Antibody Modified Gold Micro Array Electrode Based Electrochemical Immunosensor for Ultrasensitive Detection of Cortisol in Saliva and ISF

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Abstract

Dithiobis(succinimidyl propionate) (DTSP) self-assembled monolayer (SAM) functionalized gold microelectrode array has been used for covalent immobilization of monoclonal cortisol antibody (C-Mab). Binding interactions of cortisol to EA/C-Mab/DTSP/Au electrode was tested using impedimetric technique. This Bio-electrode was used to estimate the concentration of cortisol in samples of saliva and Interstitial Fluid (ISF). Sensors were exposed to solutions with different cortisol concentration and label-free electrochemical impedance (EIS) technique was used to make cortisol measurements. EIS results (change in charge transfer resistance, Rct) confirmed that EA/C-Mab/DTSP/Au based biosensor can accurately detect cortisol in the range of 1 pM to 1 μM has exhibit detection limit of 1 pM. In-vitro measurement of cortisol levels in saliva and ISF shows good correlation with the values obtained with commercial ELISA experiments.

Keywords: Cortisol; Self-assembled monolayer; Electrochemical impedance; Immunosensor; Dithiobis(succinimidyl propionate); Diaposable biosensor; Interstitial Fluid (ISF).

1. Introduction

Association of several human diseases (cancer, drug addiction, heart disease) to regular exposure to psychosocial stress has led to growing interest in finding new sensors capable of sensitive and specific detection of stress. Cortisol, a stress biomarker, is a steroid hormone which under normal physiological limit level is important for the regulation of blood pressure, glucose levels, and carbohydrate metabolism, however under elevated level causes numerous diseases [1-3]. In addition, while excess cortisol levels contribute to the development of Cushing’s disease with the symptoms of obesity, fatigue and bone fragility, decreased cortisol levels lead to Addison’s disease which is manifested by weight loss, fatigue, and darkening of skin folds and scars [1,3]. Hence, a sensor capable of measuring cortisol is required to monitor the stress level. Cortisol in blood primarily exists in a bound state with corticosteroid-binding globulin (CBG). It has been reported that while nearly 90% of cortisol is bound, about 10% of it exists in a free biologically active form [4] and can also be found in bodily fluids like saliva, urine and interstitial fluids (ISF).

Recently it has been shown that, there is a good correlation in the amount of free cortisol present in the saliva and the total cortisol present in the blood (5), however, free cortisol levels in saliva is up to 100-fold lower than in serum [6,7]. Thus measurement of free cortisol requires reliable and accurate collection of any body fluid, e.g., blood, saliva, or urine. Blood collection, however, requires trained medical personnel and the trauma of venipuncture that results in much reduced patient participation. Hence, researchers have been exploring minimally
invasive techniques and these efforts have resulted in completely non-invasive sampling: (a) saliva or (b) urine for the free cortisol concentration estimation. The need for the patient to collect samples at odd hours, however, adds to additional stress, thereby adding a bias constituent to the results. Collection of ISF is a technique, which while minimally invasive, does not require the patient’s compliance as a simple ISF harvesting device, attached to the patients can be utilized to harvest ISF continuously. These harvesting devices can harvest ISF continually over 2-3 days without any additional compliance from the patient [8, 9]. The use of ISF collection approach necessitates development of a wearable technique that can accurately measure cortisol in ISF.

Current techniques used for estimation of free cortisol, e.g. High Performance Liquid Chromatography; fluorometric assay; and reverse phase chromatography give precise results however; require long analysis time, are expensive, and cannot be implemented at point of care. Alternate techniques of cortisol detection including radioimmunoassay, flow immunoassay and enzyme-linked immunosorbent assay [1, 4, 10-12] are though reliable and accurate, have problems of laborious, time-consuming, large sample volume requirement and cumbersome. Recently, various biosensors have been reported for cortisol estimation [1, 3, 13-15]. However, all of these involve complicated system, tedious fabrication, and indirect cortisol estimation. Biosensor development using combination of self-assembled monolayers (SAM) which provide molecular level interface for binding of biomolecule and electrochemical techniques that known for their enhance sensitivity, fast response, low cost and portability have gained increased attention in recent years [16-19]. In particular, use of electrochemical impedance spectroscopy (EIS) coupled with SAMs is very promising to result in label free biosensing of cortisol as they allow the recording of direct binding event occurring at the electrode surfaces through changes in capacitance and resistance. In addition, the data points are generated using a small perturbation in signal, which reduces the matrix interference [20].

