Toward a Label-Free Electrochemical Impedance Immunosensor Design for Quantifying Cortisol in Tears

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ABSTRACT: Cortisol is a viable biomarker for monitoring physiological, occupational, and emotional stress and is normally present in tear fluid at approximately 40 nM, or higher as a result of stress. We present characterization and quantification of cortisol via several electrochemical methods versus the standard enzyme-linked immunosorbent assay, commonly known as ELISA. We also present a prototyped design of a disposable test strip and handheld sensor based on label-free electrochemical impedance spectroscopy to quantify cortisol levels in tear fluid within approximately 90 seconds. Electrochemical characterization of the cortisol molecule was conducted using cyclic voltammetry, amperometric i-t, and square wave voltammetry. Lower limits of detection for these techniques were not sufficient to quantify cortisol and phycological tear ranges: 0.1 M, 0.23 M, and 193 M for cyclic voltammetry, amperometric i-t, and square wave voltammetry, respectively. However, electrochemical impedance spectroscopy (EIS) was to be the best mode of cortisol quantification and comparison to ELISA technique (detection range of ~ 138 pM – 552 nM). The initial EIS biosensor obtained a lower limit of detection of 59.76 nM with an approximate 10% relative standard deviation. The cortisol assay and tear collection prototype presented here offer a highly reproducible and ultra-low level of detection with a label-free and rapid response.

KEY WORDS: cortisol, stress, point-of-care technology, electrochemistry, electrochemical impedance spectroscopy

ABBREVIATIONS: Amp-it, amperometric i-t; **CV**, cyclic voltammetry; **DC**, direct current; **DI**, deionized; **EDC**, n-(3-dimethylaminopropyl)-n′-ethylcarbodiimide; **EIS**, electrochemical impedance spectroscopy; **ELISA**, enzyme-linked immunosorbent assay; **GDE**, gold disk electrode; **LLD,** lower limit of detection; **IgE**, immunoglobulin E; **IgG**, immunoglobulin G; **16-MHDA**, 16-mercaptohexadecanoic acid; **NHS**, n-hydroxysulfosuccinimide sodium salt; **PBS**, phosphate buffer saline; **SEBS**, styrene-ethylene-butylene-styrene; **SNR**, signal-to-noise ratio; **SWV**, square wave voltammetry

I. INTRODUCTION

Stress is a general and broadly defined state of being that has been linked to varying severities of mental health disorders, cardiovascular disease, inflammation, infection, and general physiological health.[1–3] Several biological markers have been studied to quantify stress: leptin and cortisol hormones, cardiovascular activity, fibrinogen, C-reactive protein, amyloid A, serum lipids, and inflammation molecules, among many others.[1–4] It is the goal of present study to develop a tear-based noninvasive platform to test for biomarkers of stress, which can in turn help users manage their occupational, emotional, or other stress.

Detecting cortisol in tears is an ideal substitute for blood and serum tests since tears lack many species that interfere with the sensing mechanisms in traditional blood tests (e.g., red blood cells).[5,6] Furthermore, tear levels have been shown to have less variation from subject to subject compared to serum or saliva tests.[7] Asymptomatic concentrations of cortisol in tears, saliva, and serum are on average 13.4, 15.4 and 154.8 ng/mL, respectively.[7] Though cortisol levels in stressed subjects are expected to increase, tear cortisol concentrations approach the lower limit of enzyme-linked immunosorbent assay (ELISA) and present a technical challenge to rapidly and minimally invasively quantify cortisol levels.[8]

Wearable technologies have made recent breakthroughs using optical, conductance, and accelerometer measurements to quantify stress. However, noninvasive electrochemical stress-monitoring devices allow for the direct measurement of stress-re-

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lated biomolecules and their physiological effects, compared to indirect measurements from most wearable technologies. A trend towards tear-based biosensing has gained particular acceptance in diabetes glucose monitoring to replace painful finger pricking.[9,10]

