False Sensitivities in Cortisol Immunosensors Fabricated on Gold-Screen-Printed Electrodes

1st Aishath N. Naeem
Department of Electrical and Electronic Engineering
University College London, London, United Kingdom
aishath.naeem.21@ucl.ac.uk

2nd Sara S. Ghoreishizadeh
Department of Electrical and Electronic Engineering
University College London, London, United Kingdom
s.ghoreishizadeh@ucl.ac.uk

***Abstract*—Cortisol electrochemical sensors are extensively studied since the ability to measure cortisol in real-time would provide information about abnormalities in cortisol levels and how they may affect a variety of processes in which cortisol is involved in the body. The main base layer material and redox mediator utilized in cortisol immunosensors in the literature are gold and ferri/ferrocyanide, respectively. However, this electrolyte-electrode combination is known to cause false positive or negative results, limiting the accuracy of measurements. In this work, three ways through which this electrolyte-electrode system can cause false sensitivities are experimentally demonstrated. The results show that the adsorption of chloride ions and ferri/ferrocyanide redox probe to the surface can alone be responsible for up to 40% of normalized change in sensor output. The ferri/ferrocyanide redox couple can also cause faulty readings by interfering with the sensor structure and etching the gold electrode surface.**

***Index Terms*—gold screen printed electrodes, false sensitivities, cortisol immunosensors.**

I. INTRODUCTION

Quantification of analytes is important for different applications including medical diagnostics and environmental monitoring. Electrochemical biosensors are widely studied for such applications, owing to their sensitivity, ability to selectively bind to the target molecules, and their inherent ability for miniaturization [1], [2].

Electrochemical biosensors consist of biological receptors that specifically bind to the target molecules and convert the chemical information (*i.e.* concentration of analyte) to electrical signals [3]. Gold (Au) is readily used as the sensing electrode material due to its excellent conductivity, inertness, and biocompatibility [4]. Additionally, the use of Au allows simple functionalization of bioreceptors through gold-thiol covalent bonds to form self-assembled monolayers (SAM) [5].

If the analyte does not have a redox center that can undergo oxidation or reduction upon applying a specific voltage, an external redox probe or a redox mediator is utilized in these sensors to both characterize the stepwise fabrication of sensors and for the evaluation of the sensor's analytical performance. One of the most widely used redox mediator in biosensor development is the ferricyanide/ferrocyanide couple [6].

However, the use of Au electrodes together with ferri/ferrocyanide redox couple requires careful consideration of a number of phenomena that will otherwise interfere with and negatively affect the sensor performance. These include the formation of polymeric complexes at the electrode surface due to the interaction of ferrocyanide with gold [7], [8], the adsorption of chloride ions from phosphate buffer saline (PBS) on the electrode surface [9], the instability of thiol monolayers at the gold surface due to the interaction with the ferri/ferrocyanide redox couple [10], and the dissolution and etching of gold surfaces caused by ferri/ferrocyanide complex [11].

We use electrochemical cortisol immunosensor as an example in this work to demonstrate these effects. The use of electrochemical sensing techniques for rapid and frequent measurements of cortisol (also known as the "stress hormone") has been increasingly studied over the years to better understand the relationships between cortisol, well-being, and experience and the factors affecting cortisol levels and disease processes [12]. Since cortisol lacks a redox center, ferri/ferrocyanide in PBS is used most commonly as the redox mediator and gold is the most popular choice for the electrode material in cortisol immunosensors [12]. However, the phenomena mentioned above have not yet been talked about in the cortisol sensors.

In this work, we demonstrate the presence of false sensitivities in electrochemical cortisol immunosensors. We further investigate the factors that could give rise to false sensitivities and affect the sensor performance, including (i) adsorption of ions in the redox solution to the gold electrode surface, (ii) etching of gold caused by the redox mediator, and (iii) effect of chloride ions in PBS.

II. MATERIALS AND METHODS

A. Electrodes, chemicals, and equipment

Gold screen printed electrodes (C220AT) (Au-SPE) with a working electrode diameter of $4mm$ were purchased from DropSens. A platinized titanium anode from Ti-Shop was used as an external counter electrode (CE) with the Au-SPEs. Gold Quartz Crystal Microbalance (QCM) sensors (QCM 5 MHz 14 mm Cr/Au) were acquired from QuartzPro.

