root of the scan rate ($\sqrt{\nu}$) was observed, indicating a diffusion-controlled redox process. The data points at the highest scan rates were not considered, as their deviation from linearity was attributed to ohmic drop. The SPCE/PYw4 electrode (Figure 4.40b) showed a linear relationship between the intensity of peaks II' $\cap$ X and II $\cap$ X', and the scan rate (ν), consistent with an adsorption-controlled process. These results suggest that the PYw4 modification enhances the interaction between Fe (III) and the electrode surface, shifting the dominant mechanism from diffusion to adsorption [44, 157, 158].

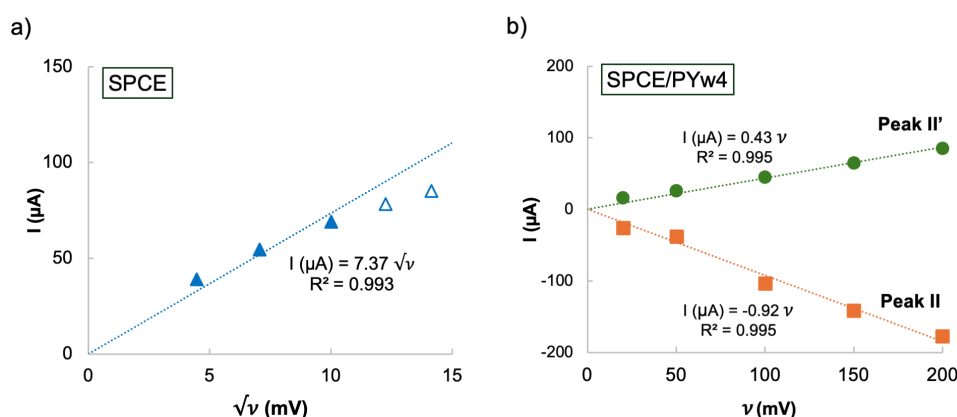


Figure 4.40. Relationship between current I (mA) and scan rate ($\text{mV} \cdot \text{s}^{-1}$) for: a) SPCE and b) SPCE/PYw4 in 5 mM Fe(III) probe solutions.

4.3.3.4 Oxidation range

The SPCE/PYw4 response in the anodic potential range was investigated by CV, evaluating several experimental parameters, including the presence or absence of a Fe(III) redox probe, scan rate, and incubation time, as summarized in Figure 4.41. This study aimed to optimize the experimental conditions for cortisol detection by identifying the most responsive electrochemical signals.

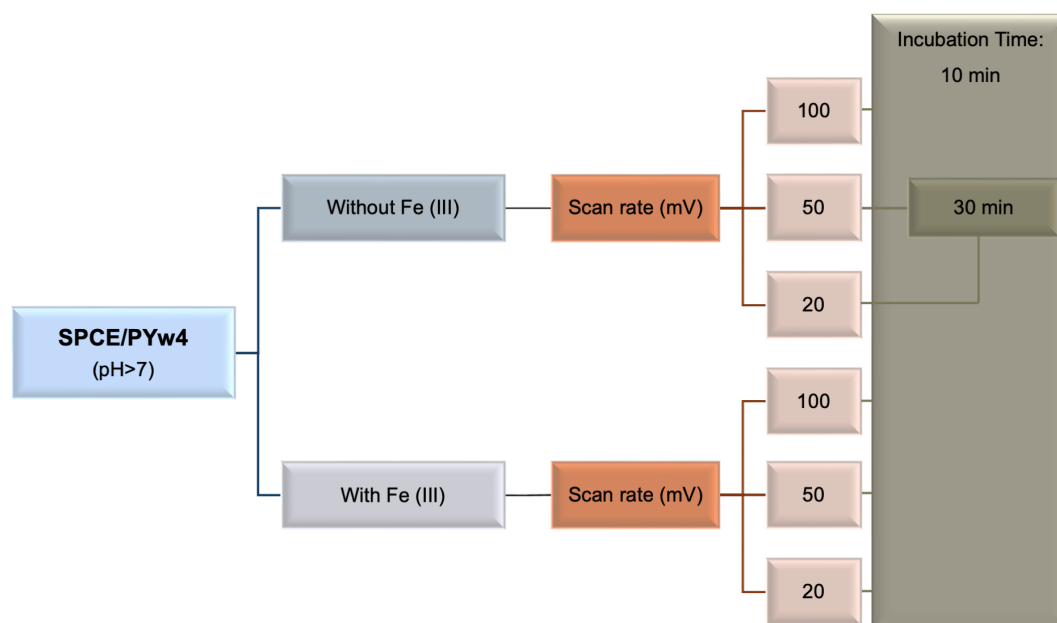


Figure 4.41. Diagram of the experimental approach used to investigate the SPCE/PYw4 response in the oxidation region by cyclic voltammetry (CV).

Figure 4.42 shows the SPCE/PYw4 response incubated 10 min over a range of cortisol concentrations at a scan rate of $50 \text{ mV} \cdot \text{s}^{-1}$. Only the intrinsic PY peaks are observed in both cycles, while no cortisol-related peaks are visible. The paradoxical increase in Peak I current in the absence of a redox probe

suggests that cortisol induces a molecular reorientation of the PY layer, locally enhancing electroactive site accessibility. Conversely, the signal attenuation in the presence of Fe(III) confirms a predominant surface-blocking mechanism, where the cortisol-PY complex hinders the flux of the external mediator. No other peaks exhibited a consistent response to cortisol.

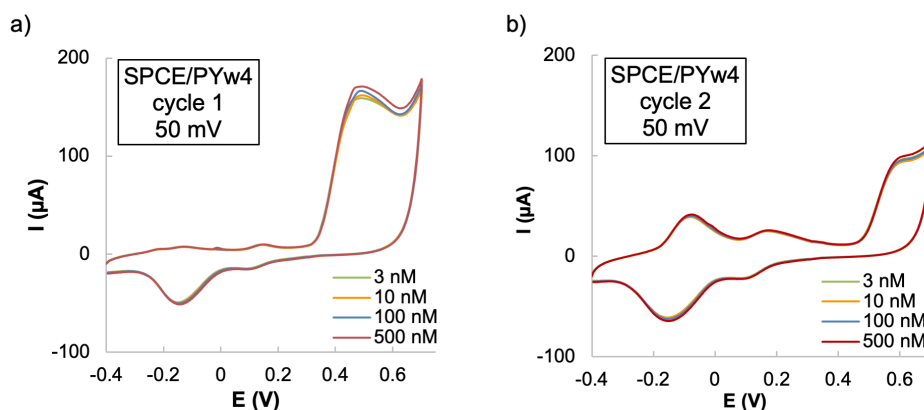


Figure 4.42. CV response of SPCE/PYw4 incubated for 10 min with cortisol concentrations (nM) at a scan rate of $50 \text{ mV} \cdot \text{s}^{-1}$ during cycles 1 (a) and 2 (b).

Other experimental parameters, including scan rate and incubation time, were also evaluated (Figures S4.20–S4.23 and S4.25–S4.27). The results are summarized in Table 4.5. As expected for a surface-confined system, the peak current increased with scan rate. Variation in incubation time resulted in a slight shift in peak position in cycle 1, along with a small decrease in peak current intensity for most peaks in both cycles, except for peaks II and II', which remained unchanged. At scan rates of 20 and $100 \text{ mV} \cdot \text{s}^{-1}$, no significant sensitivity toward cortisol was observed. In general, low slope values and poor linearity were observed, particularly at $20 \text{ mV} \cdot \text{s}^{-1}$, which may be associated with slower electron-transfer kinetics and a lower signal-to-noise ratio under these conditions. Among the tested conditions, peak I showed the most favorable response at $50 \text{ mV} \cdot \text{s}^{-1}$ after 10 min incubation, with a slope of $4.79 \mu\text{A}/\text{dec}$ and $R = 0.997$. The peak current increased with cortisol concentration, even though cortisol is not electroactive at this potential. This behavior likely reflects subtle interfacial effects, such as adsorption of cortisol near or onto the PY layer, which may locally perturb the arrangement or orientation of PY molecules, enhancing electron-transfer efficiency at certain accessible sites. However, this effect was not consistently reproducible, likely due to heterogeneity in PY coverage and variability in functional group accessibility across the electrode surface.

Table 4.5. Response of the characteristic peaks of SPCE/PYw4 over a range of [CORT] at different incubation times (min), respective equations (μA) and R .

Peak	Incubation time (min)	Scan rate ($\text{mV} \cdot \text{s}^{-1}$)		
		100	50	20
I	10	$I (\mu\text{A}) = -3.00 \log[\text{CORT}] + 282; R = 0.620$	$I (\mu\text{A}) = 4.79 \log[\text{CORT}] + 151; R = 0.997$ $I (\mu\text{A}) = -1.78 \log[\text{CORT}] + 162; R = 0.126$	$I (\mu\text{A}) = 0.24 \log[\text{CORT}] + 68.6; R = 0.063$
	30		$I (\mu\text{A}) = 0.65 \log[\text{CORT}] + 142; R = 0.045$	$I (\mu\text{A}) = 0.23 \log[\text{CORT}] + 65.9; R = 0.122$
III'	10	$I (\mu\text{A}) = -0.31 \log[\text{CORT}] + 50.2; R = 0.305$	$I (\mu\text{A}) = -0.31 \log[\text{CORT}] + 50.2; R = 0.305$ $I (\mu\text{A}) = -0.35 \log[\text{CORT}] + 30.1; R = 0.110$	$I (\mu\text{A}) = 0.10 \log[\text{CORT}] + 9.13; R = 0.114$
	30		$I (\mu\text{A}) = -0.35 \log[\text{CORT}] + 30.1; R = 0.110$	$I (\mu\text{A}) = 0.15 \log[\text{CORT}] + 6.6; R = 0.285$

Figure 4.43 shows the SPCE/PYw4 response in the presence of 5 mM Fe(III) redox probe over a range of cortisol concentrations (10 min incubation) at a scan rate of $100 \text{ mV} \cdot \text{s}^{-1}$. Only the characteristic PY and Fe peaks are visible, with no additional peaks related to cortisol. The intensity of the PY oxidation peak I (cycle 1) and peak III' (cycle 2) decreases with increasing cortisol concentration. This behavior may be attributed to partial electrode surface blocking by cortisol and/or to competition between cortisol and the redox probe for access to electroactive sites, leading to a decrease in current response. No other peaks showed a reliable or quantifiable response to cortisol. Peak I exhibited the most defined response at $100 \text{ mV} \cdot \text{s}^{-1}$, with a slope of $-7.33 \mu\text{A}/\text{dec}$ and R of 0.997. The negative slope, in contrast to the positive trend observed in the absence of the probe, further supports the influence of the Fe(III) mediator on interfacial charge-transfer processes, enhancing cortisol's surface-blocking effect. SPCE/PYw4 electrodes were also tested at a scan rate of $50 \text{ mV} \cdot \text{s}^{-1}$ in the presence of the Fe(III) probe, with no response improvement (Figures S4.24 and S4.28). The results are summarized in Table 4.6.

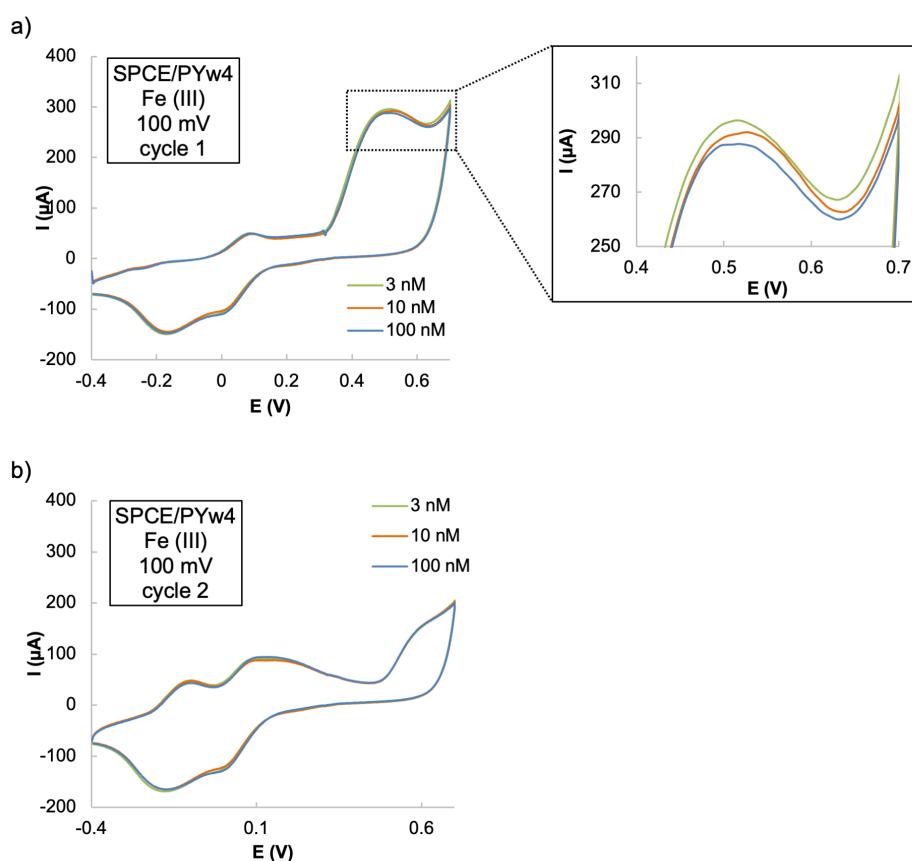


Figure 4.43. CV response of SPCE/PYw4 incubated for 10 min with cortisol concentrations (nM) in the presence of 5 mM Fe (III) at a scan rate of $100 \text{ mV} \cdot \text{s}^{-1}$ during cycles 1 (a) and 2 (b).

Table 4.6. Response of the characteristic peaks (I and III') of SPCE/PY to increasing [CORT] in the presence of 5 mM Fe (III) (10 min incubation) at different scan rates ($\text{mV} \cdot \text{s}^{-1}$), respective equations, and R .

Peak	Scan rate ($\text{mV} \cdot \text{s}^{-1}$)	
	100	50
I	$I (\mu\text{A}) = 7.33 \log[\text{CORT}] + 260$; $R = 0.997$	$I (\mu\text{A}) = -6.40 \log[\text{CORT}] + 140$; $R = 0.858$
III'	$I (\mu\text{A}) = -1.45 \log[\text{CORT}] + 31.9$; $R = 0.967$	$I (\mu\text{A}) = -0.76 \log[\text{CORT}] + 17.9$; $R = 0.910$

Overall, the electrochemical response of SPCE/PYw4 toward cortisol is characterized by changes in the intensity of intrinsic PY-related peaks rather than the appearance of new electrochemical signals. In the absence of the Fe(III) probe, an increase in oxidation peak I is observed with increasing cortisol concentration, whereas in its presence, a decrease in the intensity of peaks I and III' is detected, indicating that the sensing mechanism is strongly governed by interfacial processes. The observed dependence on experimental parameters, such as scan rate and the presence of a redox mediator, reflects the system's inherent sensitivity to interfacial and kinetic effects.

Measurable sensitivity to cortisol was observed under certain conditions, both in the absence and

presence of the Fe(III) probe. The reliability of these responses was limited, with variability observed between measurements. This variability may be partially attributed to the arrangement and accessibility of PY molecules on the SPCE surface, as well as the chemical characteristics of their functional groups. The surface modification of SPCE is likely not fully homogeneous, leading to uneven distribution and accessibility of PY groups for cortisol interaction. These results highlight the importance of controlling surface modification and measurement conditions to enhance reproducibility and analytical performance.

4.3.3.5 Reduction range

The SPCE/PYw4 response in the cathodic potential range was evaluated by DPV. Figure 4.44 shows the voltammetric responses recorded in PBS, allowing the identification of PY characteristic electrochemical peaks. Figure 4.44a presents the DPV responses obtained using two independent SPCE/PYw4 electrodes in PBS. Three characteristic peaks are observed. Peak 0, located around -0.15 V exhibits the highest intensity, while peaks I and II, located at approximately -1.1 and -1.3 V respectively, are less intense and partially overlap. The reproducibility of the signals in PBS is limited, as evidenced by variations in peak intensity and shape across measurements. This behavior can be attributed to the heterogeneity of the electrode surface, arising both from the inherent variability of commercial SPCEs, where small differences in surface structure influence the PY modification process, and from the drop-casting procedure, which may lead to non-uniform film formation, affecting the effective electroactive area and contributing to signal dispersion.

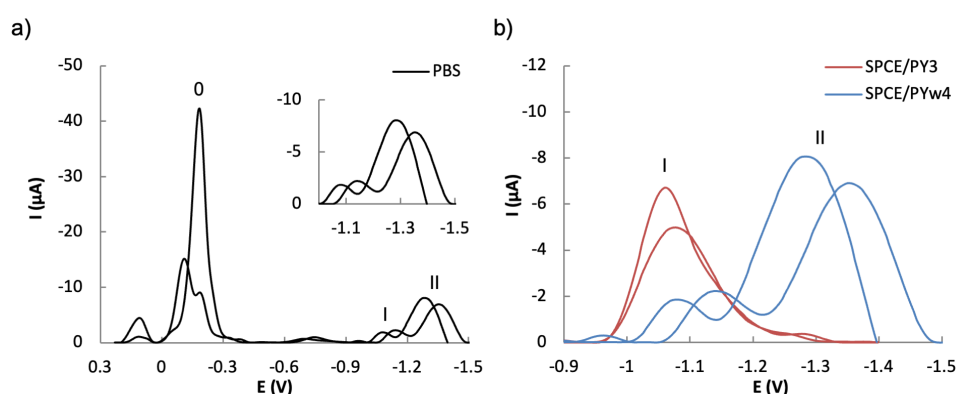


Figure 4.44. DPV responses of SPCEs in PBS modified with pyrene derivatives: (a) SPCE/PYw4; (b) comparison of SPCEs modified with aqueous (PYw4) and hydroalcoholic (PY3) PY solutions. Two replicates are shown for each measurement.