Electrochemical biosensors have become an essential component of medical diagnostics. Many electrochemical techniques have been employed to detect a multitude of biomarkers ranging from whole cells, DNA, proteins, and gases, among others.[11] The most popular techniques—cyclic voltammetry (CV), amperometric i-t (Amp-it), square wave voltammetry (SWV), and electrochemical impedance spectroscopy (EIS)—have shown promise to quantify molecules similar to cortisol, including neurotransmitters, hormones, diabetes molecules, inflammatory markers, and cardiovascular disease markers.[12–18] CV is useful for determining the general electroactivity of a molecule and needs a redox mediator or recognition element (e.g., an enzyme) to be quantified.[19] The technique used in self-monitoring blood glucose meters, amp-it, requires very little hardware and can provide sensitive quantifying results.[19] SWV is a more sensitive version of CV that can achieve better resolution but requires more signal analysis.[19] EIS is the most sensitive of the electrochemical techniques used; it can quantify molecules with better precision and limit of detection than ELISA.[20]

Label-free electrochemical techniques are ideal for sensing cortisol in tears. In the present study, the feasibility of a handheld cortisol stress sensor is explored with the aim of improving the current state of the art with respect to assay time, sensitivity, and tear collection.

II. MATERIALS AND METHODS

A. Chemical Reagents and Electrochemical Cell Configuration

All reagents were purchased from Sigma-Aldrich (St. Louis, Missouri) unless otherwise indicated. Hydrocortisone was dissolved into various concentration gradients in 10-mM phosphate buffer saline solution (EMD Chemicals, Billerica, Massachusetts) or a 50% mixture of 200-proof ethanol and chloroform (BDH solvent, VWR International, Radnor, Pennsylvania) to suit each technique. The anticortisol monoclonal antibody used in the EIS technique was aliquoted into 10 ug/mL in PBS. 100-mM Potassium Ferricyanide solution was prepared using PBS. 1-mM 16-mercaptohexadecanoic acid (MHDA) in ethanol, 10-mM N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide in PBS, 40-mM (in PBS) N-hydroxysulfosuccinimide sodium salt (Toronto Research Chemicals, Toronto, Ontario), and 1% (in DI solution) ethanolamine, were used in the EIS technique.

Commercially available three-electrode screen-printed Zensors (CH Instruments, Austin, Texas) were used in most of the CV and all of the amp-it, and SWV techniques. CV was also run on gold disk electrodes (CH Instruments, Austin, Texas). Platinum and silver-silver chloride wires (CH Instruments, Austin, Texas) were also used in the impedance technique to create a three-electrode electrochemical cell with the GDE, as described in La Belle et al.[20] Two CH Instruments electrochemical workstations were used: the CHI 1230B (for CV, amp-it, and SWV) and the CHI 660C (for CV and EIS).

A goat cortisol ELISA kit was purchased from Cusabio (Wuhan, Hubei Province, China). Potential interference included IgE (Abcam, Cambridge, United Kingdom), lactoferrin, dopamine hydrochloride, and D-glucose. A SpectraMax M2e (Molecular Devices, Sunnyvale, California) collected absorbance data.

B. CV Measurements

The parameters set for the CV assay were as follows: initial E = −1.4 V, high E = 1.4 V, low E = −1.4 V, scan rate = 0.1 V/s, 6 segments, sample interval = 0.01 V, and autosensitivity. Before a concentration gradient of cortisol was run, three blank solutions (50-mM ferricyanide) were run. The cortisol solutions were measured using 50-µL 100-mM ferricyanide (in PBS) and 50-µL hydrocortisone (in ethanol, chloroform, and PBS). The 50-µL hydrocortisone solutions were made by serial dilution with a stock solution of 100-mg hydrocortisone in 500-µL ethanol and 50-µL chloroform. The subsequent dilutions (five between 14.5 and 0.01 mg/mL) were diluted serially using PBS.

The current (A) signals from each concentration and blank solution were fitted to a natural logarithmic calibration curve with respect to concentration of cortisol at the oxidation potential. Additionally, lower limits of detection were calculated via 3.3 times the standard deviation over the slope of the calibration curve. Maximum current averaging was also employed to find the average oxidation potential over the entire cortisol gradient for use in subsequent techniques.

C. Amp-it Measurements

The parameters for the amp-it assay were set as follows: initial E = oxidation potential found from CV in V, sample interval = 0.1 s, runtime = 40 s, and sensitivity = $1E^{-5}$ A/V. Before the cortisol gradient was run, three blanks were run as described in Section IIB. The stock solution, 100-mg hydrocortisone in 500-µL ethanol and 50-µL chloroform, was serially diluted with PBS (six solutions between 3.63 and 0.004 mg/mL). The electrodes were rinsed with DI solution before each solution was tested. A calibration curve over the cortisol concentration gradient for the amp-it technique was created from a natural logarithmic fit of the current (A) versus concentration of cortisol at a selected time point. Based on the slope and R^2 at each time point in the amp-it (40 s), a minimum assay time was estimated. Similarly, the lower limit of detection (LLD) was calculated as detailed in Section IIB.