This paper reports on accurate measurement of cortisol in ISF and saliva using antibody modified gold microelectrode arrays via DTSP-SAM. ISF was extracted using a novel method that allows continuous harvesting of ISF over 72 hours. EIS is used as a characterization tool and for cortisol estimation via recording of charge transfer processes occurring at the sensor-sample interface. This research establishes the feasibility of using EIS based biosensor for a rapid disposable cortisol detector.

2. Materials and methods

2.1. Chemicals and reagents

Dithiobis(succinimidyl propionate) (DTSP) and sodium borohydride (NaBH₄) were purchased from ThermoFisher Scientific. Monoclonal cortisol antibody (anti-cortisol, C-Mab) 2330-4809 was procured from Abd Serotec. Phosphate buffered saline (PBS) tablets and hydrocortisone (cortisol) were purchased from Sigma Aldrich. ThermoFisher Scientific. Monoclonal cortisol antibody (anti-cortisol, C-Mab) 2330-4809 was procured from Abd Serotec. All other chemicals were of analytical grade and were used without further purification. Working solutions of hydrocortisone were prepared by dilution in PBS (10 mM, pH 7.4). Saliva and ISF samples were collected to mimic a 24-hour circadian rhythm and were provided by Guided Therapeutics Inc. The results of Electrochemical Impedance Spectroscopy (EIS) measurements were correlated with ELISA measurements of duplicate samples of saliva and ISF, which was performed by the Systems Laboratory of Dr. Clemens Kirschbaum in Dresden, Germany.

2.2. Measurement apparatus

EIS was utilized to characterize the bio-electrode fabrication and to estimate cortisol as a function of its concentration. EIS measurements were carried out at equilibrium potential without external biasing in the frequency range of 0.1–10⁵ Hz with a 5 mV amplitude using Autolab Potentiostat/Galvanostat (Eco Chemie, Netherlands). EIS measurements were carried out using 30 μl of PBS solution (10 mM, pH 7.4) containing mixture of 5mM Ferrocyanide and 5mM Ferricyanide i.e. 5 mM $\text{Fe(CN)}_6^{3-/4+}$ as a redox probe. Nyquist plots of impedance spectra in present studies have been exploited to study charge transfer change at sensor-solution interfaces after DTSP SAM formation, C-Mab binding and ethanol amine blocking, the change of charge resistance with changing concentration of cortisol and to measure association constant for the cortisol–C-Mab interaction. All EIS spectra were recorded in PBS containing 5 mM $\text{Fe(CN)}_6^{3-/4+}$ as a redox probe.

2.3. Fabrication of test chip, SAM Preparation and Antibody Immobilization

The biosensor chips were fabricated on oxidized silicon wafer using standard photolithography techniques [15]. Briefly, Cr/Au and Cr/Ag layers deposited using evaporation and were patterned, through lift off. Gold
microelectrode arrays with 5μm wide electrode fingers at a pitch of 15 μm were used. As a final step, SU8 chamber patterned around the electrodes using SU8 50 to create a sample well around these electrodes (Figure 1).