Figure 4.44b compares the responses obtained in PBS for SPCEs modified with PY solutions prepared in aqueous (PYw4) and hydroalcoholic (PY3) media. Despite differences in the explored potential windows, distinct electrochemical behaviors are observed. In the case of SPCE/PYw4, the peak at -1.3 V exhibits

higher intensity than the peak at -1.1 V whereas the opposite trend was previously observed for PY3-modified electrodes. These differences highlight the influence of the solvent used during the modification process on the PY organization at the electrode surface. The aqueous medium (PYw4) may promote aggregation or alternative molecular orientations, which affect electron transfer processes and favor the response at more negative potentials. Further work is needed to clarify the electrochemical behavior and analytical performance of PYw4-modified electrodes.

Several experimental parameters were systematically evaluated, including PY concentration at the electrode surface, SPCE pre-treatment prior to PY modification, and incubation time with cortisol, as summarized in Figure 4.45. Multipeak analysis was performed to determine which PY characteristic peak was the most sensitive to cortisol.

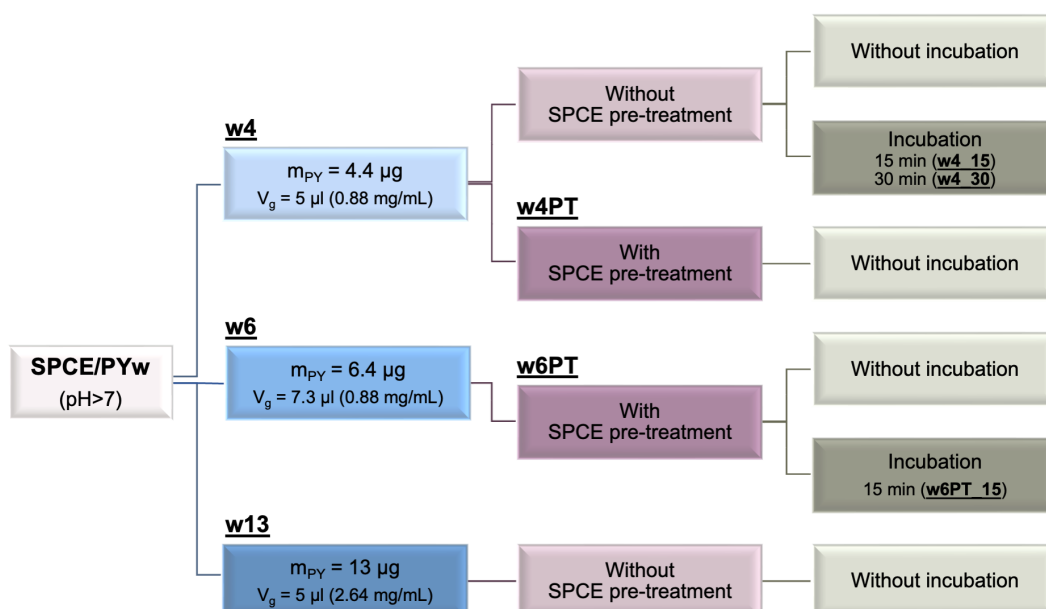


Figure 4.45. Diagram of the experimental approach used to investigate the reduction region by DPV), including variation of PY surface concentration, SPCE pre-treatment, and incubation time.

Effect of PY Solution Concentration

Electrodes modified with different PY loadings: PYw4 ($m_{PY} = 4.4 \mu g$), PYw6PT ($m_{PY} = 6.4 \mu g$, with SPCE pre-treatment), and PYw13 ($m_{PY} = 13 \mu g$), were tested to evaluate the effect of PY surface concentration on the electrochemical response.

Figure 4.46a presents the DPV responses recorded in PBS in the absence of cortisol. Three characteristic peaks are observed: peak 0, peak I, and peak II, located at approximately -0.11 V, -1.0 V, and -1.3 V, respectively. A slight shift of all peaks toward more negative potentials is observed for PYw6PT, indicative of a less favorable electron-transfer process. This can be attributed to the SPCE pre-treatment prior to PY deposition. Repeated CV cycling in PBS likely induces surface activation, leading to the for-

mation of oxygen-containing functional groups and changes in surface wettability and electroactive area. These modifications can influence the subsequent PY deposition, affecting its surface coverage and distribution and, consequently, the electron-transfer kinetics. At peak 0 (-0.11 V), the highest intensity is observed for PYw13, followed by PYw6PT and PYw4. This trend indicates that higher PY loading results in a denser, more homogeneous surface coverage, enhancing the electrochemical response at this potential. Electrodes PYw4 and PYw6PT exhibit a small overlapping peak, suggesting a less-defined surface organization. At peak I (-1.0 V), PYw13 again exhibits the highest intensity, while PYw4 shows a moderate response and PYw6PT the lowest. The enhanced response for PYw13 can be attributed to improved charge transfer associated with a denser and more continuous pyrene layer. In contrast, the lower intensity observed for PYw6PT may result from the combined effect of intermediate PY coverage and surface modification induced by the pre-treatment, which may partially hinder electron transfer by altering surface accessibility or introducing less favorable interfacial structures. At peak II (-1.3 V), PYw4 shows the highest intensity, followed by PYw6PT, while PYw13 exhibits the lowest response. Thicker pyrene layers (PYw13) may hinder charge transfer by increasing film resistance or partially blocking the electrode surface, resulting in lower current responses. In contrast, lower PY loadings (PYw4) result in thinner or less compact films, which impose less resistance to electron transfer and thus enable higher currents under these conditions. PYw6PT displays an intermediate behavior, consistent with its intermediate PY loading and the effects of surface pre-treatment on film formation.

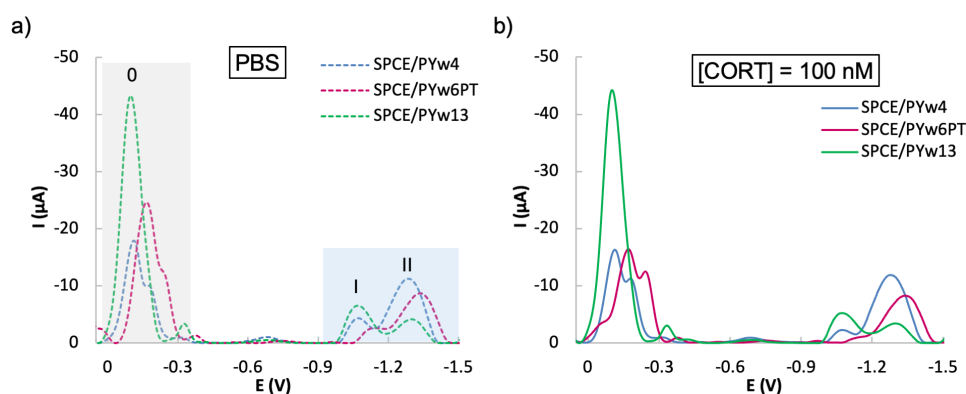


Figure 4.46. Comparison of DPV responses of PYw4 ($m_{\text{PY}}=4.4 \mu\text{g}$), PYw6PT ($m_{\text{PY}}=6.4 \mu\text{g}$), and PYw13 ($m_{\text{PY}}=13 \mu\text{g}$) tested in PBS in the absence (a) and presence of 100 nM cortisol (b).

Increasing PY concentration enhances the electrochemical response at less negative potentials (peaks 0 and I), while reducing activity at more negative potentials (peak II), reflecting a trade-off between surface coverage and electron-transfer efficiency.

Figure 4.46b presents the DPV responses of electrodes PYw4, PYw6PT, and PYw13 in the presence of 100 nM cortisol. No new peaks are observed, indicating that cortisol does not introduce additional redox

processes within the explored potential window. At peak 0 (-0.11 V), PYw13 exhibits a slight increase in current upon cortisol addition, whereas PYw4 shows a small decrease and PYw6PT a more pronounced decrease compared to their responses in PBS. This suggests that the denser pyrene layer in PYw13 is less affected by cortisol, whereas electrodes with thinner or partially compact PY layers (PYw4 and PYw6PT) are more susceptible to interfacial interactions. At peak (-1.0 V), the presence of cortisol reduces current at all electrodes, reflecting inhibition of the PY redox process. This decrease likely arises from interactions between cortisol and the pyrene molecules at the electrode interface, which hinder electron transfer. The effect is less pronounced in the denser PYw13 electrodes, where the compact pyrene layers limit PY exposure near the electrode surface, leaving the redox-active sites less affected. Peak II (-1.3 V) shows no significant change upon cortisol addition at any electrode, suggesting that processes at more negative potentials are less affected by cortisol under these conditions.

The electrochemical responses of the -modified electrodes across a range of cortisol concentrations are presented in Figure 4.47. Cortisol concentrations from 3 nM to 1000 nM were tested for electrodes PYw4 (Figures 4.47a and 4.47b) and PYw13 (Figures 4.47e and 4.47f), while electrode PYw6PT (Figures 4.47c and 4.47d) was evaluated at 3 nM and 100 nM. PY characteristic peaks 0, I, and II were monitored to assess changes in current as a function of cortisol concentration.

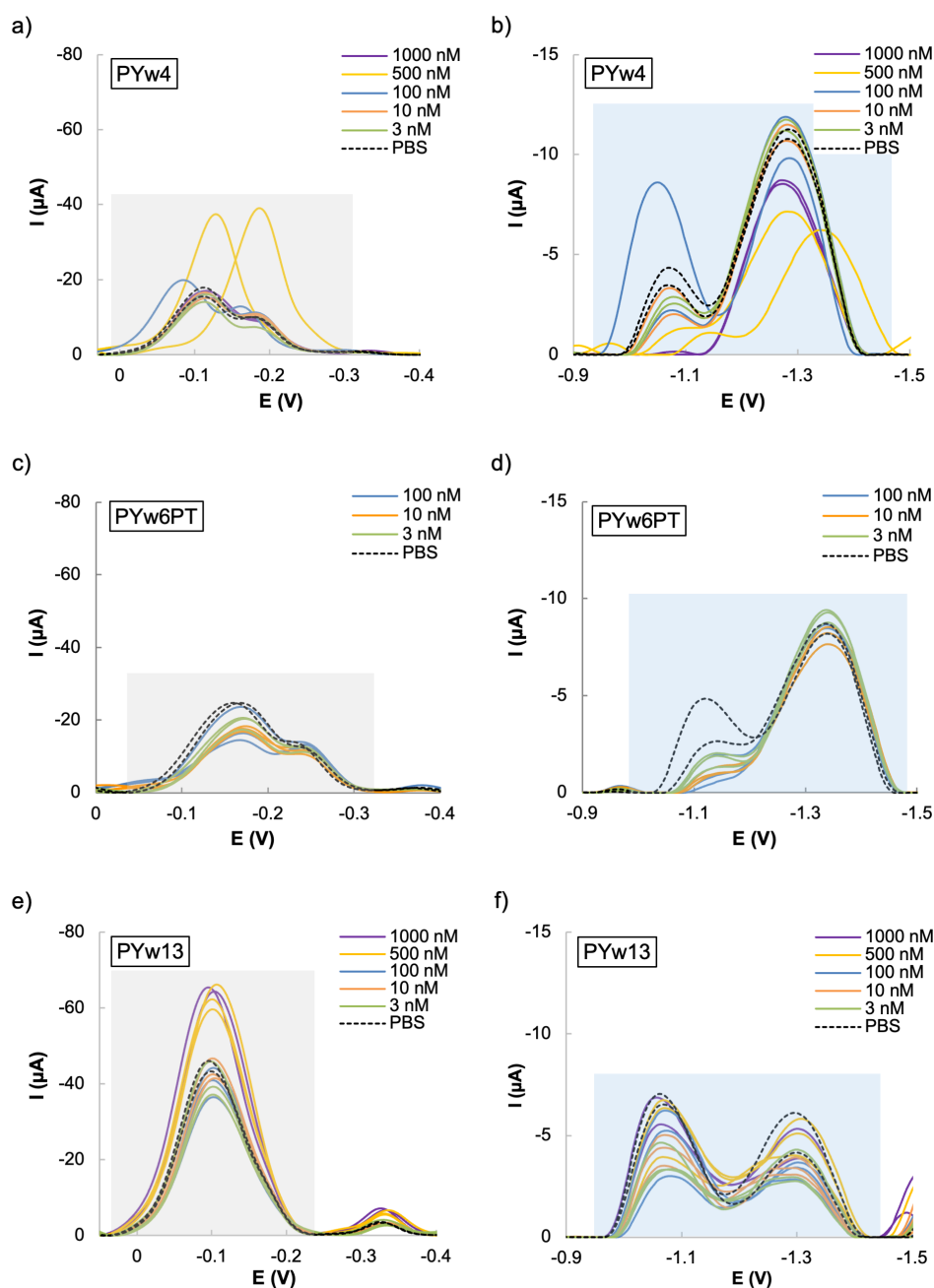


Figure 4.47. DPV response of SPCE/PYw4 (a and b), PYw6PT (c and d), and PYw13 (e and f) electrodes tested over a cortisol concentration range (nM). Subfigures (a), (c), and (e) correspond to Peak 0, whereas subfigures (b), (d), and (f) correspond to Peaks I (≈ -1.1 V) and II (≈ -1.3 V). Two replicates are shown for each measurement.

For electrode PYw4, peak 0 (-0.11 V, Figure 4.47a) shows no consistent trend in current with increasing cortisol concentration, and substantial variability is observed across replicates at the same concentration. Peak I (-1.0 V, Figure 4.47b) shows a general decrease in current with increasing cortisol, reflecting partial inhibition of the pyrene reduction process, possibly due to cortisol interactions at the electrode interface that hinder electron transfer. Peak II (-1.3 V, Figure 4.47b) also shows decreased currents at higher cortisol concentrations, but no clear concentration-dependent relationship can be established.

Electrode PYw6PT (Figures 4.47c and 4.47d) shows no clear relationship between peak currents and cortisol concentration, with substantial variability between replicates. The SPCE pre-treatment prior to PY deposition likely modified the surface chemistry and affected PY deposition, potentially reducing responsiveness to cortisol and limiting electron transfer. Electrode PYw13, with the highest PY loading, displays slightly different behavior. At peak 0 (-0.11 V, Figure 4.47e), a small increase in current is observed with increasing cortisol concentration. This change is minimal between 3 and 100 nM, followed by a more noticeable increase at 500 nM, while the response at 1000 nM remains similar to that at 500 nM. Because cortisol is electrochemically inactive at this potential, the observed changes are likely associated with interfacial effects. At lower concentrations, the limited presence of cortisol has little impact on the electrochemical response, whereas at higher concentrations, increased surface coverage may induce changes in the interfacial environment, such as slight reorganization of the pyrene layer or alterations in the electrical double layer, resulting in a measurable increase in current. The similar responses at 500 nM and 1000 nM suggest a tendency toward interfacial saturation under these conditions. Peaks I (-1.0 V) and II (-1.3 V, Figure 4.47f) show decreasing currents with increasing cortisol concentration, although variability between replicates prevents quantitative analysis. At these more negative potentials, the high PY loading may lead to a more compact or partially aggregated film that, together with cortisol adsorption, increases interfacial resistance and hinders charge transfer, resulting in lower current responses.

SPCE/PYw4 showed the best cortisol response, with a sensitivity of $1.64 \mu\text{A}/\text{dec}$, with a yet limited R value of 0.804 for peak II. The corresponding fittings for all samples are provided in Figure S4.29 (Appendix).

Effect of SPCE Pre-treatment Before Modification

To address the variability associated with the non-homogeneous surface of commercial SPCEs, a pre-treatment step (electrode PYw4PT) was applied prior to PYw4 deposition. The pretreatment involved potential cycling (-0.6 to 0.6 V at $50 \text{ mV} \cdot \text{s}^{-1}$, 20 cycles) in PBS to improve surface uniformity and enhance reproducibility [159, 160].

Figure 4.48a compares the DPV responses of SPCEs modified with PY, without (PYw4) and with pre-treatment (PYw4PT), in the absence of cortisol. Three characteristic peaks are observed: peak 0 (between -0.1 and -0.2 V), peak I (around -1.1 V), and peak II (near -1.3 V). The detrimental effect of SPCE pre-treatment on sensor performance is attributed to the electrochemical introduction of oxygenated functional groups. This modification increases surface hydrophilicity and disrupts the π - π stacking stability between the PY framework and the carbon substrate, resulting in increased interfacial resistance and suppressed cortisol interaction. The intensity of peak 0 increases after pre-treatment. This behavior may result from

changes in the film structure and small differences in PY loading, leading to higher current responses at this potential. In contrast, peak I shows only minor changes in intensity, while peak II shows a decrease in intensity, suggesting a less favorable electron-transfer process at more negative potentials, possibly due to increased disorder or partial blocking of electroactive sites. Figure 4.48b shows the DPV responses of SPCEs modified with PY, without (PYw4) and with pre-treatment (PYw4PT) in the presence of 100 nM cortisol. Peak I shows only a slight decrease in intensity. Peak 0 shows higher intensity for the pre-treated electrode, while peak II shows a significantly lower intensity than for the untreated electrode. These differences are consistent with the behavior observed in PBS and suggest that the pre-treatment alters the PY layer's structure and interfacial properties, influencing its interaction with cortisol.