D. SWV Measurements

The parameters set for the SWV assay at 15 Hz, 20 Hz, and 30 Hz were as follows: initial E = −1.4 V, final E = 1.4 V, increment E = 0.004 V, amplitude = 0.025 V, and sensitivity = $1E^{-9}$ A/V. Sample measurement and preparation mimicked the details provided in Section IIB except that the cortisol concentration gradient included six solutions serially diluted from stock between 363 and 0.004 ug/mL. A calibration curve over the cortisol concentration gradient at each frequency for the SWV technique was created from a natural logarithmic fit of the current of the average oxidation potential (A) versus concentration of cortisol (ug/mL). LLD were calculated for each frequency.

E. EIS Measurements

The electrochemical cell consists of a GDE functionalized with an anticortisol antibody, a Pt wire, and an Ag/AgCl wire (Fig. 1A).[21] Each GDE was polished using Al2O¬3 pads, followed by sonication in water. A quality check using CV and EIS was performed to ensure GDE cleanliness and to determine its formal potential (Fig. 1B). An alkane-thiol (1-mM 16-MHDA in ethanol) was incubated on the GDE surface at room temperature for one hour. Another quality check was carried out using EIS at the formal potential to ensure proper deposition of the 16-MHDA monolayer (Fig. 1C). The GDE was rinsed with DI and incubated the 10 mM EDC/80 mM NHS in PBS solution for one hour to prepare the 16-MHDA for protein attachment. After another rinse with DI, 10-µg/mL anticortisol in PBS was incubated on the GDE surface for one hour at room temperature. After a gentle PBS rinse, 1% ethanolamine in DI solution was placed on the GDE surface for 30 minutes. The GDEs were stored in PBS at 4°C until needed.

Real-time measurements of impedance magnitude and phase at each stimulating frequency were recorded for each solution. Nyquist plots were generated and used as a visual cue for experimental data quality. Each GDE produced impedance data for a concentration gradient of cortisol. A calibration curve was created for each GDE at a specified frequency; the complex impedance (Ω) versus concentration of cortisol (pg/mL) was fitted with a natural logarithmic curve. Based on the slope and R^2 for the fit at each frequency, an optimal frequency was determined for each GDE. Replication of experiments allowed for an averaging of optimal binding frequencies to generally represent the population of GDEs from experiment to experiment (Section IIID). Once an optimal frequency was selected, a more precise calibration curve was created by normalizing the average impedance data (ratio of the average impedance at a given concentration divided by the average impedance of the blank concentration). Such a procedure allowed for the average baseline differences (e.g., slight variation in 16-MHDA coverage) in the measurements to be factored out.

