Reagent-less Low-Concentration Cortisol Detection Enabled By Laser-Induced Graphene Electrodes

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Abstract— **Electrochemical sensors are crucial for medical diagnostics and on-body biomarker monitoring due to their high sensitivity, low cost, and miniaturization capability. This study presents a novel reagent-less molecularly imprinted polymer (MIP)-based cortisol sensor using laser-induced graphene (LIG) electrode. The LIG electrodes were fabricated by converting polyimide substrates with a CO_2 laser and modified with Prussian Blue (PB) stabilized by Nickel Hexacyanoferrate (NiHCF). The high surface area and porosity of LIG enhanced the stability and adhesion of PB nanoparticles, leading to exceptional redox signal stability in neutral pH. Compared to a conventional screen-printed carbon electrode (SPCE), the LIG-based sensor exhibited higher sensitivity, a lower limit of detection (173.3 pM), and a wider dynamic range (1 nM – 100 µM) for cortisol detection, as demonstrated by square wave voltammetry (SWV). The LIG sensor effectively detected cortisol at 1 nM below the physiological range, suitable for real-time stress monitoring and medical diagnostics. This study highlights LIG as a superior transduction material, enhancing the MIP-based sensors' capability to detect low-concentration analytes. The findings offer a cost-effective and scalable approach for advanced biosensing applications.**

Keywords— Electrochemical sensors, Laser-induced Graphene (LIG), Molecularly imprinted polymer (MIP), Cortisol detection

I. INTRODUCTION

Electrochemical sensors have become ubiquitous in healthcare applications, including point-of-care (POC) diagnostic devices and wearable biosensors, due to their ability to provide rapid, cost-effective, and miniaturized detection systems [1]. However, to meet the increasing demands for detecting low-concentration analytes in biofluids, there is a growing interest in advanced electrode materials to enhance the sensitivity [2]. Graphene-based electrodes have emerged as promising candidates due to their exceptional properties, such as high electrical conductivity, large surface area, and chemical stability [2]. Nevertheless, traditional graphene production methods, like chemical vapor deposition (CVD) and chemical exfoliation, are often complex and costly, limiting their widespread adoption in electrochemical sensor fabrication [3].

Recently, laser-induced graphene (LIG) has emerged as a cost-effective and scalable technique for fabricating graphene electrodes [4][5]. This method uses a CO_2 laser to carbonize polyimide into graphene, offering a simpler and more efficient alternative to conventional techniques [4]. LIG electrodes show great promise in various electrochemical sensing applications due to their high surface area and excellent electrical conductivity [6]. Similarly, molecularly imprinted polymers (MIPs) have gained attention as a promising analyte affinity layer [7]. MIPs are synthetic receptors tailored to recognize specific target molecules through the polymerization of functional monomers in the presence of template molecules (i.e., the analyte), thus creating complementary cavities in a polymer matrix after template removal [8]. They offer several advantages over natural recognition elements, including high stability, low cost, and ease of integration with various transduction methods [9]. However, most MIP-based sensors rely on solution phase redox probes to signal the analyte binding, which is not feasible for continuous on-body monitoring in biofluids [10].

Leveraging LIG's and MIPs' advantages, we present a novel reagent-less electrochemical sensor for cortisol detection. Cortisol, a steroid hormone involved in the body's stress response, has a physiological range of 22.1 nM to 386 nM in sweat and 2.76 nM to 30.4 nM in interstitial fluid [11,12]. The low concentration range makes it very challenging to detect with conventional electrochemical sensors [12]. To construct the reagent-less sensor, we utilized electrodeposited Prussian blue (PB) on the LIG electrode as the internal redox probe for signal

generation. To improve PB's stability in neutral-pH solutions, a PB-analogue nickel hexacyanoferrate (NiHCF) was introduced to form a stabilized solid solution layer on the PB surface, enhancing PB's chemical robustness and preventing its leaching [13]. A MIP layer is then fabricated on top of the PB/NiHCF layer by electropolymerization and a subsequent template removal process. During detection, the rebinding of cortisol to MIP cavities modulates the electron transfer by blocking access of potassium ions to the PB layer [14]. The redox signal decrease can be measured and correlated with the cortisol concentrations.