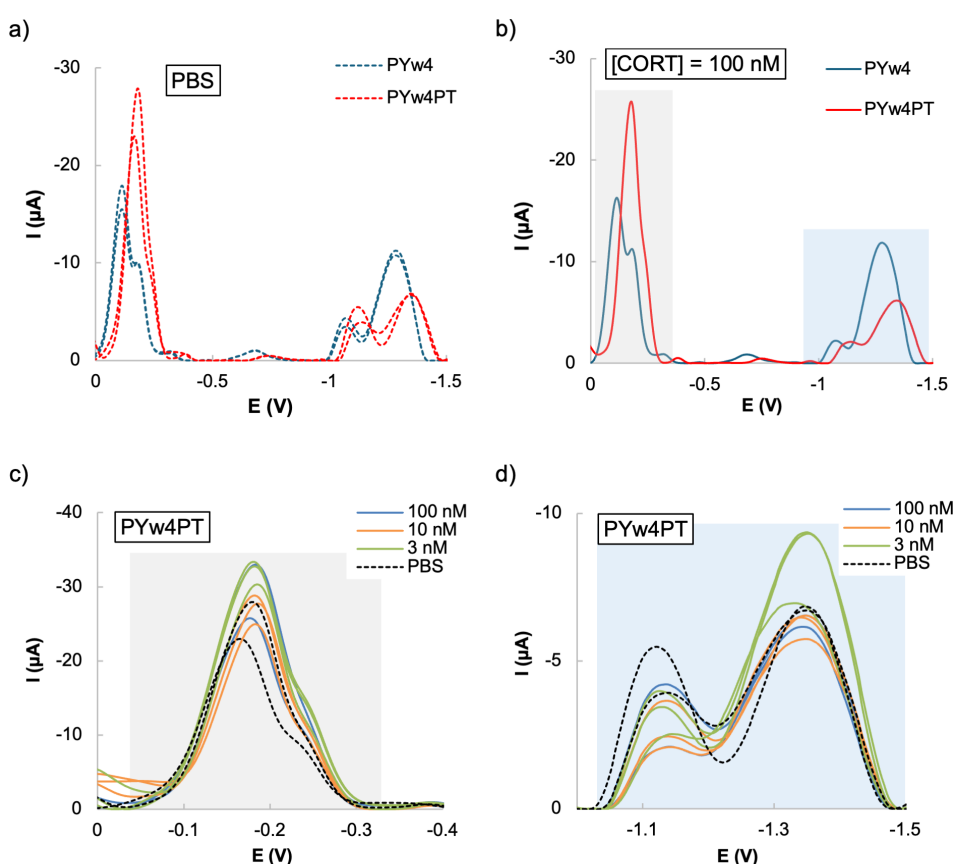


Figure 4.48. Comparison of DPV responses from SPCE/PYw4 ($m_{PY} = 4.4 \mu g$) without (PYw4) and with (PYw4PT) pre-treatment of the SPCE, tested in PBS in the absence (a) and presence of 100 nM cortisol (b); c) and d) Comparison of DPV responses from SPCE/ PYw4PT over increasing cortisol concentration (nM). All replicates are shown.

Figures 4.48c and 4.48d further show the response of the pre-treated electrode (PYw4PT) over a range of cortisol concentrations. No clear relationship is observed between cortisol concentration and the intensities of peaks 0 and I. Although a slight decrease in peak II intensity is observed with increasing cortisol concentration, it is not sufficiently consistent to establish a reliable correlation. The corresponding fits are provided in the Figure S4.30 (Appendix). These results suggest that pre-treatment negatively

affects sensor performance, possibly by influencing the amount of PY deposited, its interaction with the electrode surface, and the orientation or accessibility of its functional groups, ultimately hindering effective interaction with cortisol.

Effect of Incubation Time with Cortisol

To reduce variability in sensor response and improve signal stability, SPCEs modified with 4.4 μg of PY (PYw4) were incubated for 15 and 30 minutes in solutions containing different cortisol concentrations. Figure 4.49a shows the DPV responses of SPCE/PYw4 electrodes incubated in PBS in the absence of cortisol. With increasing incubation time, peak 0 remains essentially constant in intensity, with a slight shift toward more positive potentials. This behavior may indicate a gradual reorganization of the PY layer at the electrode interface, possibly driven by interactions with the medium (e.g., hydration), leading to a more stable electrochemical environment.

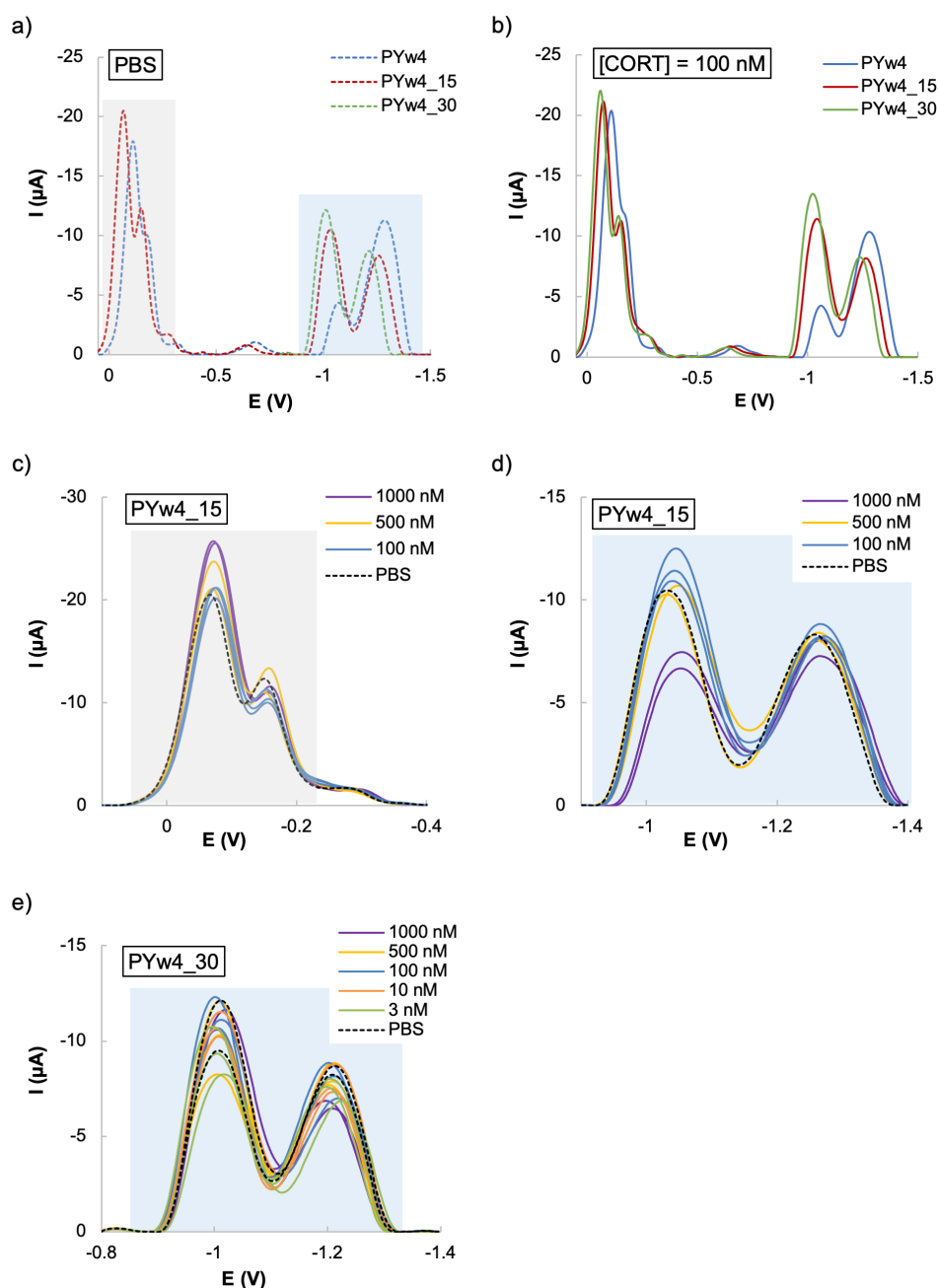


Figure 4.49. a) DPV responses of SPCE/PYw4 electrodes ($m_{\text{PY}} = 4.4 \mu\text{g}$) after incubation in PBS for 0, 15, and 30 minutes, in the absence of cortisol; b) corresponding responses in the presence of 100 nM cortisol; c) and d) DPV responses obtained after 15 minutes of incubation (PYw4_15) at different cortisol concentrations (nM); e) DPV responses obtained after 30 minutes of incubation (PYw4_30) at different cortisol concentrations (nM). All replicates are shown.

Peak I shows a clear increase in intensity from 0 to 15 minutes, followed by a smaller increase up to 30 minutes, suggesting that incubation promotes a more favorable arrangement of the PY layer for electron transfer. In contrast, peak II decreases in intensity and shifts toward less negative potentials, suggesting that parts of the PY layer become less accessible over time, possibly due to film reorganization or partial blocking. Figure 4.49b shows a similar response in the presence of 100 nM cortisol, indicating

that the main effect of incubation time is due to changes in the PY layer rather than a direct electrochemical contribution from cortisol.

In Figures 4.49c and 4.49d, the DPV responses corresponding to peaks 0, I, and II, respectively, are analyzed as a function of cortisol concentration during a 15 min incubation. Peak 0 increases slightly with increasing cortisol concentration. As cortisol is electrochemically inactive at this potential, this behavior is more likely related to changes at the electrode interface, such as adsorption-induced modifications of the PY layer or local environment. In contrast, peaks I and II show a decrease in current with increasing cortisol concentration, with the effect more pronounced for peak I. This trend suggests that cortisol interacts with the PY layer through nonspecific adsorption or partial blocking of electroactive sites, partially hindering electron transfer. The best cortisol response was obtained for SPCE/PYw4 incubated for 15 minutes, with a sensitivity of $4.36 \mu\text{A}/\text{dec}$, R value of 0.862 (peak 0), and $3.84 \mu\text{A}/\text{dec}$, R value of 0.856 (peak I).

For a 30 minute incubation (Figure 4.49e), peaks I and II show increased variability between replicates and no clear dependence on cortisol concentration. This indicates that longer incubation does not improve sensitivity and may negatively affect reproducibility. This effect may be associated with continued reorganization of the PY layer, possible swelling or partial desorption, or changes in film adhesion, leading to less stable electrochemical responses. The corresponding fittings for all samples are provided in Figure S4.31 (Appendix).

To further evaluate the effect of incubation time, additional experiments were performed at 1000 nM and 100 nM cortisol using different incubation periods (0, 1, 5, 10, and 15 minutes for 1000 nM; 0, 15, and 30 minutes for 100 nM).

For peak 0 (Figure S4.32a), at 1000 nM cortisol, the current shows a slight increase at short incubation times followed by stabilization. This likely reflects changes at the electrode interface due to cortisol adsorption or rearrangement of the PY layer, which modify the local environment and measured current. At 100 nM, the signal remains nearly constant, suggesting either weak interaction or rapid establishment of equilibrium without significant interfacial changes.

For peak I (Figure 4.50), at $1 \mu\text{M}$ cortisol, the current increases during the first minutes of incubation and stabilizes after approximately 10 minutes. At 100 nM, the current shows a more gradual variation, extending up to 30 minutes. This behavior indicates that the increase in current is at least partially driven by time-dependent reorganization of the PY layer in PBS, which improves electron transfer. The presence of cortisol appears to modulate this process: at higher concentrations, the system reaches equilibrium more rapidly, likely due to faster occupation of available sites, while at lower concentrations, both PY reorganization and cortisol interaction contribute over a longer timescale.

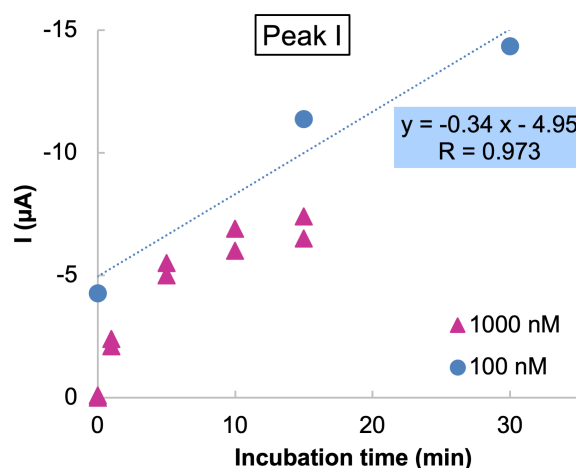


Figure 4.50. Comparison of the intensity of peak I with the increase in incubation time for different cortisol concentrations (● – 100 nM and ▲ – 1000 nM).

For peak II (Figure S4.32b), the current decreases during the initial minutes of incubation and then stabilizes. This trend is consistent with early-stage film reorganization or partial blocking of less accessible electroactive regions, followed by stabilization of the interface. The similar behavior observed at both concentrations suggests that this peak is primarily governed by structural changes within the PY layer rather than by direct interaction with cortisol.

To evaluate whether incubation improves the response of pre-treated electrodes, SPCEs modified with PYw6PT (6.4 μg) were incubated for 15 minutes. Figure 4.51a shows that in the absence of cortisol, incubation shifts all peaks toward less negative potentials and increases peak I intensity, while peaks 0 and II remain largely unchanged. This suggests that incubation promotes some reorganization of the PY layer, particularly affecting the sites associated with peak I, possibly due to medium-induced rearrangement. In the presence of 100 nM cortisol (Figure 4.51b), similar behavior is observed. The increase in peak 0 intensity is consistent with the changes already observed and is therefore more likely related to PY layer reorganization than to cortisol interaction.

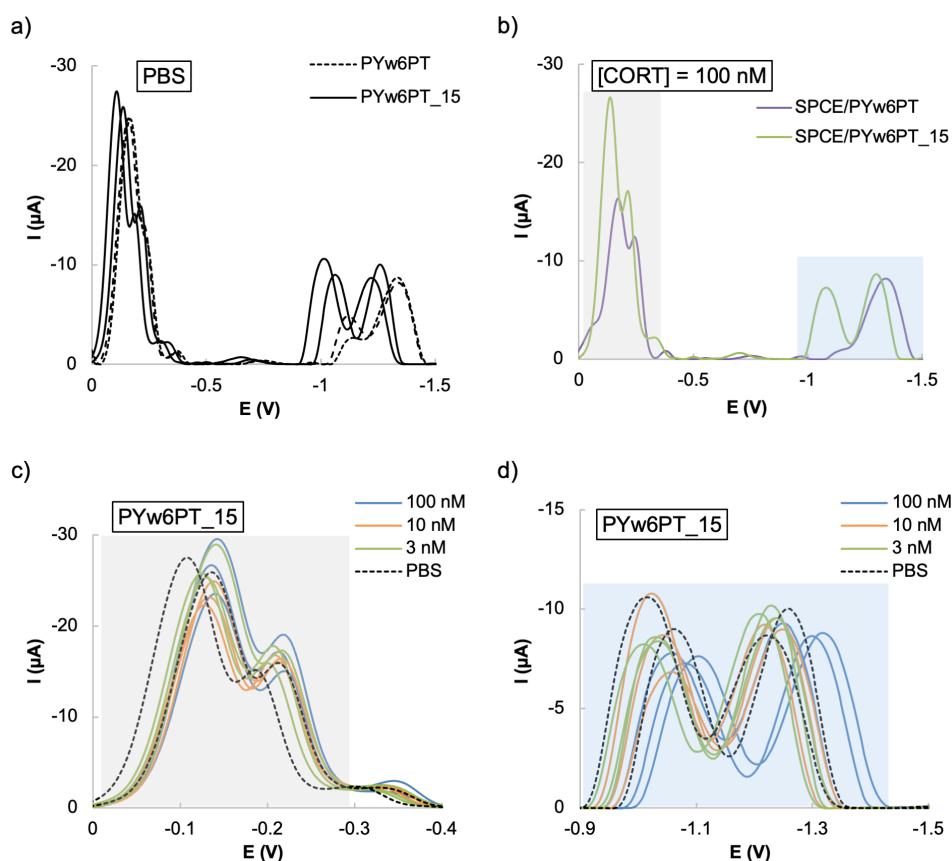


Figure 4.51. a) DPV responses of SPCE/PYw6PT electrodes ($m_{\text{PY}} = 6.4 \mu\text{g}$) after incubation in PBS for 0 and 15 minutes, in the absence of cortisol. Two replicates are shown for each measurement; b) corresponding responses in the presence of 100 nM cortisol; c) and d) DPV responses obtained after 15 minutes of incubation (PYw6PT_15) at different cortisol concentrations (nM). Three replicates are shown for each measurement.

Figures 4.51c and 4.51d show that peaks 0 and I do not present a clear dependence on cortisol concentration. Peak II shows a tendency toward lower currents at higher cortisol concentrations, although variability remains significant. This variability likely reflects differences in PY layer organization introduced by the pre-treatment, as discussed previously, including possible changes in film adhesion, structure, and accessibility of functional groups. Although incubation may stabilize the interface, it does not significantly improve the analytical response to cortisol (Figure S4.33, Appendix).

Figure 4.52 summarizes the quantitative analysis of the electrochemical response obtained for SPCE/PYw4 ($4.4 \mu\text{g}$), PYw6PT ($6.4 \mu\text{g}$ with pre-treatment), and PYw13 ($13 \mu\text{g}$) electrodes, accounting for PY loading, SPCE pre-treatment, incubation time, and the selected electrochemical peak.