PBS tablets, sodium sulfate (Na_2SO_4), potassium ferricyanide(III) and potassium hexacyanoferrate(II) trihydrate, 3,3'-dithiodipropionic acid di(N-hydroxysuccinimide ester) (DTSP), sodium borohydride ($NaBH_4$) anti-cortisol antibody (Anti-CAb), ethanolamine hydrochloride (EA-HCl), and cortisol were purchased from Sigma-Aldrich. Dimethyl sulfoxide

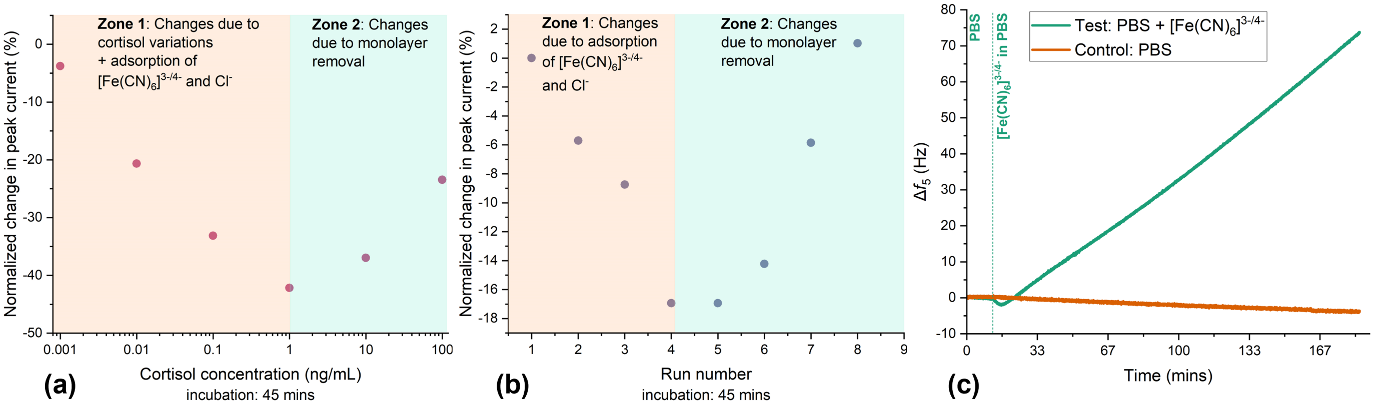

Fig. 1: The normalized change (with respect to zero cortisol concentration) in DPV peak currents for (a) sensor tested with varying cortisol concentrations (in 5 mM $[Fe(CN_6)]^{3-/4-}$ in PBS and incubation time of 45 mins at each concentration,) and (b) control sensor after repeated 45 minute incubation runs in same solution without cortisol. (c) QCM-D results depicting changes in frequency over time for the test sensor with $[Fe(CN_6)]^{3-/4-}$ in PBS and for the control sensor with just PBS.