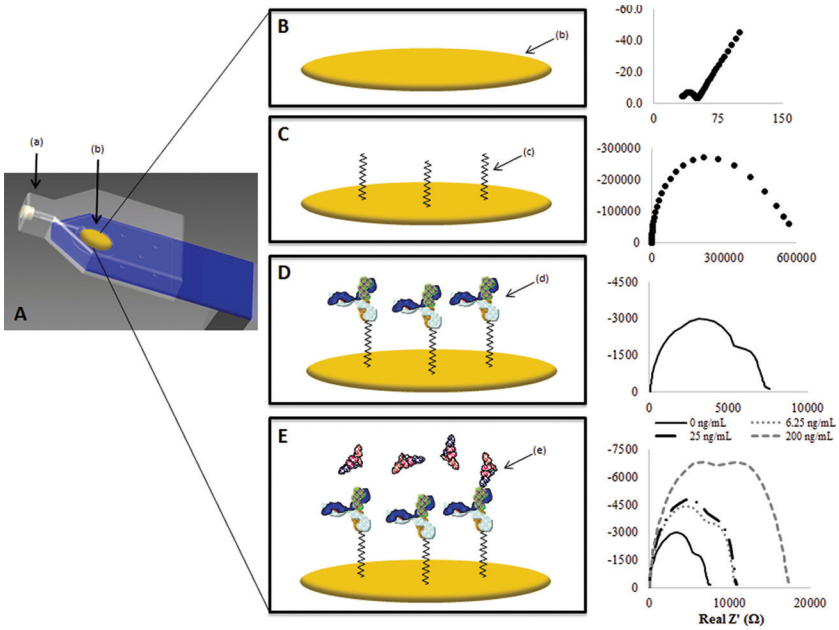

FIG. 1: (A) SolidWorks rendering of the envisioned tear capture device: (a) eye interface and (b) thin gold layer where immobilization occurs. (B) Clean gold surface before immobilization produces the second Nyquist curve; the 16-MHDA layer (c) depicted in (C) produces the third Nyquist curve. (D) After the EDC/NHS step, the anticortisol antibody is deposited onto the gold; the fourth Nyquist curve shows the EIS signal of a blank solution after the ethanolamine step. (E) Interaction of the cortisol molecule (e) with the anticortisol antibody; the final Nyquist curves shows impedance changes with cortisol concentration; the *y*-axis on the Nyquist plots (*right*) is imaginary Z (Ω).

F. ELISA Measurements

The ELISA experiments were run in accordance with the instructions provided in the competitive inhibition cortisol ELISA kit (Cusabio, Houston, Texas). Deviations from the protocol specified by Cusabio were as follows: the plate was split into two halves for $n = 2$; thus six columns of the plate were used on different days. The columns were assigned as follows: 1: a gradient of the hydrocortisone used in the EIS experiments (0–200 ng/mL); 2: lactoferrin (0–100 ng/mL); 3: IgE (0–100 ng/mL); 4: dopamine (0–100 ng/mL); 5: glucose (0–100 ng/mL); and the standard (0–200 ng/mL) gradients. The last solution in each column was a physiologically relevant concentration of the analyte determined by the naturally occurring concentrations found in tear fluid (except the standard column, which was made per the instructions): 1: physiological concentrations were 0–200 ng/mL, 2: 2.4 mg/mL, 3: 300 ng/mL, 4: 500 µg/mL, 5: 5 µg/mL, respectively.

The optical density of the solutions was measured at 450-nm wavelengths and the corrective 570-nm wavelengths (450 nm subtracted from the 570-nm signal). The data for each species were compared to the average cortisol signal for SNR analysis. The SNR calculation used was

$$\text{SNR} = \left(\frac{\text{amplitude of signal}}{\text{amplitude of noise}}\right)^2$$

where amplitude of the signal was the corrected optical density of cortisol, and the amplitude of noise was the corrected optical density of the interferent at the same concentration as the cortisol signal. These calculations were used to develop the bar graph in Section IIIE.

III. RESULTS

A. Cyclic Voltammetry

Figure 2 shows the electroactivity of cortisol in 100-mM ferricyanide solution. It appears that the

single electron reduction and oxidation of the cortisol molecule is a reversible reaction. CV is a simple technique and ideally suited for a handheld device if its detection limits reach the required concentration range. The lower limit of detection (using the lowest concentration of cortisol) found in these (n = 2) data was only 0.1 M. The observed LLD was not sufficient to detect physiological ranges of cortisol in tear fluid; however, the average oxidation (0.32V) and reduction (0.03V) potentials proved to be useful information in the subsequent experiments.

B. Amp-it

The graph in Fig. 3 shows a calibration curve that would be used to program the handheld device to convert measured current to concentration of cortisol. Amp-it is a relatively straightforward technique often used in self-monitoring blood glucose meters. It is preferred for its simplicity of stimulation, making it easy to manufacture. The oxidation potential (from cyclic voltammetry) was applied as a DC voltage, while the resulting current was measured. From the data collected on cortisol concentrations optimized for amp-it, the lower limit of detection at the lowest concentration of cortisol was 0.23 M. As shown in Fig. 3, the lowest concentration of cortisol had the highest standard deviation, making the LLD too high to detect cortisol in tear fluid.