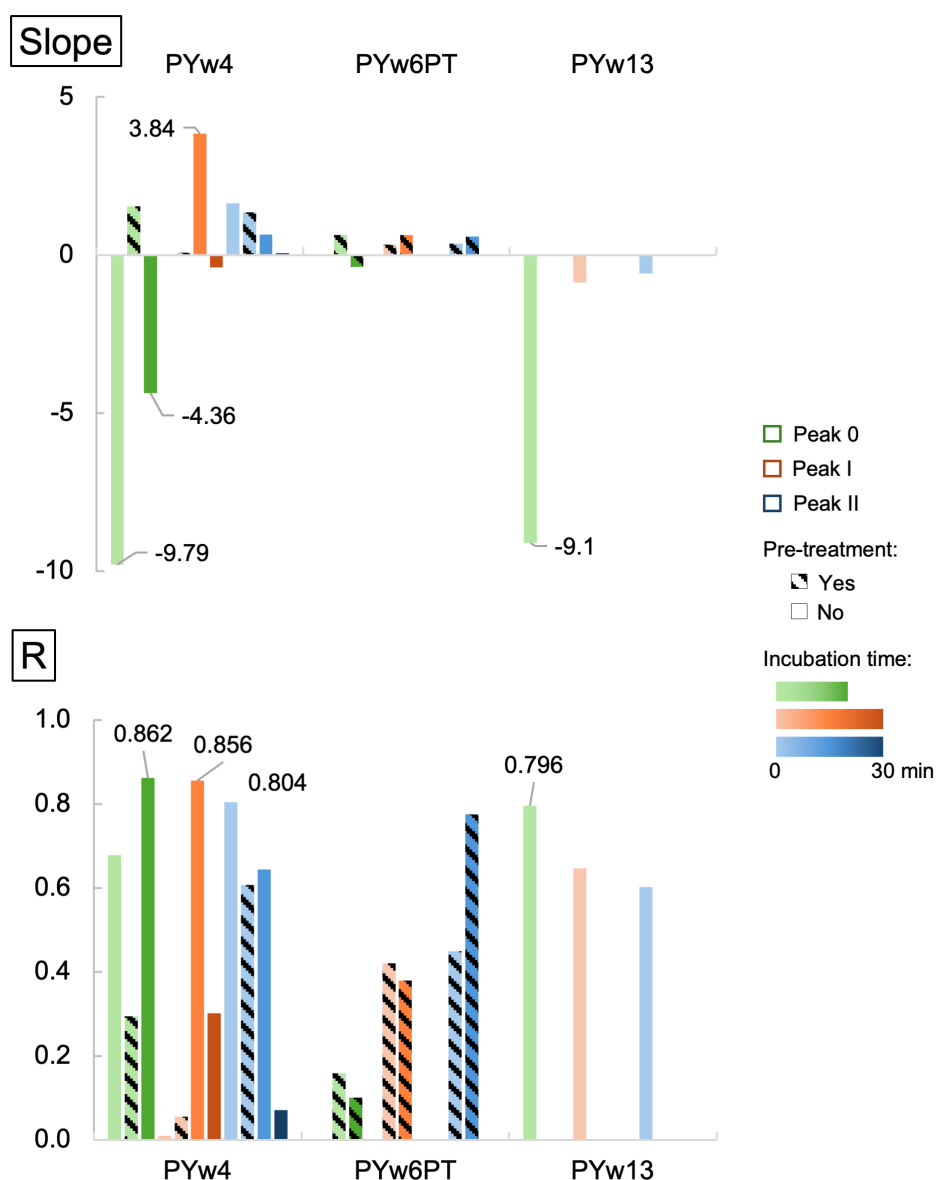


Figure 4.52. Comparison of slope and R values of PYw4, PYw6PT, PYw13 electrodes for peaks 0 (green), I (orange), and II (blue), under incubation time (0, 15, and 30 minutes, color gradient) without and with SPCE pre-treatment (dashed pattern).

The best overall response is obtained for the intermediate PY loading (PYw4), although with some limitations in linearity. This behavior may be associated with partial interface saturation, charge-transfer limitations, or adsorption effects at higher cortisol concentrations. Pre-treatment of the SPCE consistently leads to poorer performance, as observed for PYw4PT and PYw6PT. Incubation time also influences the response, particularly for peaks I and II. A 15 minute incubation improves the response for peak I, likely due to stabilization and reorganization of the PY layer at the electrode interface. In contrast, longer incubation (30 minutes) leads to increased variability and reduced sensitivity. For peak II, the highest response is generally observed without incubation, suggesting that prolonged exposure may reduce accessibility of electroactive regions contributing to this peak.

4.3.4 Conclusion

The electrochemical response of PY depends strongly on the applied potential, with cortisol detection reflected in changes in intrinsic PY peak intensities rather than new signals, highlighting interfacial effects.

In the anodic region, the PY oxidation peak shows opposing trends under different experimental conditions. At 0.5 V, peak currents increase slightly with cortisol in the absence of the Fe(III) mediator, likely due to minor adsorption-induced rearrangements of PY that enhance electron transfer at accessible sites. This behavior is not consistently reproducible, probably due to heterogeneity in PY coverage and functional group accessibility. In the presence of Fe(III), peak currents decrease with increasing cortisol, indicating that the mediator amplifies surface-blocking effects by competing for access to electroactive sites.

In the cathodic region, SPCE/PYw4 electrodes with 15 minute incubation showed the best balance of sensitivity and reproducibility at -1.1 V. Higher PY loadings and SPCE pre-treatment reduced performance, likely due to changes in film structure and reduced accessibility of functional groups. Shorter incubation stabilized the PY layer, while longer exposure increased variability and decreased sensitivity.

In summary, aqueous-alkaline deposition is a scalable, environmentally friendly route to uniform sensing interfaces. We demonstrate that optimal performance is achieved by balancing PY loading ($4.4\ \mu\text{g}$) with a controlled incubation time (15 min), yielding a sensitivity of $4.36\ \mu\text{A}/\text{dec}$. These findings provide a blueprint for the fine-tuning of non-affinity electrochemical sensors.

4.3.5 Appendix

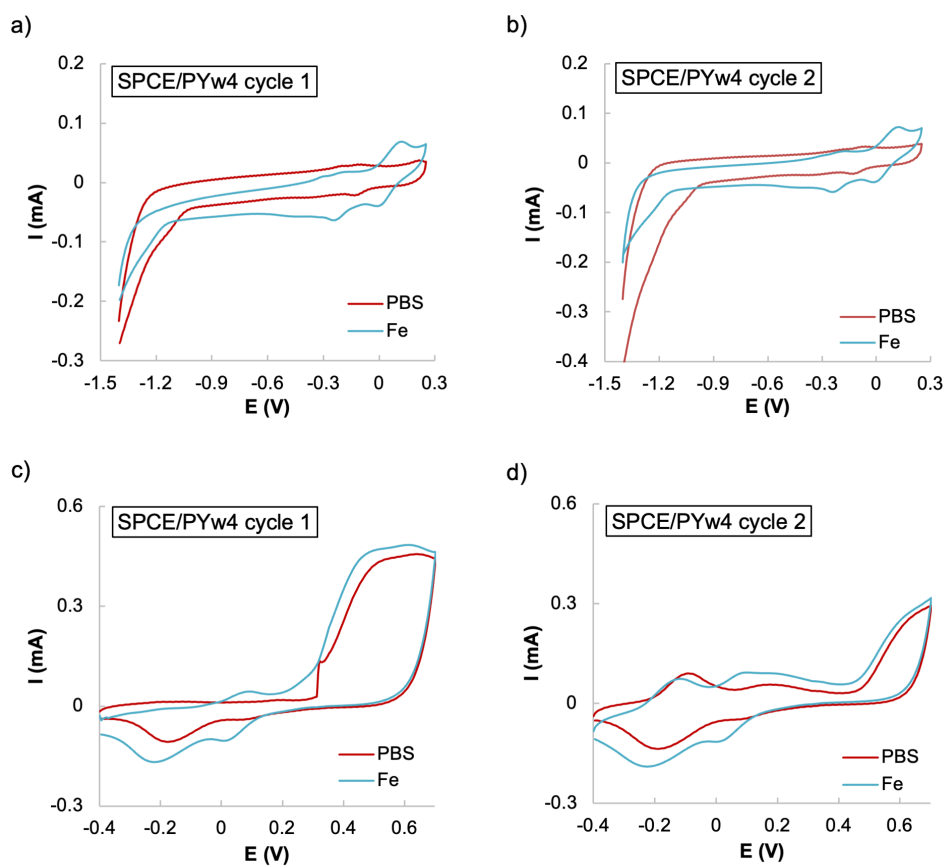


Figure S4.19. Comparison of the CV response of SPCE/PYw4 in the absence (PBS) and presence of 5.0 mM Fe (III) redox probe within the reduction potential range of -1.5 to 0.25 V for cycles 1 (a) and 2 (b), and oxidation potential range of -0.4 to 0.7 V for cycles 1 (c) and 2 (d).

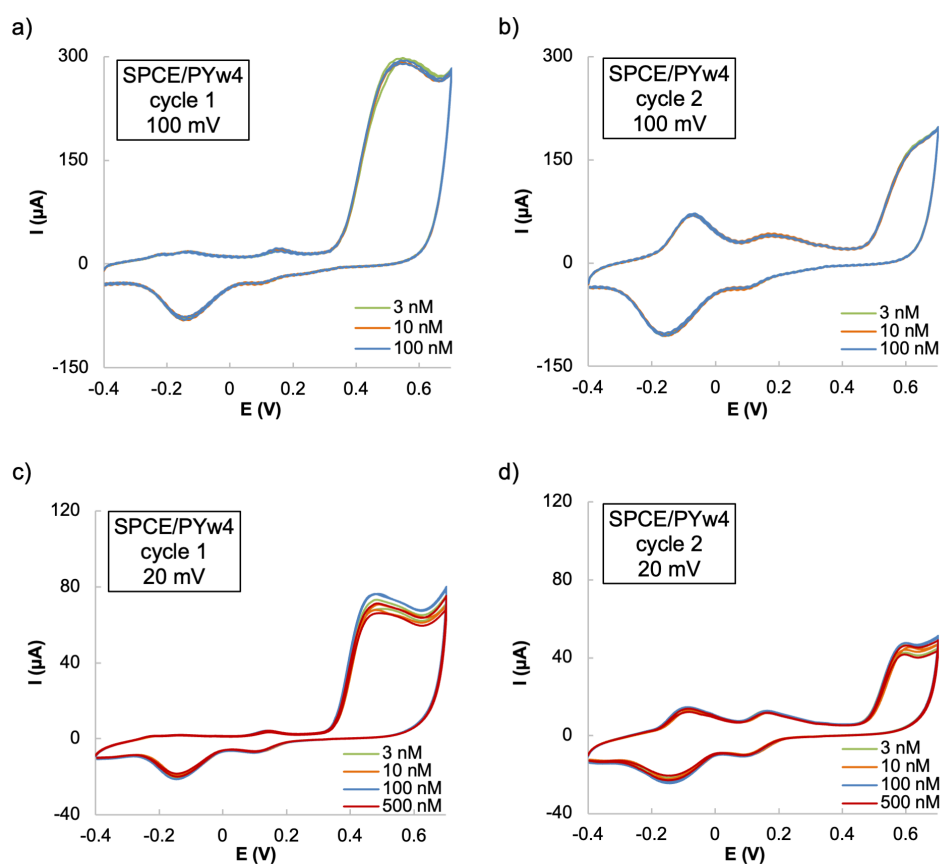


Figure S4.20. CV response of SPCE/PYw4 incubated for 10 min with cortisol concentrations (nM) at a scan rate of $100 \text{ mV} \cdot \text{s}^{-1}$ (a and b) and $20 \text{ mV} \cdot \text{s}^{-1}$ (c and d) during cycles 1 and 2, respectively. Two replicates are shown for each measurement.

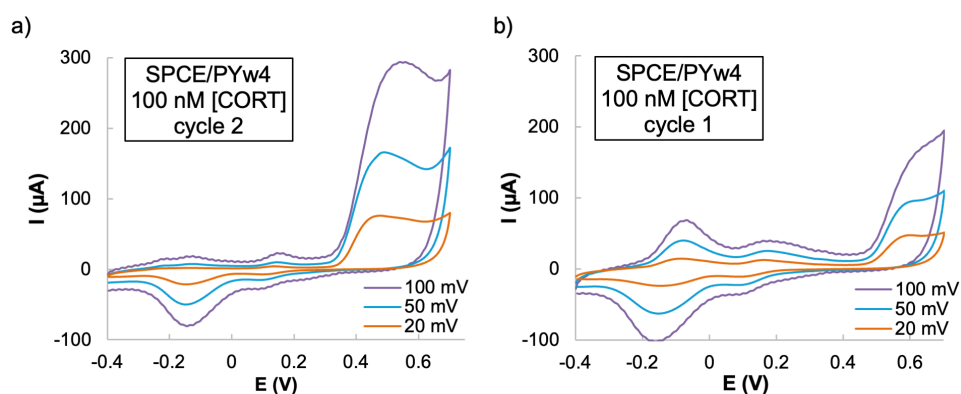


Figure S4.21. Effect of the scan rate ($100, 50, 20 \text{ mV} \cdot \text{s}^{-1}$) on the CV response of SPCE/PYw4 during cycles 1 (a) and 2 (b), tested with 100 nM of cortisol (10 min incubation).

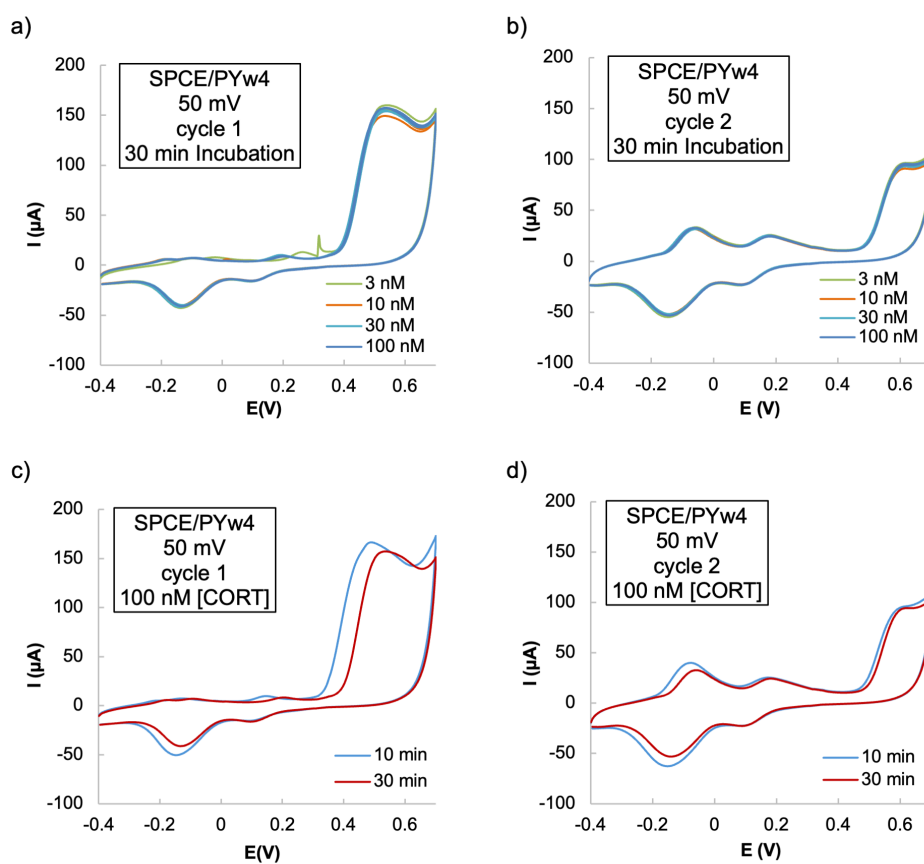


Figure S4.22. CV response of SPCE/PYw4 incubated for 30 min with cortisol ranging from 3 to 100 nM during cycles 1 (a) and 2 (b) at a scan rate of $50 \text{ mV} \cdot \text{s}^{-1}$. Two replicates are shown for each measurement. Effect of incubation time (10 and 30 min) of 100 nM of cortisol on the CV response of SPCE/PYw4 during cycles 1 (c) and 2 (d).

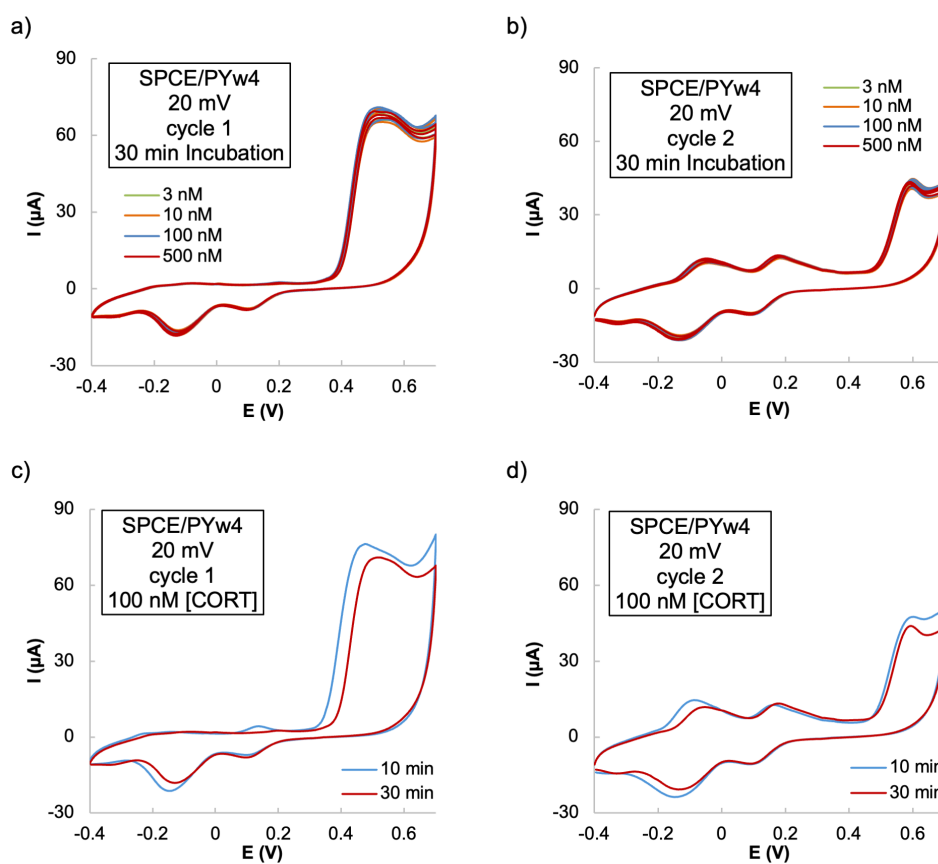


Figure S4.23. CV response of SPCE/PYw4 incubated for 30 min with cortisol ranging from 3 to 100 nM during cycles 1 (a) and 2 (b) at a scan rate of $20 \text{ mV} \cdot \text{s}^{-1}$. Two replicates are shown for each measurement. Effect of incubation time (10 and 30 min) of 100 nM of cortisol on the CV response of SPCE/PYw4 during cycles 1 (c) and 2 (d).