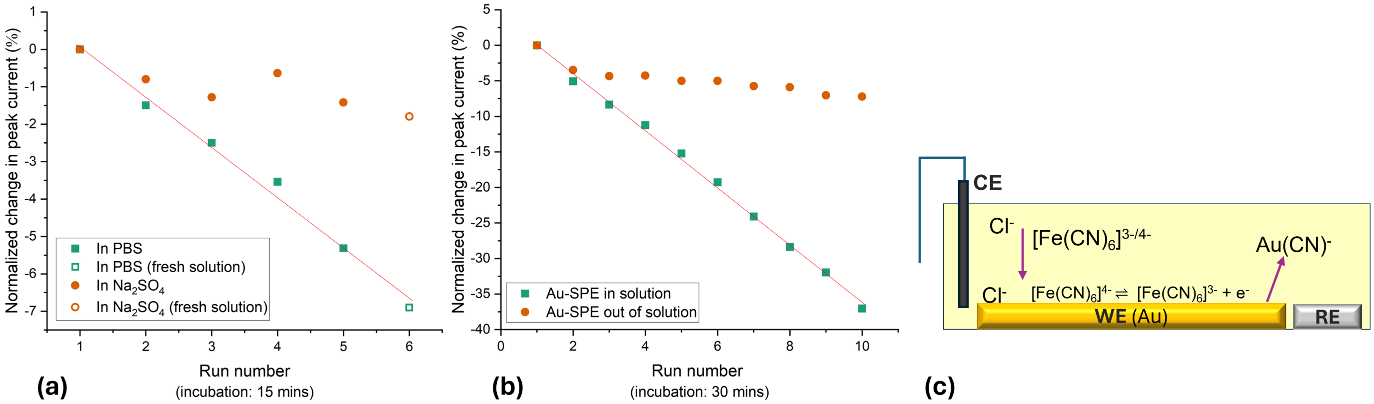

Fig. 2: Normalized change in peak currents for (a) Au-SPEs tested in $[Fe(CN_6)]^{3-/4-}$ in PBS vs. in Na_2SO_4. Run no. 6 was carried out in a fresh redox solution, and (b) Au-SPEs tested where one electrode was incubated in the $[Fe(CN_6)]^{3-/4-}$ in PBS, and the other outside in dry conditions. (c) An illustration of adsorption phenomena of $[Fe(CN_6)]^{3-/4-}$ in PBS and CN^- based etching on Au electrode surface.

(DMSO) was from Fisher Scientific. Electrochemical measurements were performed with a potentiostat (Autolab, Metrohm), and quartz crystal microbalance with dissipation (QCM-D) measurements were done in Q-Sense E4 instrument (Biolin).

B. Sensor fabrication and measurements

Cortisol immunosensors were fabricated using a recipe previously published [13]. In summary, 2 mg/mL DTSP in DMSO was reduced with 10 mg/mL $NaBH_4$ in DI water before being deposited on Au-SPE for 2 hrs for SAM formation. Then, 10 μg/mL Anti-CAb in PBS were covalently immobilized by incubation with the sensors for 1.5 hrs. Finally, the unreacted DTSP was blocked with 0.1 M EA-HCl for 10 mins. The sensors were stored in PBS at 4°C while not in use.

Differential pulse voltammetry (DPV) was performed to evaluate the sensors/electrodes in the potential range of -0.1 to 0.4 V (step volatge: 0.005V, interval time: 0.5s) in 5 mM $[Fe(CN)_6]^{3-/4-}$in PBS, which was the concentration used in the literature for the evaluation of cortsiol immunosensors. The normalized change in response (ΔI) (%) was calculated using the below equation where I_i is the initial current and I_f is the current measured after each respective incubation.

$$\Delta I = \frac{I_f - I_i}{I_i}.100 \qquad (1)$$

The changes in resonant frequency in the fifth overtone (Δf_5) were obtained for QCM-D measurements and the results were analyzed using the Sauerbrey's relation (Eqn. 2), which states that the frequency (Δf) decreases proportionally to the mass added on to the sensor (Δm) for a given mass sensitivity constant (C) and an overtone number (n).

$$\Delta m = -C.\frac{\Delta f}{n} \qquad (2)$$

III. RESULTS AND DISCUSSION

The dose-response curve of electrochemical cortisol immunosensors reported in the literature is often based on using a single sensor that undergoes incubation and test runs with increasing concentrations of cortisol [14], [15]. The same method was used in this work to calibrate the sensor.

Cortisol immunosensors were dipped in 10 mL PBS solution with 5 mM $[Fe(CN)_6]^{3-/4-}$. The solution was spiked with cortisol to achieve concentrations between 1 pg/mL to 100 ng/ml, and incubated for 45 mins, before running the DPV. As cortisol concentration increases, a decrease in peak current is expected as the binding of more cortisol to the antibodies would increase the hindrance of mass transport of the redox couple to the electrode surface, and hence the transfer of electrons.

A control experiment was also conducted in the absence of cortisol, where a sensor was placed in the $[Fe(CN)_6]^{3-/4-}$ solution and DPV measurements were taken every 45 mins at zero cortisol concentration.

Both sensors followed the same trend of decreasing peak current followed by an increase after the fourth measurement (see Fig 1a. and 1b.). This clearly indicates that the change in sensor current is not caused by the binding of cortisol alone, and there are compounding effects. Comparing the the variations in zone one of the two graphs in Fig 1a. and 1b. indicate a substantial 17% out of overall 42% of variations in the output of the cortisol sensor in this zone is not due to cortisol variations at all. This represents a 40% contribution from compounding effects. The existence of zone two in both plots, indicate removal of material from the sensor or sensor etching. To investigate the possible causes of these errors, the impact of three distinct phenomena: ferri/ferro related etching, ferri/ferro adsorptions, and chloride ion adsorption are experimentally studied in this work.