C. Square Wave Voltammetry

The calibration curve in Fig. 4 shows the expected positive correlation between cortisol concentration and measured current, as shown in the CV data. Of the three frequencies, 15 Hz was found to provide the best correlation between cortisol concentration and the resulting current. The SWV technique is certainly more sensitive than CV (LLD = 192.8 M), and should be sensitive enough to detect physiological concentrations of tear-fluid cortisol. However, Fig. 4 shows that the technique is excessively sensitive and detects differences in sensor materials. Though the two Zensors were from the same sealed package, it is clear that they produced different signals. In fact, the variation in commercial screen-printed electrodes is well known, and is the reason that glucose test strips are batch-assessed based on their electrochemical signal.[22] As evidenced by the unexpectedly high LLD and the variation, the practical use of SWV would also require batch-assessed Zensors. Moreover, the SWV stimulating signal is more complicated and requires more hardware in a handheld device. Additional math must be done to

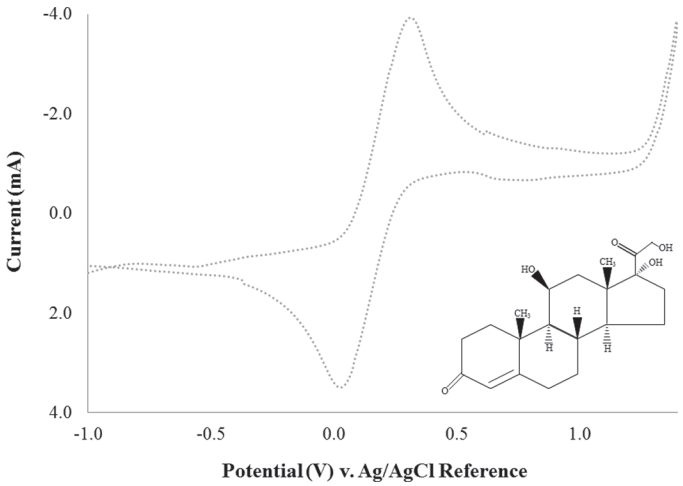

FIG. 2: CV of 50-µL 100-mM ferricyanide and 50-µL hydrocortisone at 145 mg/mL 0.04 M (effective concentration of 72.5 mg/mL) on a Zensor; (*right*) chemical structure of hydrocortisone.

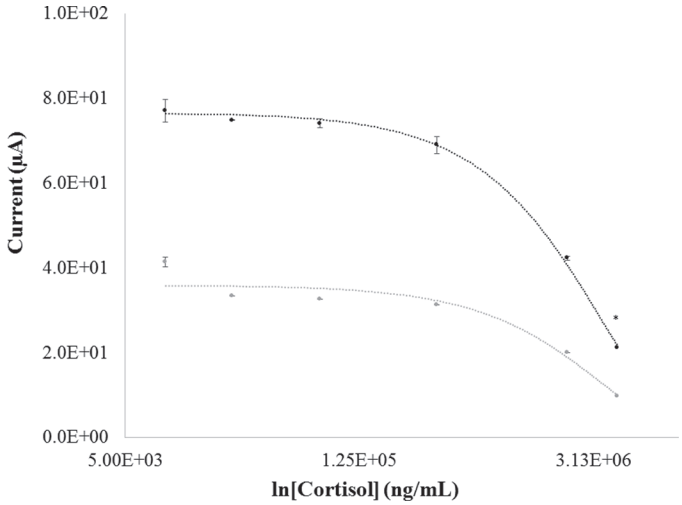

FIG. 3: Amp-it calibration curve plotting current cortisol concentration versus time: (A) 0.1 s and (B) 1.0 s; exponential fits at 0.1 s and 1.0 s have slopes of 76.64 and 35.92 µA/(M) and R^2 of 0.9981 and 0.9739, respectively. The final point in both data sets is $N = 1$ (no error bars).

obtain the differential current, thereby delaying the final result.

D. Electrochemical Impedance Spectroscopy

EIS requires molecular recognition elements (monoclonal antibody) to increase the specificity of the assay. As the previous cortisol experiments were performed in PBS solution (analogous to tear fluid), there was not a distinctive need to use antibodies that would increase the cost of the end device. However, molecular recognition elements are required in EIS to distinguish cortisol signals from noise created by salt ions and similar molecular structure interferents. The proposed technique rivals the sensitivity