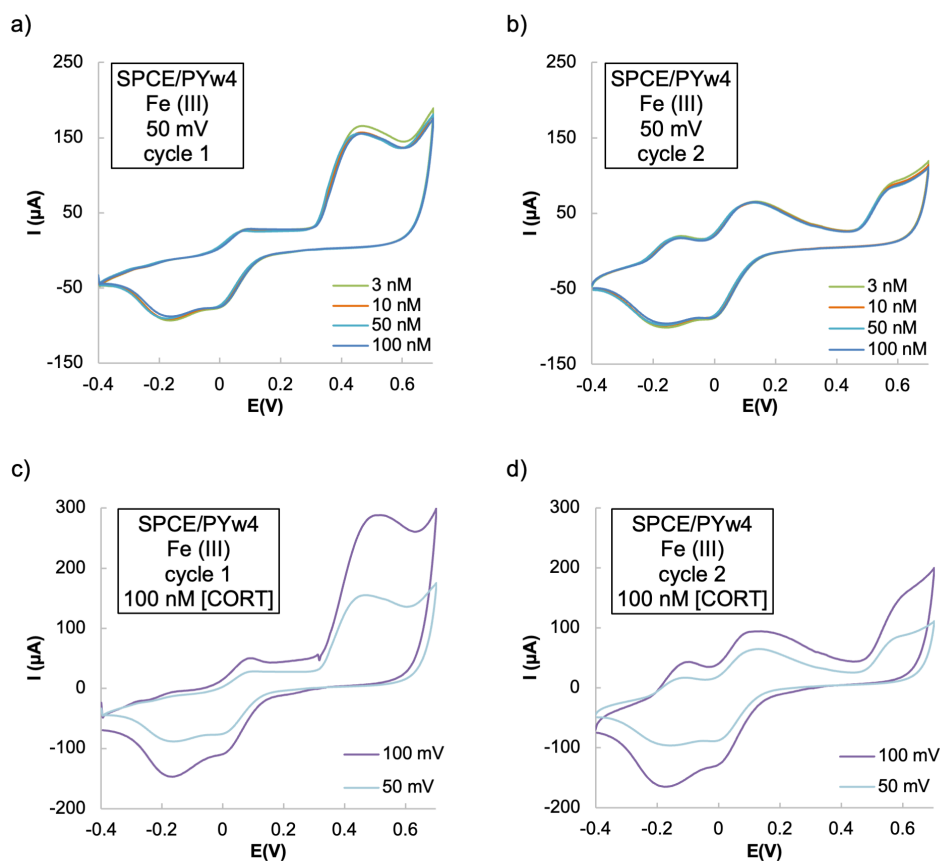


Figure S4.24. CV response of SPCE/PYw4 incubated for 10 min with cortisol concentrations in the presence of 5 mM Fe (III) at a scan rate of $100 \text{ mV} \cdot \text{s}^{-1}$ during cycles 1 (a) and 2 (b). Effect of the scan rate (100 and $50 \text{ mV} \cdot \text{s}^{-1}$) on the CV response of SPCE/PYw4 during cycles 1 (c) and 2 (d), tested with 100 nM of cortisol (10 min incubation).

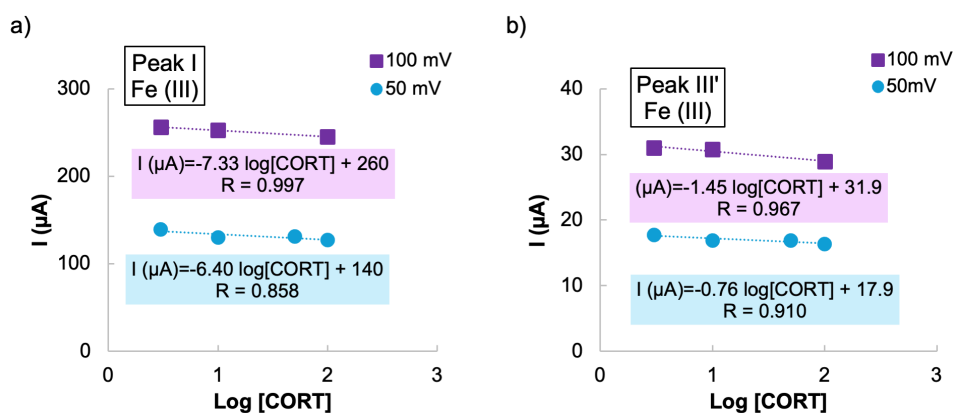


Figure S4.25. Effect of scan rate ($100, 50, 20 \text{ mV} \cdot \text{s}^{-1}$) on the SPCE/PY4w intensity peaks I (a) and III' (b) across cortisol concentrations (nM), with an incubation time of 10 min.

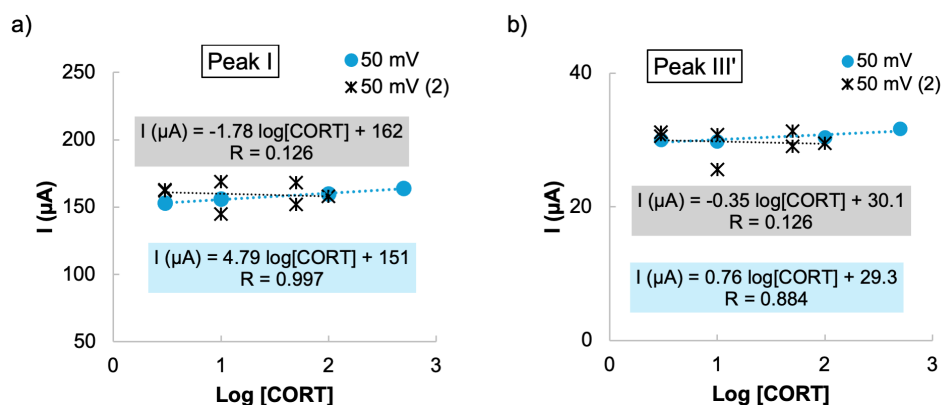


Figure S4.26. Reproducibility of peak I (a) and III' (b) intensities across cortisol concentrations (nM) at 50 mV · s⁻¹ (10 min incubation), based on two replicates.

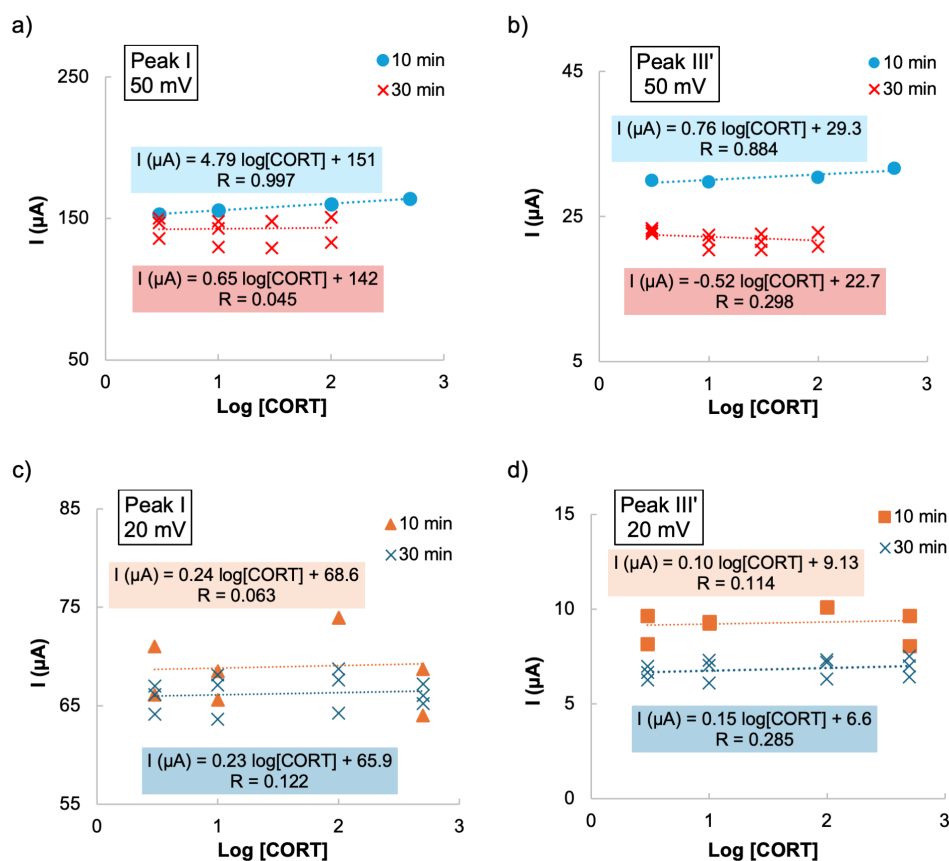


Figure S4.27. Effect of incubation time (10 and 30 min) on peak I and III' intensities at SPCE/PY4w across cortisol concentrations (nM) at 50 mV · s⁻¹ (a, b) and 20 mV · s⁻¹ (c, d), respectively. All replicate measurements are shown.

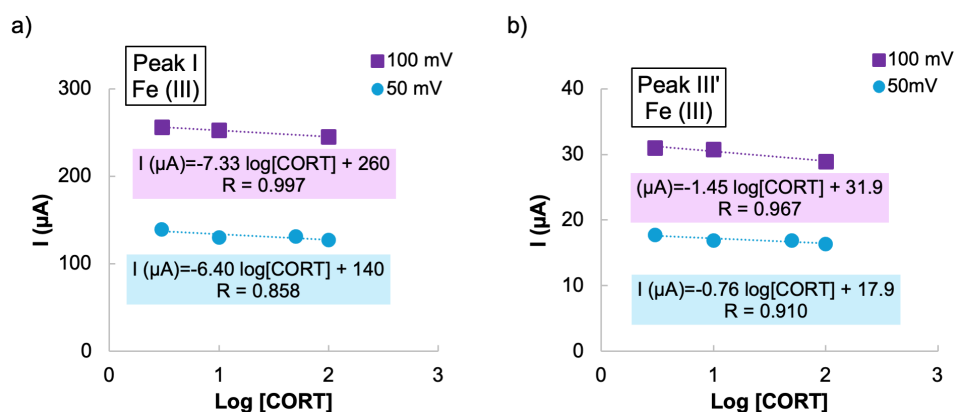


Figure S4.28. Effect of scan rate (100 and 50 $\text{mV} \cdot \text{s}^{-1}$) on peak I (a) and III' (b) intensities over nM cortisol range (10 min incubation) in the presence of 5 mM Fe(III).

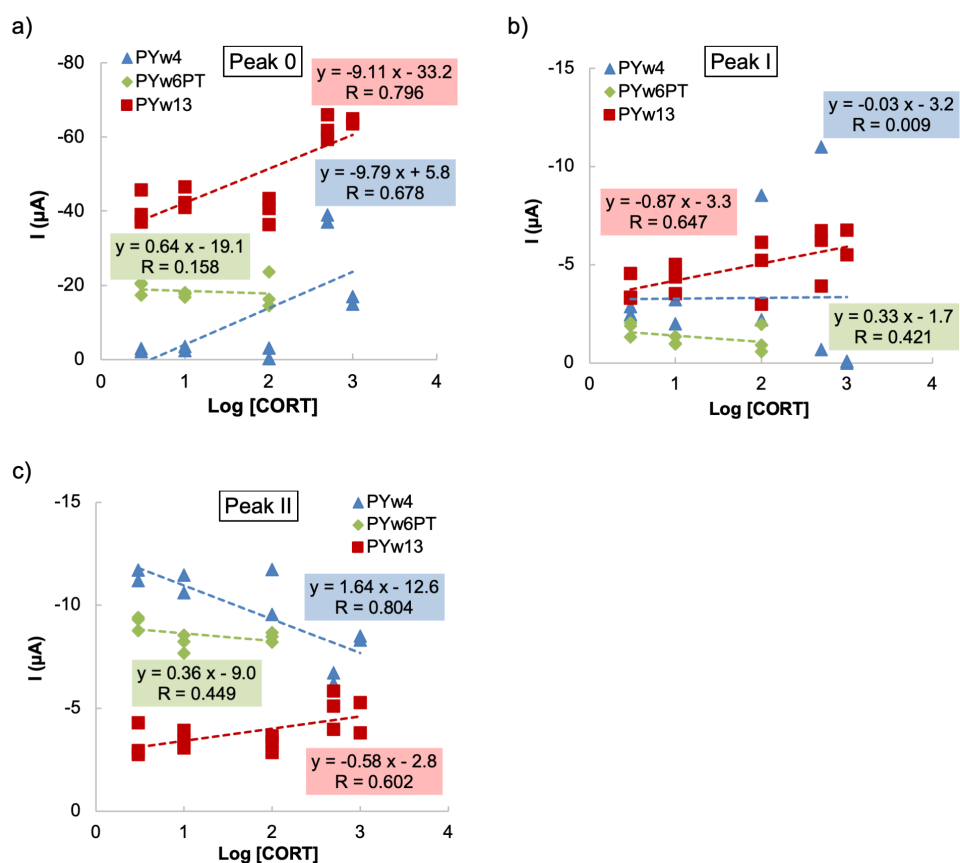


Figure S4.29. Effect of PY concentration in the SPCE/PYw electrodes on peak 0, I, and II intensities across cortisol concentrations (nM). All replicate measurements are shown.

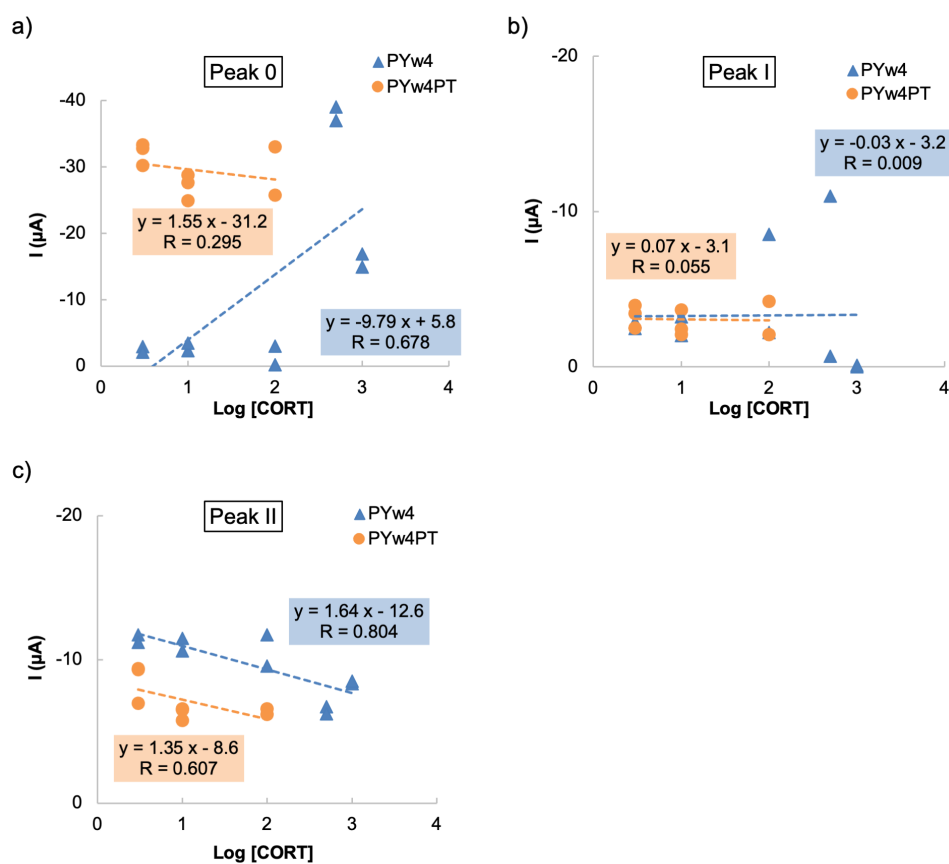


Figure S4.30. Effect of pre-treatment in the SPCE/PYw4 electrodes on peak 0, I, and II intensities across cortisol concentrations (nM). All replicate measurements are shown.