A. Etching of the Au electrode

The ability of $[Fe(CN)_6]^{3-/4-}$ solution to etch the Au electrode surface was investigated using QCM-D, where PBS was flowed over a gold QCM-D sensor to obtain a baseline, followed by 5 mM $[Fe(CN)_6]^{3-/4-}$. The results show (see Fig 1c.) an initial dip in frequency because of the adsorption of ions but is then quickly followed by a continuous increase in frequency, which demonstrates the removal of initial mass on the sensor. This experiment confirms the continual dissolution and etching of gold when exposed to $[Fe(CN)_6]^{3-/4-}$.

B. Effect of chloride ions

To verify whether the chloride ions in PBS buffer cause drift, Au-SPEs were tested with $[Fe(CN)_6]^{3-/4-}$ in 0.1 M PBS and in a chloride-free buffer solution: in 0.1 M Na_2SO_4. The electrodes were incubated with their respective buffer (either PBS or Na_2SO_4 but without the redox couple) for 15 mins then immersed into a 10 mL of 5 mM $[Fe(CN)_6]^{3-/4-}$solution (in either PBS or Na_2SO_4) where DPV was carried out. This was repeated five times. The results are presented in Fig 2a. Au-SPEs that were kept and measured in the chloride-free buffer show a 1.4 % change in current from run 1 to 5 (total 75 mins incubation) compared to 5.3% in PBS. Both electrodes were then tested with a fresh solution in the end (run number 6 in Fig. 2a). This did not cause the peak current to increase, indicating that it is indeed changes to the surface of the electrodes that cause the changes in current, rather than any changes or degradation in the redox solution. The adsorption of chloride ions from PBS onto the Au surface can also be inferred from the control QCM-D experiment shown in Fig 1c. where exposure to PBS led to a decrease in the frequency.

C. Estimation of errors using bare Au-SPEs

Unmodified Au-SPEs were tested to identify the extent of adsorption or etching of the gold surface by the ferri/ferrocyanide and chloride ions together. One electrode was kept in $[Fe(CN)_6]^{3-/4-}$ solution and DPV measurements were taken every 30 mins, while the other electrode was rinsed with DI water and kept dry for 30 mins between measurements. The change in sensor response is reported in Fig 2b., showing the signal of Au-SPE that was kept in solution decreased by 40%, compared with just 7% for the electrode that was kept dry. This generally agrees with previous work that shows the interaction of redox solution with the Au electrode surface can lead to substantial errors due to the adsorption of redox probe and chloride ion to the surface [7]. The results show that this effect increases almost linearly with the overall time the electrode spends in the solution. However, zone 2 was not observed here after 4.5 hrs in the solution, unlike the cortisol immunosensors in Fig 1a. and 1b. The existence of zone 2 in the immunosensor may be because of thiol SAM instability in the presence of $[Fe(CN)_6]^{3-/4-}$ and the desorption of molecules due to multiple measurements, in addition to or instead of the direct etching of gold. These adsorption phenomena and CN^- induced etching are depicted conceptually in Fig 2c.

IV. CONCLUSION

Several factors can interfere with the performance of cortisol immunosensors causing false sensitivities in the sensor. This work highlights the importance of taking into account the interaction between the redox couple and gold electrodes, the effect of incubation in the buffer in between measurements, and the composition of the buffer used. In analyzing electrochemical cortisol immunosensors, it is important that the electrodes are not exposed to the ferri/ferro redox couple or to chloride ions for a long time to minimize the effects of ion adsorption and etching. Despite the seemingly common practice of using a single sensor to achieve dose-response curves, this work demonstrates how long incubation times inversely impact the readings. We recommend that a fresh sensor is used for each measurement to minimize the sensor-electrolyte contact time and thus the several sources of errors involved. Finally, it is imperative to run multiple control experiments in parallel to account for the false changes caused by these effects.

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REFERENCES

[1] M. Li, A. A. S. Gill, A. Vanhoestenberghe, and S. S. Ghoreishizadeh, "An integrated circuit for galvanostatic electrodeposition of on-chip electrochemical sensors," in *2022 29th IEEE International Conference on Electronics, Circuits and Systems (ICECS)*, 2022, pp. 1–4.