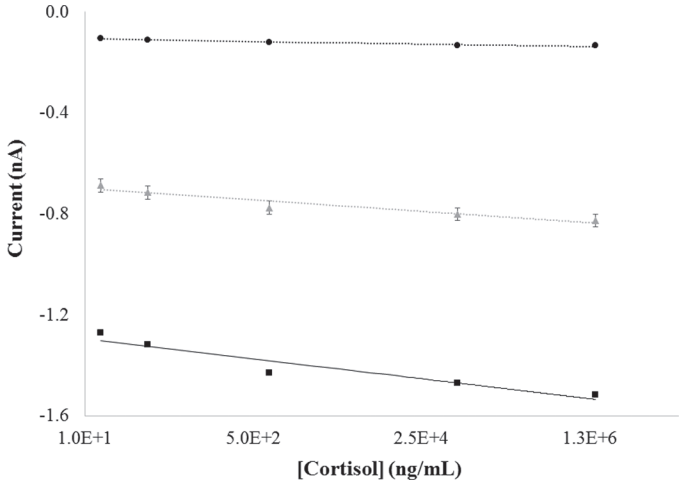

FIG. 4: 15-Hz SWV to determine cortisol concentration (ng/mL) versus current (nA) at the 0.184-V oxidation peak. The natural log fit of the top data (Zensor 1) has a −0.003-nA/(ng/mL) slope and R^2 of 0.9156; the bottom data log fit (Zensor 2) has a −0.021-nA/(ng/mL) slope and R^2 of 0.9134. The average of these data sets is in the middle (gray) with a slope of −0.011 nA/(ng/mL) and R^2 of 0.9178.

of ELISA with an LLD of 20.75 ng/mL. Additionally, once the electrodes are prepared (they could eventually be purchased in a ready-to-use state), the assay time is only 90 seconds with little to no sample preparation.

The calibration curve in Fig. 5A shows the relationship between the average impedance of $N = 6$ electrodes measuring the cortisol concentration gradient at 1172 Hz. However, the blank solution (no cortisol) can add variation for several reasons, including differences in temperature of the electrode surface between runs (the electrodes were stored in 4°C until needed). To remove any error caused by the blank and sensor-to-sensor variation, the graph in Fig. 5B shows the normalized average impedance data. The average impedance reading at each concentration of cortisol was divided by the average impedance reading of the blank solution (instead of subtracted due to the naturally logarithmic data).

E. ELISA

One reason for running ELISA was to determine which of the molecules commonly found in tear fluid may nonspecifically bind to the EIS antibody or cause interference. It was used as the gold standard to compare the sensitivity of the EIS results. Via a signal-to-noise analysis, it was found that the four molecules commonly found in tear fluid did not produce a significant signal compared to the cortisol signal (Fig. 6).

The graph shown in Fig. 7 is similar to the Clarke error grid, where one method (EIS) is compared to the gold standard (ELISA). The same concentration values were plugged into the model of each method and plotted against the other method. If the new method exactly correlated to the standard method, the points would lie on the gray (45°) line. Figure 7 shows the potential of EIS to work as well as ELISA, with the opportunity to become a handheld point-of-care stress diagnostic. EIS requires neither sample preparation nor labels. Once the electrodes are prepared (as commercial test strips would be sold), a cortisol result can be obtained in 90 seconds.

F. Proposed Handheld Stress Diagnostic

The proposed disposable test strip (Fig. 1A) resembles the microfluidic test strip design used in our previous research.[9,10] Such fluidic designs to capture tears have been vetted in animal models and show efficacy in glucose detection.[9,10] The basic composition of the device includes a screen-printed and sputtered substrate functionalized for EIS measurements, on top of which is glued a microfluidic tear capture device made of a cast soft plastic known as SEBS (styrene-ethylene-butylene-styrene). The soft plastic contains a piece of foam that initially collects the tear fluid to minimize the required contact with the eye surface (~ 2 s) and then transfers the fluid to the sensing area.