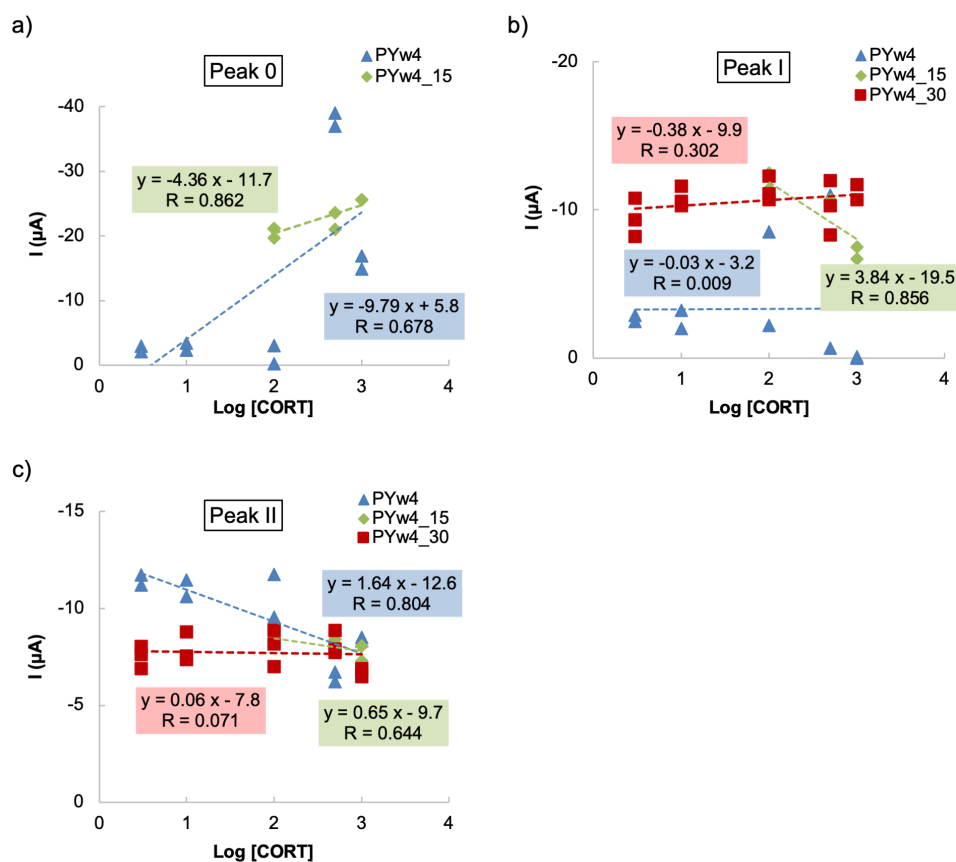


Figure S4.31. Effect of incubation time (0, 15, and 30 minutes) in the SPCE/PYw4 electrodes on peak 0, I, and II intensities across cortisol concentrations (nM). All replicate measurements are shown.

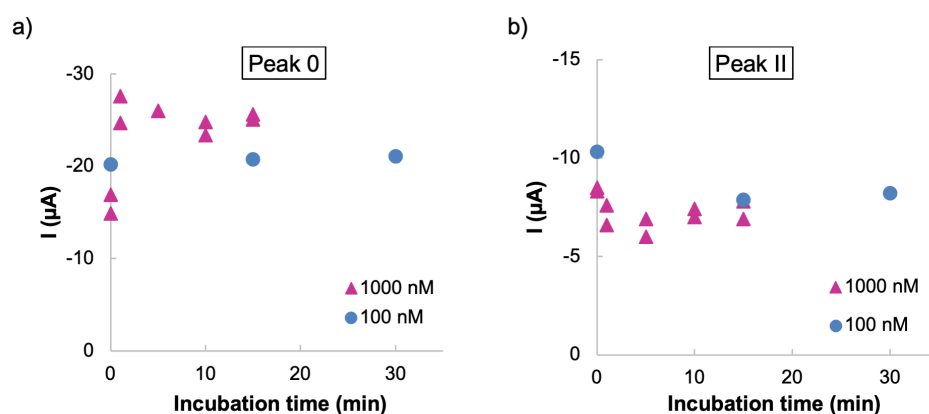


Figure S4.32. a) and b) Comparison of the peak intensities 0 and II, respectively, with the increase in incubation time for different cortisol concentrations (● – 100 nM and ▲ – 1000 nM). All replicate measurements are shown.

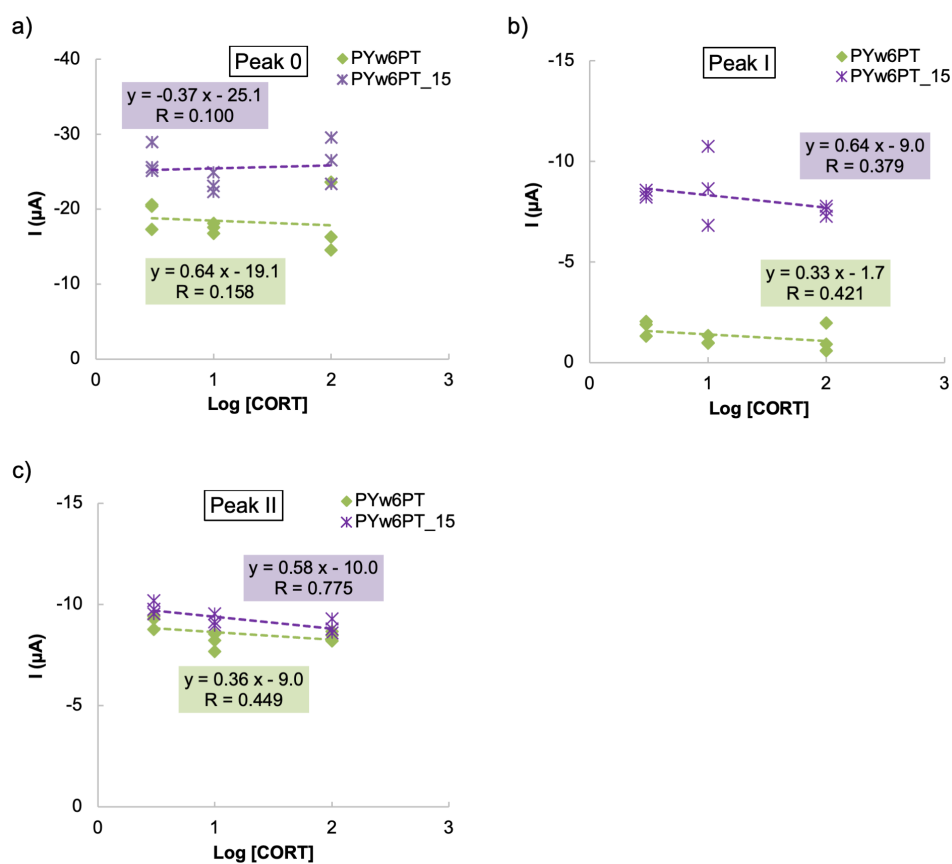


Figure S4.33. Effect of incubation time (0 and 15 minutes) in the SPCE/PYw6PT electrodes on peak 0, I, and II intensities across cortisol concentrations (nM). All replicate measurements are shown.

Chapter 5

Tailoring the Electrochemical Interface for Cortisol Sensing: A Comparative Study of MWCNT and Carbon Black Hybrid Architectures Functionalized with Pyrene Derivatives

This chapter is based on the article:

Santos, A. R., Bento, F., Paiva, M. C., Silva, A. F., & Viana, J. C. "Tailoring the Electrochemical Interface for Cortisol Sensing: A Comparative Study of MWCNT and Carbon Black Hybrid Architectures Functionalized with Pyrene Derivatives". (in preparation)

5 Tailoring the Electrochemical Interface for Cortisol Sensing: A Comparative Study of MWCNT and Carbon Black Hybrid Architectures Functionalized with Pyrene Derivatives

Building on a previously developed electrochemical platform based on a pyrene derivatives (PYs), this study explores the integration of carbon nanomaterials, namely multi-walled carbon nanotubes (MWCNTs) and carbon black (CB). The hybridization of screen-printed carbon electrodes (SPCEs) with these materials significantly increases the electroactive surface area for cortisol detection. Electrochemical evaluation using differential pulse voltammetry (DPV) demonstrated that both nanomaterials enhance sensitivity in the nanomolar range. The CB/PY configuration achieved a sensitivity of $4.13 \mu\text{A}/\text{dec}$, surpassing that of the MWCNT/PY platform ($2.15 \mu\text{A}/\text{dec}$). This improvement is attributed to the high defect density and heterogeneous structure of CB, which favorably influences the orientation of the pyrene-based sensing layer.

5.1 Introduction

Cortisol is a steroid hormone commonly used as a biomarker of physiological stress, and its detection at low concentrations is crucial in complex biological samples. Electrochemical sensing platforms based on SPCEs are widely used for this purpose due to their low cost, disposability, and compatibility with miniaturized devices [161]. However, bare SPCEs have an inherently heterogeneous surface, which limits the availability of well-defined active sites and efficient electron transfer, leading to reduced sensitivity and poorly resolved electrochemical signals [161, 162]. To overcome these limitations, SPCE surfaces are often modified with carbon nanomaterials to enhance electrochemical performance. MWCNTs are among the most used modifiers, providing high electrical conductivity and a large surface area that promote efficient electron transfer and improved current responses [151, 162–166]. Their graphitic structure also allows π – π interactions with aromatic molecules such as pyrene derivatives, facilitating the formation of stable sensing interfaces. More recently, CB has emerged as a cost-effective alternative [167–169]. CB combines fast charge-transfer properties with a high electroactive surface area. Its quasi-spherical particles can form uniform films on the electrode surface, increasing surface roughness and introducing additional electrochemically active sites, which further enhance electrocatalytic performance.

In our previous work, a cortisol-sensing platform based on a modified PY was developed, demon-

strating promising electrochemical performance [101]. In the present study, carbon nanomaterials were incorporated into this PY-based sensing platform. MWCNTs and CB were combined with the PY sensing layer to fabricate modified SPCE. MWCNT/PY dispersions were first investigated by UV–Vis spectroscopy to evaluate their interaction and stability. The electrochemical responses of MWCNT/PY and CB/PY-based electrodes were evaluated by DPV at nanomolar cortisol concentrations. The influence of the cortisol incubation time on the sensor response was assessed.

5.2 Experimental section

Multi-walled carbon nanotubes (MWCNT, NC700) were purchased from Nanocyl. Four solutions containing 0.002 g of MWCNT and 0.05 mM PY in 100 mL of ethanol were prepared. Each one (MWCNT/PY 0.5h, MWCNT/PY 1h, and CNT/PY 3h) was subjected to ultrasonic tip treatment (Hielscher UP200Ht, 50% speed) for 0.5, 1, and 3 h, respectively. Sonication was performed in an ice bath to prevent overheating.

UV-visible analysis was performed using a Shimadzu UV-2401PC to assess solution dispersion efficiency. The UV spectra of PY were recorded in ethanol, while those of CNT/PY were obtained using a PY solution of the same concentration (0.05 mM).

Absorbance (A) was used as a relative measure of concentration. According to the Beer–Lambert law:

$$A = \epsilon lc \quad (5.1)$$

where ϵ is the extinction coefficient, l the optical path length, and c the concentration. For a constant optical path length and fixed experimental conditions, ϵ and l remain constant, resulting in $A \propto c$.

After sonication, MWCNTs non-covalently functionalized with PY were obtained either by solvent evaporation (MWCNT/PYe) or by filtration using a porous nylon membrane (45 μm) to remove excess PY (MWCNT/PYf), and then dried in a vacuum at 60°C.

Aqueous suspensions ($pH > 7$) of 1 mg/mL MWCNT/PYe and MWCNT/PYf were prepared, and 5 μL were drop-cast onto SPCEs. The electrodes were allowed to dry under yellow light before measurements.

A CB suspension (1 mg/mL) was prepared by mixing 0.01 g CB in a 10 mL sodium dodecyl sulfate (SDS, 3.47 mM)/water (1:9) solution. The mixture was then ultrasonicated for 1 h (UP 100H, 100% A, 0.9 cycle). SPCEs were modified with 6.5 μL of CB, dried under yellow light, and then 5 μL of PY (4.4 ng) was deposited and dried at room temperature. The effects of 15 and 30 min of cortisol incubation were examined.

The modified SPCE/MWCNT/PYe, SPCE/MWCNT/PYf, and SPCE/CB/PY were tested over a wide

range of cortisol concentrations. PBS and cortisol solutions preparation and electrochemical measurements were performed as described previously [101].

The modified electrode's surface morphology was characterized by scanning electron microscopy (SEM) analysis using a NanoSEM-200 apparatus from FEI Nova. The samples were coated with 9 nm of gold (Au)/palladium (80/20 %W).

5.3 Results

5.3.1 SPCE/MWCNT/PY

5.3.1.1 Optical characterization (UV–Vis Spectroscopy)

The stability of the MWCNT/PY dispersions was evaluated by UV–Vis analysis. Figure 5.1a compares the UV–Vis spectra of PY solution (reference) and MWCNT/PY suspension sonicated for 0.5 h. The spectrum of the MWCNT/PY suspension retains the characteristic absorption bands of PY, although with reduced intensity. The intrinsic absorption of the MWCNTs shifts the spectrum to higher absorbance values, resulting in an increased background signal. This shift to higher absorbance indicates the presence of MWCNTs in suspension.

Figures 5.1b and 5.1c show the evolution of the absorbance at 275 nm and 500 nm, respectively, for CNT/PY dispersions prepared by ultrasonication for 0.5, 1, and 3 h and monitored over time. The wavelength at 275 nm corresponds to a characteristic absorption region of PY and includes a contribution from dispersed MWCNTs. Variations in absorbance at this wavelength therefore reflect changes in the amount of dispersed MWCNT/PY complexes present in the optical path.

As shown in Figure 5.1b, the absorbance decreases during the first 24 h for all dispersions, indicating progressive aggregation or sedimentation of MWCNTs in suspension. The signal at 500 nm is mainly attributed to light absorption and scattering by dispersed MWCNTs. As shown in Figure 5.1c, the absorbance of all dispersions rapidly decreases within the first 24 h, indicating progressive sedimentation of MWCNTs.

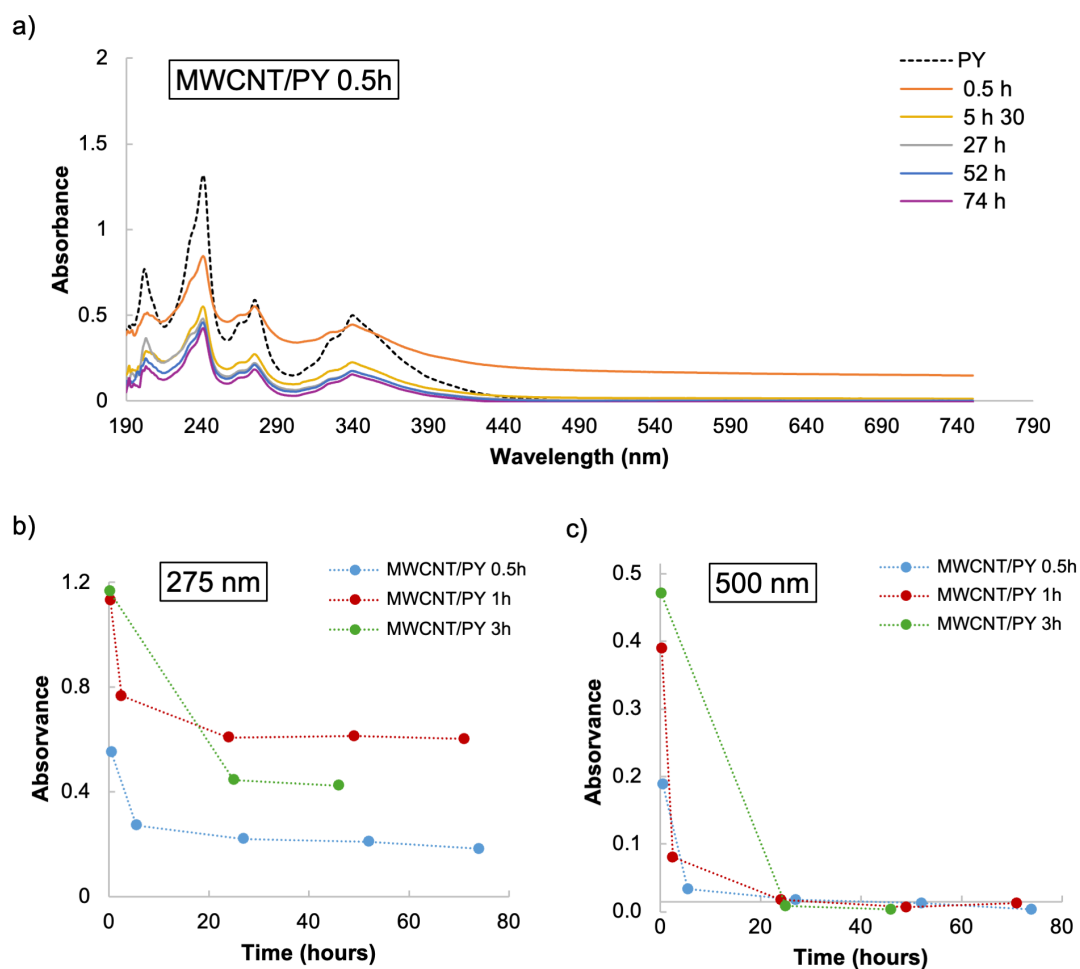


Figure 5.1. (a) UV–Visible spectra of PY and MWCNT/PY suspension prepared by ultrasonication for 0.5 h; the suspension was maintained at rest and the UV-vis spectra were obtained along the shelf-time. Evolution of the absorbance at (b) 275 nm and (c) 500 nm for MWCNT/PY suspensions prepared with ultrasonication times of 0.5, 1, and 3 h, monitored over time.

The dispersion prepared with 3 h of ultrasonication exhibited the highest initial absorbance, indicating a more effective initial dispersion of MWCNTs in the PY solution, and was therefore selected for the preparation of MWCNT/PY-modified electrodes in subsequent electrochemical experiments. The MWCNT/PY dispersions prepared under these conditions were used for electrode modification, allowing a few hours of stability without significant loss of suspended MWCNTs.

5.3.1.2 Morphological characterization

SEM analysis of the SPCE/MWCNT/PYe interface revealed a uniform coating where the typical PY ribbon-like structures were absent (Figure 5.2). This suggests that strong $\pi - \pi$ interactions between the pyrene rings and the graphitic walls of the MWCNT prevent the self-aggregation of PY into ribbons, favoring a more dispersed molecular state.