[2] N. J. Ronkainen, H. B. Halsall, and W. R. Heineman, "Electrochemical biosensors," *Chem. Soc. Rev.*, vol. 39, pp. 1747–1763, 2010. [Online]. Available: http://dx.doi.org/10.1039/B714449K

[3] D. R. Thévenot, K. Toth, R. A. Durst, and G. S. Wilson, "Electrochemical biosensors: recommended definitions and classification1international union of pure and applied chemistry: Physical chemistry division, commission i.7 (biophysical chemistry); analytical chemistry division, commission v.5 (electroanalytical chemistry).1," *Biosensors and Bioelectronics*, vol. 16, no. 1, pp. 121–131, 2001. [Online]. Available: https://www.sciencedirect.com/science/article/pii/S0956566301001154

[4] T. Xiao, J. Huang, D. Wang, T. Meng, and X. Yang, "Au and au-based nanomaterials: Synthesis and recent progress in electrochemical sensor applications," *Talanta*, vol. 206, p. 120210, 2020. [Online]. Available: https://www.sciencedirect.com/science/article/pii/S0039914019308434

[5] M. Zamani, C. M. Klapperich, and A. L. Furst, "Recent advances in gold electrode fabrication for low-resource setting biosensing," *Lab on a Chip*, vol. 23, no. 5, pp. 1410–1419, 2023.

[6] P. Krishnaveni and V. Ganesh, "Electron transfer studies of a conventional redox probe in human sweat and saliva bio-mimicking conditions," *Scientific reports*, vol. 11, no. 1, p. 7663, 2021.

[7] J. Lazar, C. Schnelting, E. Slavcheva, and U. Schnakenberg, "Hampering of the stability of gold electrodes by ferri-/ferrocyanide redox couple electrolytes during electrochemical impedance spectroscopy," *Analytical Chemistry*, vol. 88, no. 1, pp. 682–687, 2016, pMID: 26618671. [Online]. Available: https://doi.org/10.1021/acs.analchem.5b02367

[8] A. Bogomolova, E. Komarova, K. Reber, T. Gerasimov, O. Yavuz, S. Bhatt, and M. Aldissi, "Challenges of electrochemical impedance spectroscopy in protein biosensing," *Analytical Chemistry*, vol. 81, no. 10, pp. 3944–3949, 2009, pMID: 19364089. [Online]. Available: https://doi.org/10.1021/ac9002358

[9] B. L. Garrote, A. Santos, and P. R. Bueno, "Perspectives on and precautions for the uses of electric spectroscopic methods in label-free biosensing applications," *ACS Sensors*, vol. 4, no. 9, pp. 2216–2227, 2019, pMID: 31394901. [Online]. Available: https://doi.org/10.1021/acssensors.9b01177

[10] R. B. Channon, Y. Yang, K. M. Feibelman, B. J. Geiss, D. S. Dandy, and C. S. Henry, "Development of an electrochemical paper-based analytical device for trace detection of virus particles," *Analytical Chemistry*, vol. 90, no. 12, pp. 7777–7783, 2018, pMID: 29790331. [Online]. Available: https://doi.org/10.1021/acs.analchem.8b02042

[11] X. Hua, H.-L. Xia, and Y.-T. Long, "Revisiting a classical redox process on a gold electrode by operando tof-sims: where does the gold go?" *Chemical Science*, vol. 10, no. 24, pp. 6215–6219, 2019.

[12] A. N. Naeem, S. Guldin, and S. S. Ghoreishizadeh, "Electrochemical sensors for cortisol: A review," *IEEE Sensors Journal*, vol. 24, no. 5, pp. 5746–5758, 2024.

[13] S. K. Pasha, A. Kaushik, A. Vasudev, S. A. Snipes, and S. Bhansali, "Electrochemical immunosensing of saliva cortisol," *Journal of The Electrochemical Society*, vol. 161, no. 2, p. B3077, 2013.

[14] S. K. Arya, G. Chornokur, M. Venugopal, and S. Bhansali, "Dithiobis (succinimidyl propionate) modified gold microarray electrode based electrochemical immunosensor for ultrasensitive detection of cortisol," *Biosensors and Bioelectronics*, vol. 25, no. 10, pp. 2296–2301, 2010.

[15] K. A. Sunil, C. Ganna, V. Manju, and B. Shekhar, "Antibody modified gold micro array electrode based electrochemical immunosensor for ultrasensitive detection of cortisol in saliva and isf," *Procedia Engineering*, vol. 5, pp. 804–807, 2010, eurosensor XXIV Conference. [Online]. Available: https://www.sciencedirect.com/science/article/pii/S1877705810007770