To illustrate the LIG-enabled high sensitivity of the designed PB/NiHCF-MIP-based cortisol sensor, we fabricated sensors using in-house made LIG electrodes and commercially available screen-printed carbon electrodes (SPCE) following similar fabrication procedures and compared their sensing performance. The LIG electrode significantly enhanced sensor sensitivity to enable 1 nM cortisol detection compared to 10 µM achieved by SPCE, making it promising for on-body cortisol detection. This work highlights the potential of LIG as a superior transduction material for developing reagent-less MIP-based sensors for low-concentration analytes monitoring. This advancement is essential for health management, early diagnosis, and timely intervention in medical applications [15].

II. Methods

A. LIG Electrode Fabrication

The LIG electrodes were fabricated using 75 µm polyimide HN (Dupont) substrates. The polyimide substrates were first cleaned with ethanol and then rinsed with deionized (DI) water. Following the cleaning process, direct ink writing was utilized to print the Ag Metalon electrode pads, connections, and the reference electrode. The printed silver ink was then cured at 80°C for 1 hour. Subsequently, a 60 W CO_2 laser (Universal Laser Systems) was used to engrave the graphene working and counter electrodes. The laser engraving parameters were optimized with a power setting of 4%, a speed of 12%, and a PPI (pulses per inch) of 1000. Each electrode underwent three laser engraving passes.

B. PB and NiHCF Deposition

Both the SPCE and the LIG electrodes underwent modification with PB electrodeposition followed by stabilization with NiHCF. For the SPCE, PB was deposited by performing eight cycles of CV in a potential range from -0.2 to 0.6 V at a scan rate of 50 mV/s. The deposition solution contained 2.5 mM $FeCl_3$, 2.5 mM $K_3Fe(CN)_6$, 0.1 M HCl, and 0.1 M KCl. Following the deposition of PB, the electrode is denoted as SPCE-PB. It was immersed in a solution of 5 mM $NiCl_2$, 5 mM $K_3Fe(CN)_6$, 0.1 M HCl, and 0.1 M KCl. To further stabilize SPCE-PB/NiHCF, 100 CVs were performed in the potential range from -0.2 to 0.8 V at a scan rate of 50 mV/s. After rinsing the electrode, an additional 150 CVs were performed in a solution of 0.1 M HCl and 0.1 M KCl, within the potential range from -0.2 to 0.6 V at a scan rate of 50 mV/s.

For the LIG electrode, a similar fabrication process was followed with some adjustments in the number of CV cycles. For the LIG electrode, PB was deposited by performing only two cycles of CVs in the same potential range with the same deposition solution as the SPCE. After the deposition of PB, the LIG-PB was immersed in the $NiCl_2$ and $K_3Fe(CN)_6$ solution, and 50 CVs were performed from -0.2 to 0.8 V at 50 mV/s to achieve a stable PB response. No further stabilization in 0.1 M HCl and 0.1 M KCl was required. The as-prepared electrode is denoted as LIG-PB/NiHCF.

C. MIP Electropolymerization, Template Elution, and Regeneration Overoxidation

The electropolymerization of a cortisol-binding MIP was conducted on the stabilized SPCE-PB/NiHCF and LIG-PB/NiHCF electrodes. This process involved performing five CVs in a potential range from -0.2 to 0.9 V at a scan rate of 25 mV/s, using a solution containing 10 mM cortisol (the template), 25 mM 3-aminophenylboronic acid (3-APBA), and 75 mM pyrrole in 0.1 M KCl. Following MIP polymerization, the electrodes were thoroughly rinsed with DI water to remove unpolymerized monomers and unbound template molecules. Template elution was performed by incubating the electrodes in 8% acetic acid for 10 minutes and rinse thoroughly with DI water. To regenerate the sensor after detection, overoxidation was employed by running ten CVs in 0.1 M KCl within a potential range from -0.2 to 0.8 V at a scan rate of 50 mV/s.