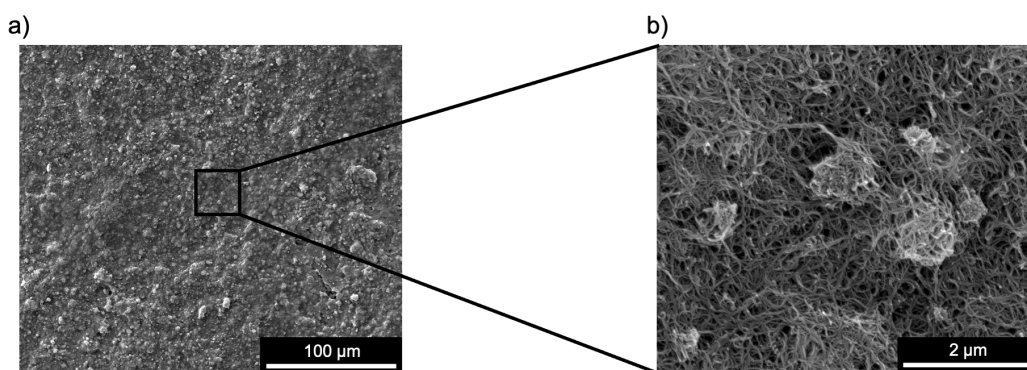


Figure 5.2. SEM images of the surface of SPCE/MWCNT/PYe at different magnifications.

5.3.1.3 Voltammetric characterization of the sensor response

The electrochemical response of MWCNT/PY was evaluated by DPV. The sensing strategy relies on the intrinsic electrochemical activity of PY, which exhibits characteristic cathodic peaks. Previous work demonstrated that cortisol interacts with PY functional groups, decreasing the intensity of the PY electrochemical signal. To verify whether this behavior is maintained in the MWCNT/PY electrode, its electrochemical response was initially evaluated at relatively high cortisol concentrations (380 and 760 μM). At 760 μM cortisol (Figure 5.3), the SPCE/PY-CNTe electrodes exhibit erratic voltammetric profiles characterized by broad and partially overlapped peaks appearing at different potentials, with low peak currents ($-0.3 < I_p < -0.45 \mu\text{A}$). Variability between electrodes tested at the same cortisol concentration is likely related to differences in the distribution of the drop-cast nanomaterial layer on the SPCE surface. As previously demonstrated by molecular simulations [101], cortisol interacts with PY through multiple non-covalent interactions. At very high cortisol concentrations, the coexistence of PY molecules interacting with different numbers of cortisol molecules may generate electroactive species with slightly different reduction potentials, resulting in broad, partially overlapping voltammetric peaks. Extensive cortisol adsorption may also hinder electron transfer at the electrode interface, further distorting the voltammetric response. At 380 μM cortisol (Figure 5.3), the voltammetric response becomes more defined, with two peaks appearing at approximately -1.1 V ($I_p \approx -1.25 \mu\text{A}$) and -1.35 V ($I_p \approx -0.25 \mu\text{A}$). The peak at -1.1 V corresponds to the characteristic reduction peak of PY and is more intense than that observed at 760 μM cortisol. This behavior is consistent with the previously reported PY–cortisol interaction, where increasing cortisol concentration leads to a progressive decrease in the intensity of the PY reduction peak. SPCE/PY-CNTf electrodes were also tested, but no significant improvement in response was observed (Figures SS5.1, Appendix).

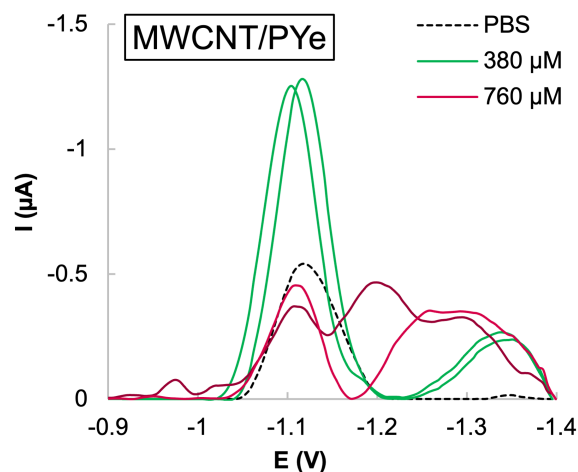


Figure 5.3. DPV responses of SPCEs modified with MWCNT/PYe for [CORT] = 0, 380, and 760 μM . Two replicates are shown for each measurement.

The electrochemical behavior of the sensor was further investigated at lower cortisol concentrations. Figure 5.4a presents the voltammetric response of SPCE/PY-CNTe electrodes for cortisol concentrations between 1 and 100 μM . In this concentration range, the electrodes exhibit a well-defined reduction peak at approximately -1.15 V (Peak I) corresponding to the intrinsic electrochemical response of PY. In some electrodes, a slight shift of this peak toward -1.2 V is observed, which can be attributed to small variations in electrode surface morphology, MWCNTs dispersion, and PY surface coverage. The peak current reaches $-9\text{ }\mu\text{A}$, significantly higher than those observed for 380 and 760 μM . As cortisol concentration increases within this range, a gradual decrease in peak current is observed. This behavior indicates that cortisol affects the electroactive PY sites at the electrode surface, attenuating the intrinsic PY signal.

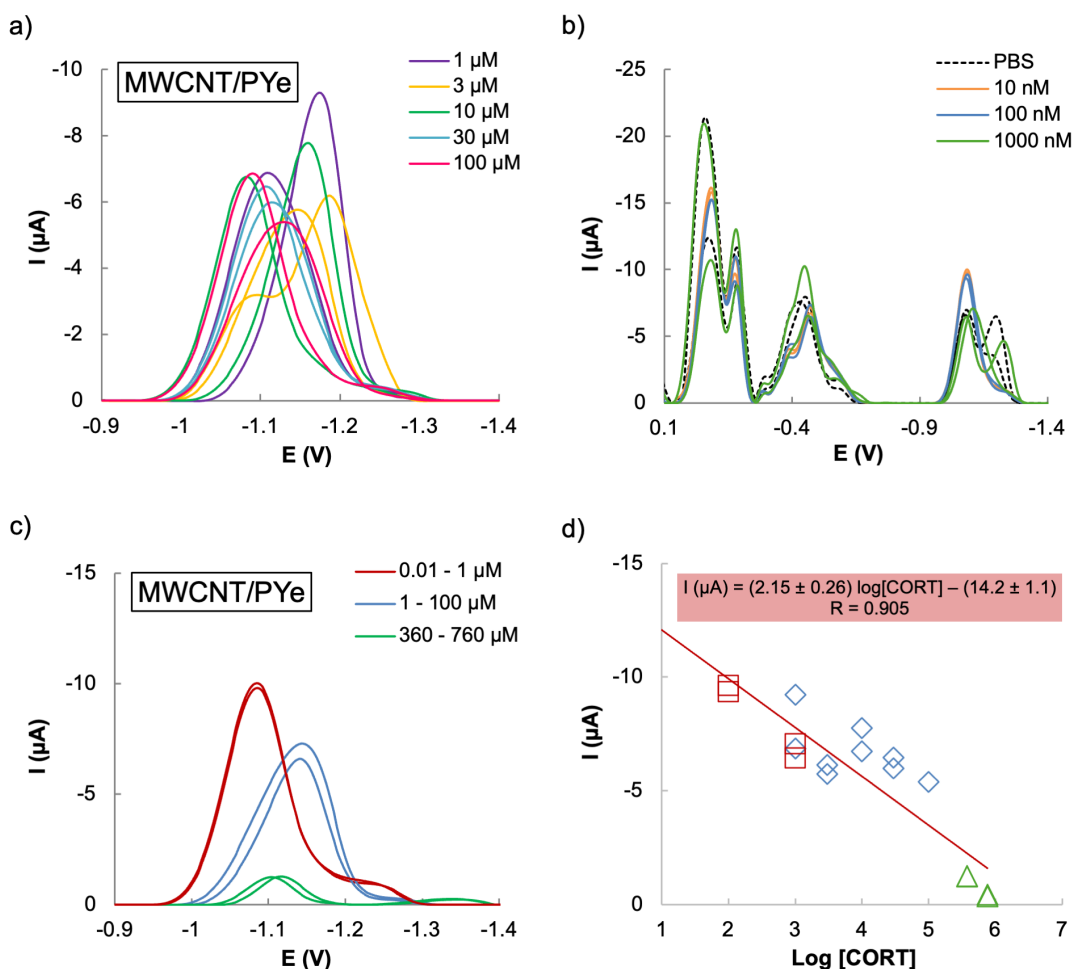


Figure 5.4. DPV of SPCE/MWCNT/PY electrodes. a) Response at high (μM) cortisol concentrations; b) Response at low (nM) cortisol concentrations; c) Comparison of peak I current intensity and potential across different cortisol concentration ranges (0.01–1 μM , 1–100 μM , 360–760 μM); d) Calibration curve showing peak I current as a function of cortisol concentration (nM). All replicates are shown.

The voltammetric behavior of the sensor was also evaluated in the nanomolar concentration range (10–1000 nM), as shown in Figure 5.4b. In phosphate-buffered saline (PBS), several PY characteristic peaks are observed at approximately 0, -0.1, -0.45, and -1.1 V (Peak I). In some samples, a small peak (Peak II) overlaps with peak I around -1.25 V, reflecting small variations in surface morphology and PY distribution across the electrode. The presence of cortisol decreases the intensity of the reduction peak at -1.1 V (Peak I). This effect is evident in Figure 5.4c, which compares the position and intensity of peak I across different cortisol concentration ranges, showing a clear decrease in peak intensity with increasing cortisol concentration. The corresponding calibration curve (Figure 5.4d) shows a sensitivity of 2.15 $\mu\text{A}/\text{dec}$ with a correlation coefficient of $R = 0.905$. The moderate correlation is primarily attributed to potential shifts in the PY reduction peak across different electrodes, resulting from slight variations in nanomaterial dispersion and surface coverage during the drop-casting process. Future stabilization of

the nanomaterial ink could mitigate this variability, enhancing the reproducibility of commercial wearable applications. Incubation with cortisol worsened the electrochemical response (Figure S5.2, Appendix).

5.3.2 SPCE/CB/PY

5.3.2.1 Morphological characterization

The surface of the CB/PY-modified electrode is covered by a mixture of small spherical particles and elongated ribbon-like structures, characteristic of CB and PY [135, 138], respectively (Figure 5.5a). Regions with a higher density of CB particles can also be observed. The PY ribbon-like structures, with measured widths ranging from approximately 100 to 500 nm (Figure 5.5b), appear partially collapsed, and some degree of overlap between ribbons is observed.

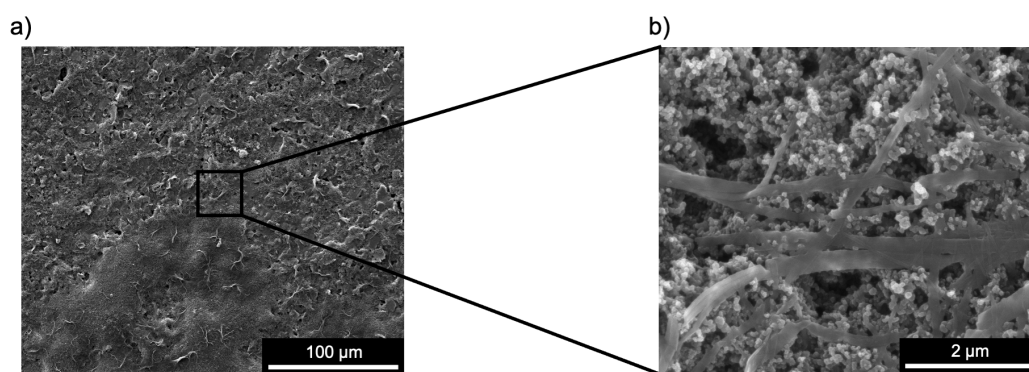


Figure 5.5. SEM images of the working electrode (WE) surface of SPCE/CB/PY at different magnifications.

5.3.2.2 Voltammetric characterization of the sensor response

The effect of incubation time on the electrochemical response of SPCE/CB/PY electrodes was evaluated. Figure 5.6a shows the intrinsic electrochemical response of the CB/PY-based electrode after 0, 15, and 30 min incubation in PBS in the absence of cortisol. For each incubation time, several SPCE/CB/PY replicas were analyzed. The electrode without incubation time exhibits two characteristic PY reduction peaks: peak I at approximately -1.05 V and peak II at -1.27 V. The intensity of peak I varies between 0.44 and 4.87 μA , whereas peak II ranges from -12.67 to -14.49 μA , showing higher intensity and lower variability than peak I.

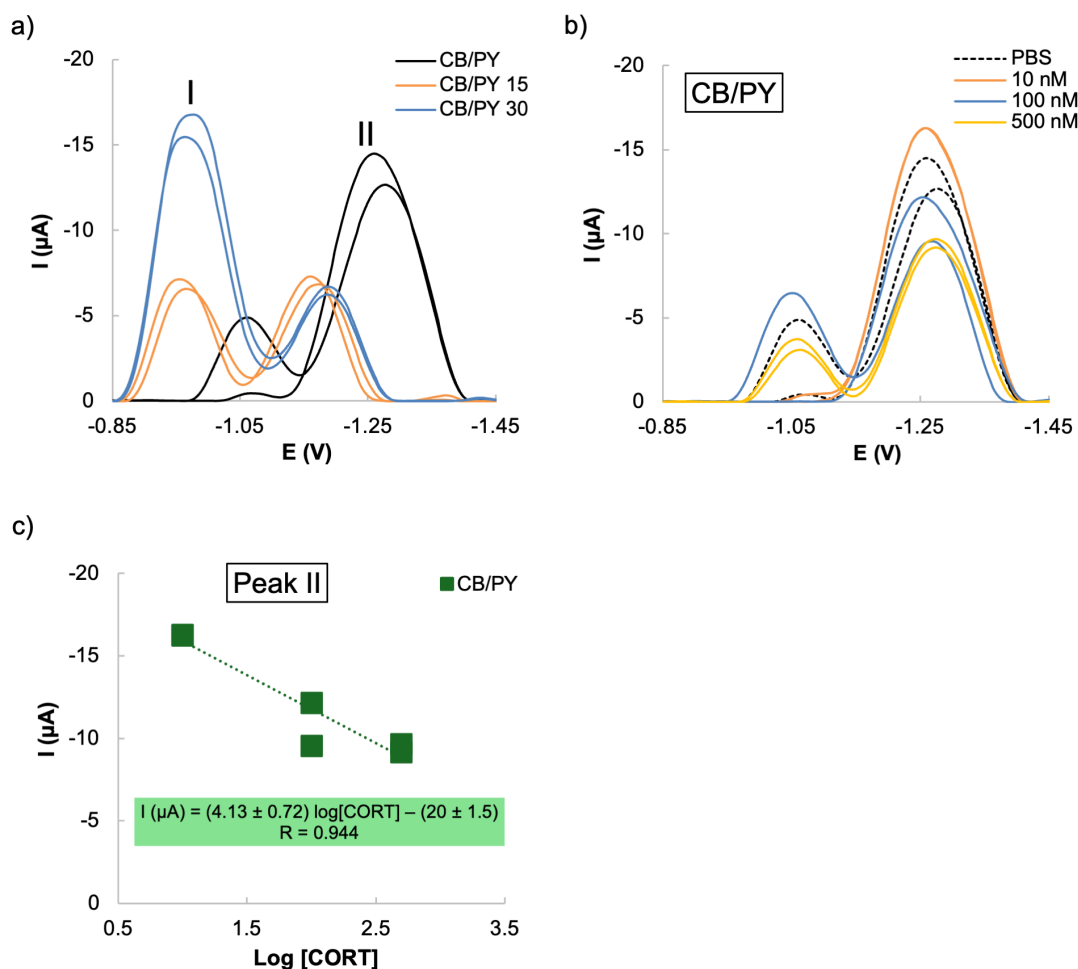


Figure 5.6. DPV of SPCE/CB/PY electrodes: a) Intrinsic electrochemical response after 0, 15, and 30 min incubation in the absence of cortisol; b) Response of the electrode to cortisol concentrations from 10 to 500 nM without incubation; c) current intensity of peak II with increasing cortisol concentrations (nM). All replicates are shown.

After 15 min of incubation, the intensity of peak I increases while peak II decreases, resulting in peaks with similar current values (6.56–7.29 μA). For a 30 min incubation time, peak I becomes dominant, with intensities ranging from 15.5 to 16.8 μA , whereas peak II decreases to 6.22–6.7 μA .

In addition, both peaks shift toward more positive potentials with increasing incubation time. This behavior may be associated with structural rearrangements of the PY layer at the electrode surface or with changes in the interaction between PY and the CB matrix, driven by contact with the aqueous electrolyte. Such processes may improve the accessibility of PY electroactive sites, favoring the reduction process associated with peak I and leading to the observed increase in its current intensity.

The DPV response of SPCE/CB/PY electrodes for cortisol concentrations ranging from 10 to 500 nM, without incubation, is shown in Figure 5.6b. In this case, the intensity of peak II decreases with increasing cortisol concentration. This behavior is consistent with previous studies [101], which showed that the PY reduction peak decreases in the presence of cortisol due to multisite noncovalent interactions, as

confirmed by molecular dynamics simulations. The corresponding calibration curve (Figure 5.6c) shows a sensitivity of $4.13 \mu\text{A}/\text{dec}$ with a correlation coefficient of 0.944. In contrast with the previously reported PY- and MWCNT/PY-based electrode described in this work, where peak I was the most sensitive to cortisol, the CB/PY electrode shows a stronger response in peak II. This difference may be related to the surface properties of CB, which exhibit a more heterogeneous structure and a higher defect density than MWCNTs. These characteristics can influence the orientation and accessibility of PY molecules at the electrode surface, potentially increasing the number of electroactive sites involved in cortisol interaction and leading to the higher sensitivity observed for the CB/PY configuration.