IV. CONCLUSIONS

The initial characterization of the cortisol molecule in simulated tear fluid (PBS) via CV, Amp-it, and

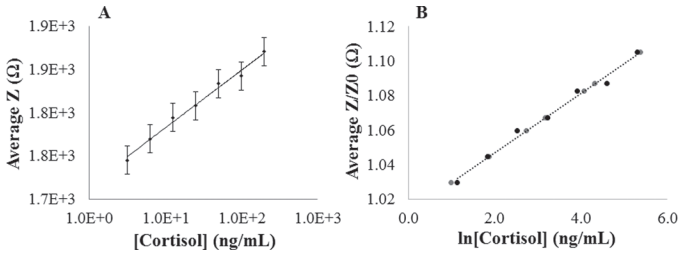

FIG. 5: (A) calibration curve ($N = 6$) with a natural log slope of 28.86 (ohms/pg/mL), and R^2 of 0.9902 at 1,172 Hz (standard error bars), with an LLD of 21.66 ng/mL of cortisol in PBS. The blank solution (0 ng/mL) has been removed to allow a log fit. (B) Black markers are the same data shown in (A) but divided by the blank solution. The normalized data are fit with a linear curve with a 0.02-Ω/ln(ng/mL) slope and R^2 of 0.9865. Gray markers show that known cortisol concentration values plugged into the normalized linear equation fall on the line as expected.

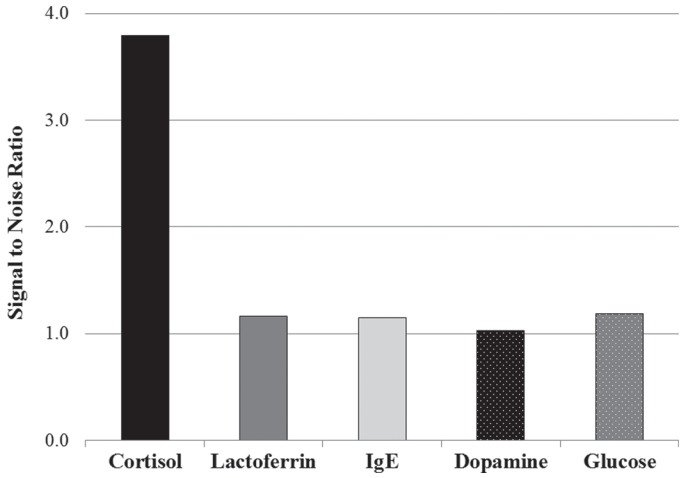

FIG. 6: SNRs resulting from use of the monoclonal IgG anticortisol antibody versus the provided standard, the EIS cortisol concentration gradient, and the tested interferents at 200 pg/mL of each respective analyte

SWV allowed transition to EIS, the most sensitive label-free electrochemical method. The prototyped EIS biosensor obtained a lower limit of detection of 59.76 nM with approximately 10% relative standard deviation. While this is not quite low enough to confidently detect the nonstressed level of cortisol in tears (40 nM), it is sensitive enough to detect cortisol in an individual with elevated stress. EIS has proven that it rivals ELISA's measurement capabilities; moreover, it provides several distinctive advantages that lend themselves to a point-of-care device. A handheld tear fluid cortisol sensor would be invaluable as a noninvasive diagnostic to monitor physiological, occupational, and emotional stress. Future work will include the use of sputtered and screen-printed substrates to reproduce these EIS results, to continue validating the tear capture fluidic designs in animal testing, and begin cortisol detection in human trials for physical stresses (e.g., after physical exercise), emotional stresses (e.g., after watching a depressing movie), and occupational stresses (e.g., after taking a timed exam).

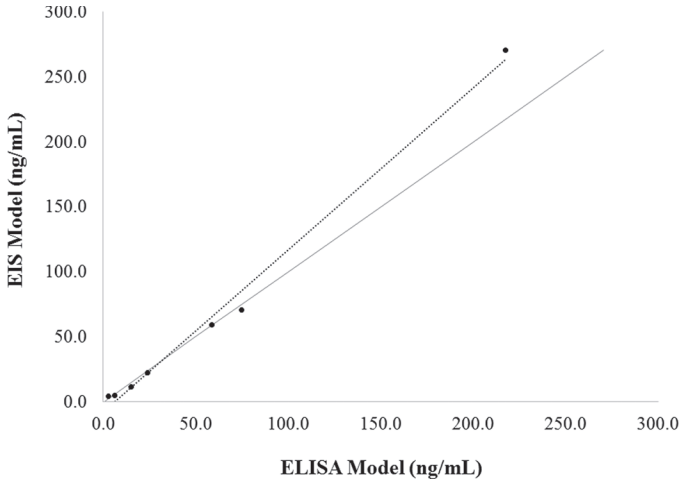

FIG. 7: Comparison of EIS model (dotted line) and ELISA (solid line). The linear relationship between the two has a slope of 1.249 and R^2 of 0.9926.

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