D. Sensor Characterization and Evaluation

Morphologies of the fabricated sensors were characterized by Field Emission Scanning Electron Microscopy (FE-SEM,

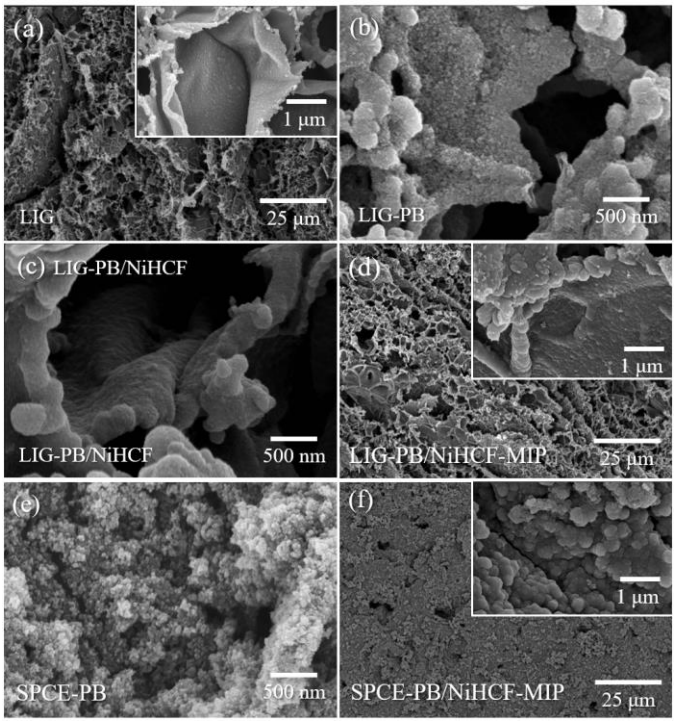

Fig. 1. FE-SEM images of (a) the LIG electrode at ×1k and ×25k magnification (inset), (b) the LIG-PB, and (c) the LIG-PB/NiHCF at ×35k magnification, (d) the LIG-PB/NiHCF-MIP at ×1k and ×25k magnification (inset), the SPCE-PB at ×35k magnification, and (f) the SPCE-PB/NiHCF-MIP at ×1k and ×25k magnification (inset).

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Hitachi S-4700). The electrochemical behavior of the modified electrodes was all evaluated on 0.1 M KCl using a CHI electrochemical workstation. Continous 50 CV cycles were carried out after PB/NiHCF modification on SPCE and LIG electrodes to assess redox signal stability in neutral pH environment. Square wave voltammetry (SWV) was employed to evaluate the sensitivity and detection range of the cortisol sensors built on SPCE and LIG electrodes.

III. Results and Discussion

A. Morphological Characterization

The morphologies of LIG-based electrodes after each modification step are presented in Fig. 1(a)-(d). The LIG electrodes in Fig. 1(a) show a highly porous 3D structure with an intricate network of pores at the microscale with numerous thin walls. After PB deposition, as shown in Fig. 1(b), a thin layer of ultrafine PB nanoparticles formed on the surface, and after NiHCF treatment (in Fig. 1(c)), the surface at the nanoscale becomes visually smoother and denser. After MIP fabrication, a layer of characteristic "cauliflower" like polymer is observed at the nanoscale while maintaining the highly porous 3D microstructure (Fig. 2(d) and inset). As a comparison, the morphologies of SPCE-based electrodes after PB deposition and the full sensor fabrication are shown in Fig. 1(e) and (f), respectively, which exhibit much flatter surfaces.