Extended incubation (15 and 30 min) strongly compromised the sensor response, with neither peak remaining reliable (Figures SS5.3 and SS5.4, Appendix). The effect may result from the disruption of the CB layer and PY adsorption, limiting the accessibility and stability of the electroactive PY sites responsible for cortisol detection. Further optimization of CB dispersion and deposition is required to reduce electrode-to-electrode variability and improve reproducibility.

5.4 Conclusions

This work demonstrates that the choice of carbon nanomaterial is a critical determinant of the sensor's analytical performance. The integration of MWCNTs and CB into the SPCE/PY platform enhances the electrochemical response toward cortisol in the nanomolar range, driven by increased electroactive surface area and improved electron transfer. The CB/PY platform stands out as a promising architecture, providing nearly double the sensitivity ($4.13 \mu\text{A}/\text{dec}$) compared to MWCNT-based designs ($2.15 \mu\text{A}/\text{dec}$). Although these modifications significantly improve the analytical signal, further optimization of fabrication conditions is required to reduce variability and ensure reproducibility. These results provide a scalable roadmap for developing label-free, non-affinity stress monitors.

5.5 Appendix

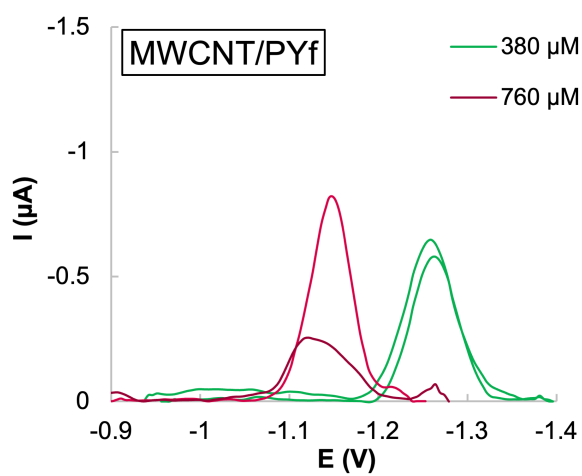


Figure S5.1. DPV responses of SPCEs modified with MWCNT/PYf for $[\text{CORT}] = 0, 380, \text{ and } 760 \mu\text{M}$. Two replicates are shown for each measurement.

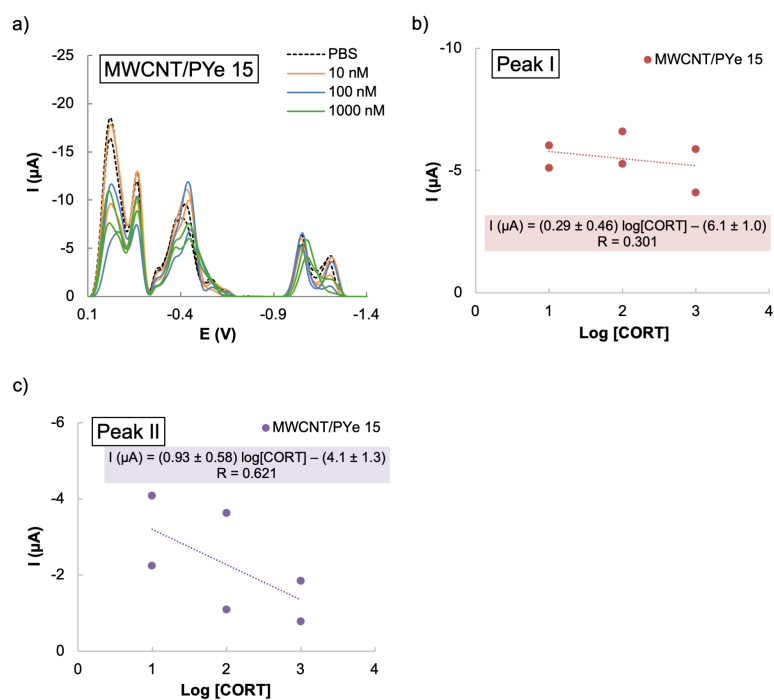


Figure S5.2. a) DPV response of SPCE/MWCNT/PYe electrodes after 15 min incubation with cortisol concentrations from 10 to 1000 nM; current intensity of peaks I (b) and II (c) with increasing cortisol concentrations (nM). Two replicates are shown for each measurement.

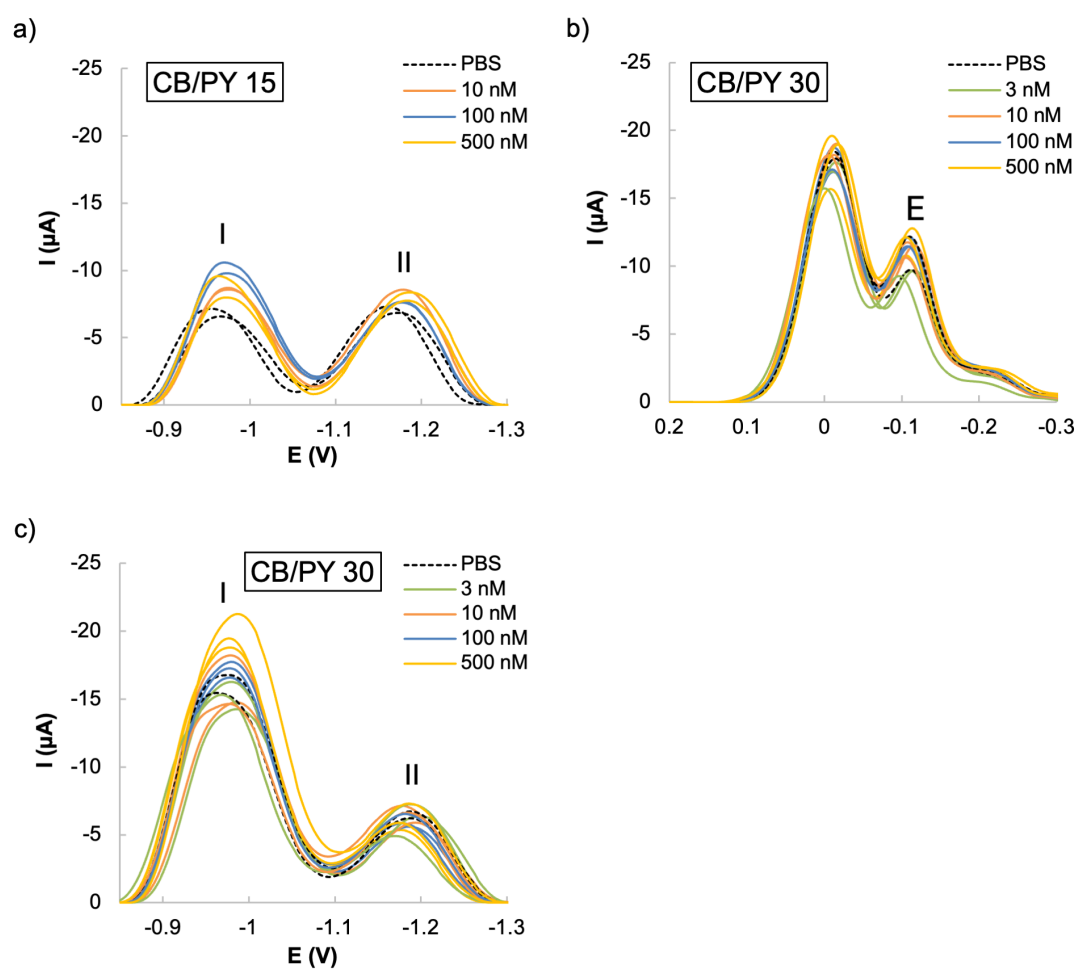


Figure S5.3. Comparison of DPV responses of SPCE/CB/PY incubated with PBS and cortisol (3 to 500 nM) for a) 15 and b) 30 min. All replicate measurements are shown.

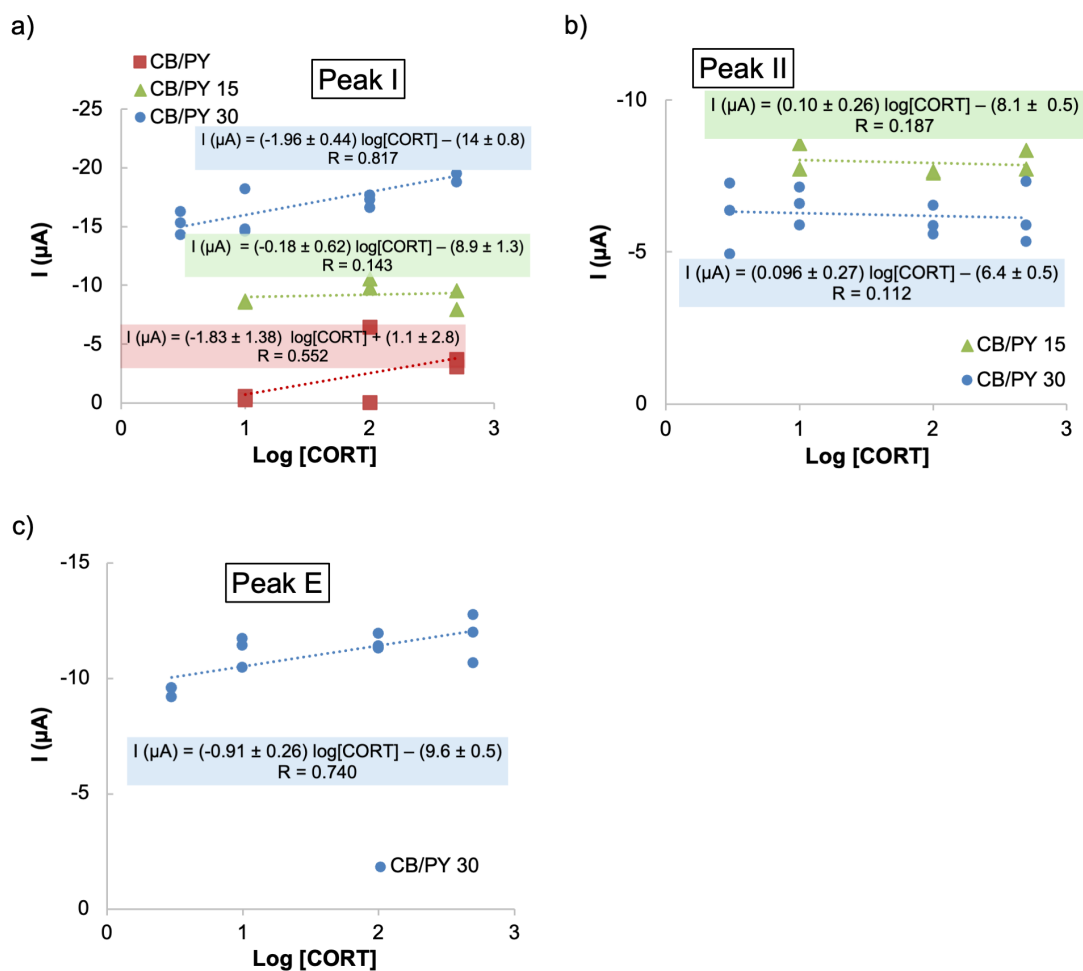


Figure S5.4. Effect of nM cortisol incubation on current peaks I, II, and E in SPCE/CB/PY: a) Peak I without incubation and after 15 and 30 min; b) Peak II after 15 and 30 min; c) Peak E after 30 min. All replicate measurements are shown.

Chapter 6

Conclusions and future work

6.1 Main conclusions

This thesis explored several material-based strategies for developing low-cost, non-affinity electrochemical cortisol sensors. The research transitioned from investigating functionalized carbon nanotubes to the optimization of pyrene-based interfaces and hybrid carbon systems.

Initial studies focused on the covalent functionalization of multi-walled carbon nanotubes with anhydride groups (MWCNT-ANI). Thermogravimetric analysis (TGA) confirmed a weight loss of approximately 28.7%, while x-ray photoelectron spectroscopy (XPS) showed a significant increase in oxygen content (C:O ratio of 81:19), proving the successful chemical modification of multi-walled carbon nanotubes (MWCNTs). Despite these structural results, the electrochemical response toward cortisol was relatively weak. The results suggest that interactions with the analyte were likely dominated by non-specific physicochemical effects rather than targeted molecular adsorption, indicating that simple covalent functionalization was insufficient for high-sensitivity detection. Consequently, the research focus shifted toward pyrene derivatives (PY)-based sensing interfaces, which proved significantly more effective for cortisol detection.

The development of these PY-based sensors was explored using two different solvent systems for electrode modification. Using a hydroalcoholic solvent system (ethanol/water mixtures), three-dimensional, micrometer-scale ribbon-like structures (1.5–4.0 μm thick) were formed across the carbon surface. A decrease in the intrinsic reduction peak of pyrene derivative (PY) at approximately -1.1 V was observed with increasing cortisol concentration. This response was linear in the micromolar range (80–760 μM), reaching a maximum sensitivity of 8.1 $\mu\text{A}/\text{dec}$ ($R = 0.980$) for the highest PY loading (PY62), while in the nanomolar range (3–500 nM), the platform achieved a limit of detection (LOD) of 3 nM and a sensitivity of 2.21 $\mu\text{A}/\text{dec}$ ($R = 0.967$). These findings indicate a sensing mechanism in which the interaction between cortisol and the PY derivative stabilizes the PY molecule and reduces the extent of its electrochemical reduction. This mechanism was further supported by theoretical simulations. Molecular dynamics (MD) simulations indicated that sensing behavior is governed by $\pi - \pi$ stacking interactions and multi-site hydrogen bonding between the functional groups of the PY derivative and cortisol. Density functional theory (DFT) calculations provided quantitative support, revealing favorable interaction energies ranging from -0.60 to -1.05 eV . A key outcome of the DFT analysis was the identification of a geometry-dependent, multi-site interaction model, in which the carbonyl groups of PY contribute most significantly to stabilization. A redistribution of atomic charges upon optimized pair configuration formation was also observed. To address challenges related to reproducibility and droplet confinement observed with organic solvents, an alternative approach using an alkaline aqueous solvent ($pH > 7$) was investigated. This approach

leveraged water's higher surface tension to improve the uniformity of the sensing layer and utilized the deprotonation of the PY carboxylic groups to facilitate dispersion. Morphological analysis via scanning electron microscopy (SEM) revealed that this aqueous-alkaline environment favors the formation of smaller, more uniform ribbon-like structures (50–300 nm) compared to the micrometer-scale structures formed in hydroalcoholic mixtures. Following this structural characterization, a comprehensive multi-peak analysis was performed across both oxidation and reduction potential regions, evaluating the influence of scan rates, incubation times, and the presence of external redox probes. This study found that the reduction peak at -1.1 V provided the most reliable response after a 15 minute incubation, yielding a significantly improved sensitivity of $4.36 \mu\text{A}/\text{dec}$ ($R = 0.862$).

The final development phase integrated the optimized PY interfaces with hybrid carbon systems. The incorporation of multi-walled carbon nanotubes and carbon black (CB) increased the electroactive surface area and improved electron transfer at the interface. Among the tested configurations, CB/PY showed the best performance, reaching a sensitivity of $4.13 \mu\text{A}/\text{dec}$ ($R = 0.944$). Although this value is close to the $4.36 \mu\text{A}/\text{dec}$ obtained for the optimized aqueous interface, both results represent a clear improvement over the initial hydroalcoholic prototypes.

In the current literature, these sensors demonstrate competitive analytical performance compared to non-affinity platforms. A limit of detection of 3 nM and sensitivities above $4 \mu\text{A}/\text{dec}$ place this work within the lower range reported for recognition-free cortisol sensors. Previously reported systems based on sulfur-doped graphene/gold nanoparticles (42 nM) and graphite/gold-leaf electrodes (10 nM) present higher detection limits. Although complex catalytic materials or antibody-based platforms can achieve lower limits of detection, they typically require more demanding fabrication steps or rely on less stable biological components. In contrast, the approach developed here combines straightforward, low-cost fabrication with the sensitivity needed for real-time cortisol monitoring in biofluids.

6.2 Future work

The developed non-affinity platforms were demonstrated at laboratory proof-of-concept level, and several aspects still need to be addressed to enable their transition to point-of-care (POC) or wearable devices.

Building on the optimization of incubation time, signal instabilities at longer exposure periods remain an aspect to be addressed. The variability and reduced sensitivity observed at extended times suggest limitations in film stability, potentially related to swelling or partial desorption. Further work could focus on establishing a relationship between PY loading and stabilization kinetics, aiming to define appropriate incu-

bation durations for optimal molecular reorganization in each case. This would support the development of a more controlled sensing interface, balancing sensitivity with electrode-to-electrode reproducibility for scalable fabrication.

Optimization of nanomaterial stability and adhesion remains important for improving device reproducibility. Future work should focus on conductive binders or surface pre-treatments to improve the mechanical stability of the hybrid layer without increasing interfacial resistance. Improved dispersion protocols for MWCNT/PY and CB/PY mixtures are also needed to reduce electrode-to-electrode variability and support scalable fabrication.

Interference and matrix effects require further investigation. Preliminary results in artificial sweat show that components such as urea, lactic acid, and salts can suppress the intrinsic redox signal of pyrene. Future work should evaluate each component separately to determine whether this effect is related to competitive adsorption, ionic strength, or pH changes. This will support the development of mitigation strategies such as protective membranes or buffering approaches.

Selectivity and cross-reactivity require further study. A major limitation of non-affinity sensors is interference from structurally similar glucocorticoids. Competitive assays with compounds such as cortisone and dexamethasone should be performed to evaluate molecular selectivity. Complementary MD simulations could further be used to explore binding behavior in multi-analyte environments. DFT calculations can also be used to compare interaction energies across different configurations and analytes, supporting the analysis of interaction strength and geometry dependence.

Finally, on-body validation and system integration are required for practical implementation. This includes integrating the sensing platform into flexible substrates for real-time sweat monitoring. Long-term stability and performance under variable conditions, such as temperature and sweat rate, should be evaluated to support continuous monitoring of chronic stress.

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