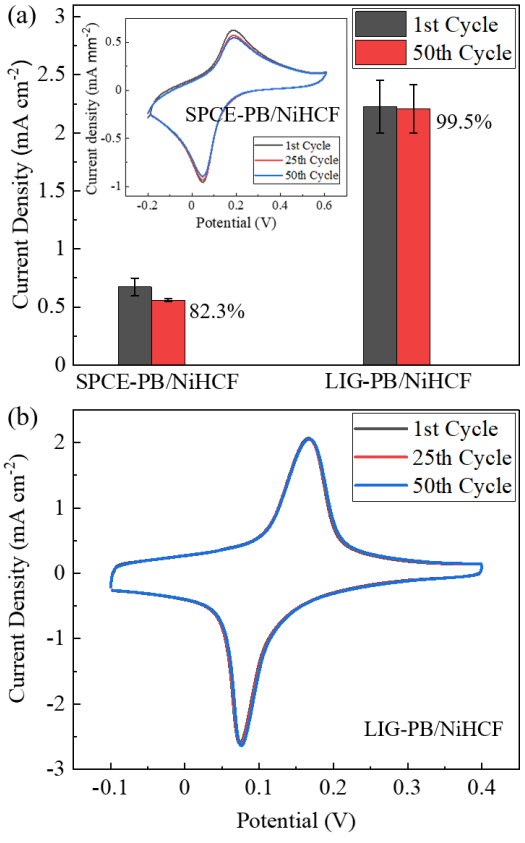

Fig. 2. (a) The bar graph comparing the current density and stability of SPCE-PB/NiHCF and LIG-PB/NiHCF electrodes (n=3) and (inset) the CVs of SPCE-PB for the 1st, 25th, and 50th cycles in 0.1 M KCl, (b) the CVs of LIG-PB for the 1st, 25th, and 50th cycles in 0.1 M KCl.

B. Signal Stability

Signal stability with minimal baseline drift is crucial for reliable detection, especially for low-concentration analytes. PB's stability depends on the electrode substrates and the pH conditions, which is often poor in neutral and alkaline environments [16]. Therefore, the signal stability of the PB/NiHCF-modified LIG and SPCE was first evaluated before MIP deposition. As shown in Fig. 2(a) and 2(b), the LIG-PB/NiHCF electrodes exhibit three times higher current density compared to the SPCE-based ones despite using 8 CV cycles of PB depositions for SPCE versus 2 cycles for LIG. This can be attributed to the porous nature of the LIG morphology, providing more surface area for PB deposition [17], as well as graphene's superior electrical conductivity which enhances the electron transfer kinetics. In addition, LIG-based electrodes demonstrated exceptional signal stability. After 50 cycles of CV in a 0.1 M KCl, SPCE-based electrodes retained an average of 82.3% of their original current density in the 1st cycle, while LIG-based electrodes maintained the current density at 99.5%, making it a great candidate for reliable signaling in biosensing. This improvement can be potentially ascribed to LIG's high conductivity, promoting efficient electron transfer and resulting in stabilized electrochemical properties [6]. In addition, the binder used in SPCE may also negatively affect the interaction with PB, leading to a significant baseline drift [18].

C. Cortisol Detection

The sensing performance of the as-prepared PB/NiHCF-MIP on LIG and SPCE was evaluated using SWV, which offers higher sensitivity than other techniques [19]. The sensing mechanism is illustrated in Fig. 3(a), which shows the binding of cortisol molecules to the cavities in the MIP could hinder electron transfer by blocking potassium ions that balance the overall ionic charge of the PB on the electrode surface [14]. This leads to a reduced PB redox signal with increasing cortisol concentration, as shown in Fig. 3(b). The three SWV blank scans were taken in 5 mL of 0.1 M KCl electrolyte followed by spiking small amounts of blank solution to confirm the sensor's signal has no drift and is not affected by the spiking motion nor small volume changes. Then, the blank solution was spiked with different cortisol concentrations and a 10 min incubation time was used to allow cortisol binding with MIP. As shown in Fig. 3(b), the LIG-based sensors achieved cortisol detection at 1 nM thanks to the highly porous structure of LIG providing a high surface area for imprinting and the promoted electron transfer rate for signal boosting. The sensor shows a broad liner range from 1 nM to 100 µM, which covers the typical physiological range in biofluids. The calculated limit of detection (LOD) is 173.3 pM based on signal-to-noise ratio=3 (noise is determined by the standard derivation of peak currents in three blank scans). This is a significant improvement compared to SPCE-based sensors, which started to show a noticeable response at 100 µM (Fig. 3(c)). LIG-based sensors' ability to detect cortisol in the nanomolar range enables their practical use for stress monitoring and medical diagnostics, holding great promise in real-time on-body cortisol monitoring.

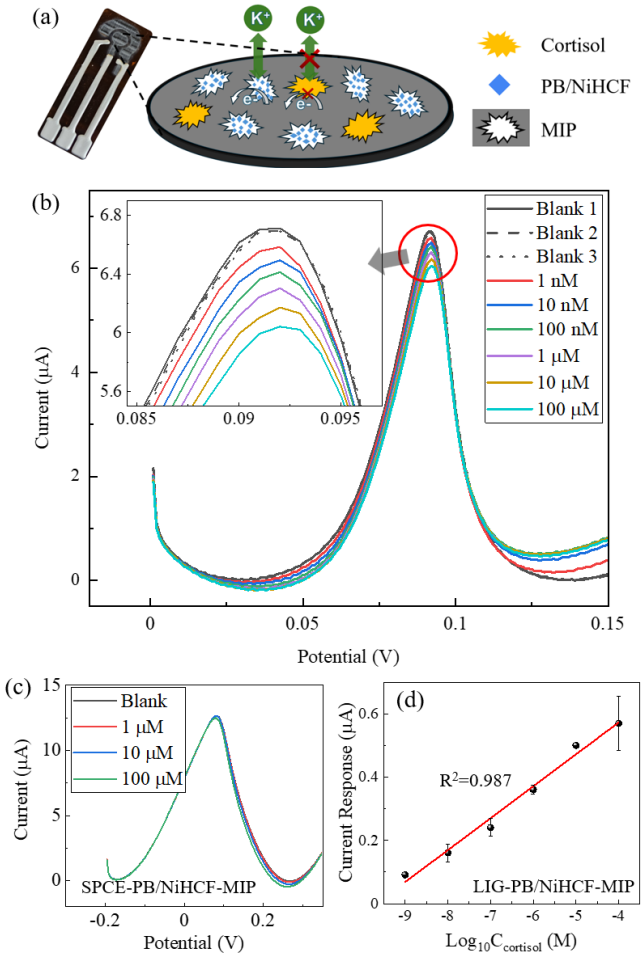

Fig. 3. (a) The schematic of the detection mechanism of LIG-PB/NiHCF-MIP-based cortisol sensors. The SWV responses to varying cortisol concentrations in 0.1 M KCl tested with the (b) LIG-PB/NiHCF-MIP electrode and (c) SPCE-PB/NiHCF-MIP electrode after baseline correction. (d) The corresponding calibration curve for the LIG-PB/NiHCF-MIP electrodes.

IV. Conclusion

In this study, we developed a novel reagent-less cortisol sensor using LIG as the transduction electrode, modified with PB and NiHCF for signaling, followed by a MIP layer for recognition. The LIG-based sensors demonstrated superior performance compared to a conventional SPCE, exhibiting remarkable signal stability, higher sensitivity, and a lower detection limit. The as-prepared LIG-based sensor successfully detected the cortisol level at 1 nM below the physiological range. Upon further optimization, sensitivity can be further improved, and testing with interferants and real biofluids will be done to push this technology towards on-body operation for stress monitoring. This work highlights the potential of LIG as a superior transduction material for developing advanced electrochemical sensors for detecting analytes at nanomolar concentrations, making them ideal for biosensing applications.

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References

[1] Zhang, Xueji, Huangxian Ju, and Joseph Wang, eds. *Electrochemical sensors, biosensors and their biomedical applications*. Academic Press, 2011.J. Clerk Maxwell, A Treatise on Electricity and Magnetism, 3rd ed., vol. 2. Oxford: Clarendon, 1892, pp.68–73.

[2] Shao, Yuyan, et al. "Graphene based electrochemical sensors and biosensors: a review." *Electroanalysis: An International Journal Devoted to Fundamental and Practical Aspects of Electroanalysis* 22.10 (2010): 1027-1036.K. Elissa, "Title of paper if known," unpublished.

[3] Novoselov, Konstantin S., et al. "A roadmap for graphene." *nature* 490.7419 (2012): 192-200.

[4] Lin, Jian, et al. "Laser-induced porous graphene films from commercial polymers." *Nature communications* 5.1 (2014): 5714.

[5] Ye, Ruquan, et al. "Laser-induced graphene formation on wood." *Advanced Materials* 29.37 (2017): 1702211.

[6] Yang, Yiran, et al. "A laser-engraved wearable sensor for sensitive detection of uric acid and tyrosine in sweat." *Nature biotechnology* 38.2 (2020): 217-224.

[7] Saylan, Yeşeren, et al. "Molecularly imprinted polymer based sensors for medical applications." *Sensors* 19.6 (2019): 1279.

[8] Bossi, A., et al. "Molecularly imprinted polymers for the recognition of proteins: the state of the art." *Biosensors and Bioelectronics* 22.6 (2007): 1131-1137.

[9] Ahuja, Tarushee, Irfan Ahmad Mir, and Devendra Kumar. "Biomolecular immobilization on conducting polymers for biosensing applications." *Biomaterials* 28.5 (2007): 791-805.

[10] Dykstra, Grace, et al. "Electropolymerized molecularly imprinted polymer synthesis guided by an integrated data-driven framework for cortisol detection." *ACS applied materials & interfaces* 14.22 (2022): 25972-25983.

[11] Russell, Evan, et al. "The detection of cortisol in human sweat: implications for measurement of cortisol in hair." *Therapeutic drug monitoring* 36.1 (2014): 30-34.

[12] Vignesh, Visesh, et al. "Advancements in Cortisol Detection: From Conventional Methods to Next-Generation Technologies for Enhanced Hormone Monitoring." *ACS sensors* 9.4 (2024): 1666-1681.

[13] Xu, Changhao, et al. "A physicochemical-sensing electronic skin for stress response monitoring." *Nature Electronics* (2024): 1-12.

[14] Dykstra, Grace, Isabel Chapa, and Yixin Liu. "Reagent-Free Lactate Detection Using Prussian Blue and Electropolymerized-Molecularly Imprinted Polymers-Based Electrochemical Biosensors." *ACS applied materials & interfaces* (2024).

[15] Joseph, Joshua J., and Sherita H. Golden. "Cortisol dysregulation: the bidirectional link between stress, depression, and type 2 diabetes mellitus." *Annals of the New York Academy of Sciences* 1391.1 (2017): 20-34.

[16] Wang, Zongwei, et al. "Stability improvement of Prussian blue in nonacidic solutions via an electrochemical post-treatment method and the shape evolution of Prussian blue from nanospheres to nanocubes." *Analyst* 139.5 (2014): 1127-1133.

[17] Yu, Zi-You, et al. "Unconventional CN vacancies suppress iron-leaching in Prussian blue analogue pre-catalyst for boosted oxygen evolution catalysis." *Nature Communications* 10.1 (2019): 2799.

[18] Suresh, Raghavv Raghavender, et al. "Fabrication of screen-printed electrodes: opportunities and challenges." *Journal of Materials Science* 56 (2021): 8951-9006.

[19] Lovrić, Milivoj. "Square-wave voltammetry." *Electroanalytical methods: guide to experiments and applications* (2010): 121-14