![Figure 1: Schematic of EA/C-Mab/DTSP/Au bio-electrode fabrication.](image)

The gold microelectrode array chips were pre-cleaned with acetone, isopropyl alcohol, and de-ionized water. Next they were exposed to pre-reduced 2mg/ml solution of DTSP in acetone for SAM formation. The DTSP SAM modified electrodes were then rinsed with acetone to remove any unbound DTSP followed by water rinsing. Cortisol antibodies were covalently attached to DTSP-SAM by incubating the electrode in antibody (C-Mab) solution. Cortisol antibodies were covalently attached to DTSP self-assembled monolayer by incubating the electrode in 30 μl of 1 μg/ml antibody in phosphate buffer saline (PBS) solution (10 mM, pH 7.4), for 1 hr. Covalent binding results from the facile reaction between amino group of C-Mab and reactive succinimidyl group of the DTSP on the SAM surface. The sensor (C-Mab/DTSP/Au) was washed thoroughly with PBS to remove any unbound biomolecules. Blocking of unreacted succinimidyl group on DTSP SAM and to remove extra unbound antibodies onto the electrode surface was achieved using ethanol amine. Figure 1 schematically illustrates EA/C-Mab/DTSP/Au bio-electrode fabrication. The fabricated bio-electrodes were characterized using the electrochemical impedance technique.

### 3. Results and discussion

#### 3.1. EIS studies

![Figure 2a: EIS spectra obtained on the EA/C-Mab/DTSP/Au bio-electrode for cortisol concentrations 1 pM–1 μM.](image)

The EA/C-Mab/DTSP/Au bio-electrode for cortisol concentrations 1 pM–1 μM. For each concentration, EIS spectra was recorded in PBS containing 5 mM $Fe(CN)_6^{3-}$ after 30 min incubation of bio-electrode in desired concentration of cortisol followed by PBS washing. Figure reveals that increasing cortisol concentration result in increase of diameter in Nyquist plots, indicating the successful interaction of cortisol with immobilized C-Mab on bio-electrode. The increase in diameter can be attributed to the decrease of electron transfer for redox probe due to resulting insulating layer of cortisol on bioelectrode surface. A linear relationship between the change in Rct values and the logarithm of cortisol concentrations was observed for the cortisol concentrations in the range of 1 pM–1 μM. This biosensing electrode reveals the sensitivity of 1.165 kΩ/M with standard deviation of 0.423 kΩ and correlation coefficient of 0.988. However, to account for the variation in initial impedance values for individual bio-electrode and to confirms that observed change in impedance was due to surface modification occurring by cortisol binding and not due to superimposed effects, all experiments were carried out using a step-by-step approach to increasing cortisol concentration and all Rct data set was normalized to [Rct for desired concentration / Rct of blank bio-electrode]. Figure 2b, shows curve for normalized data and reveals the linear
range of 1 pM–1μM. It exhibit correlation coefficient of 0.986 and standard deviation of 1.23. Fig 2b reveals that after normalization all electrodes shows similar response within the error of 4% (shown as error bars)

3.2. Selectivity studies and In-vitro saliva and ISF studies

For selectivity studies bio-electrode was investigated in relation to corticosterone (data not shown). It was found that the change in Rct for corticosterone was negligible as compared to cortisol with same concentration and can be attributed to the 10% affinity of used C-Mab for corticosterone. Thus, the bio-electrode is quite selective and can be used for selective estimation of cortisol. For in-vitro studies, bioelectrode was successfully used for in-vitro measurement of cortisol levels in saliva and ISF and results were compared with the ELISA results. From table 1 it is clear that values obtained using our system and with commercial ELISA projected a similar trend. It was observed that the EIS cortisol values can be used to get the local cortisol concentrations if normalized with a factor of 10/0.65 for ISF and 1/0.18 for saliva (Table 1).

Table 1: Estimation of cortisol concentration in real ISF and saliva samples.

<table>
<thead>
<tr>
<th>Time of sampling</th>
<th>Sample</th>
<th>Sub</th>
<th>Rct (1000xdl ISF) sample/base (A)</th>
<th>ISF Cortisol Value after normalization (6(Ax10)/0.65)(nM) [Elisa value]</th>
<th>Rct (1000xdl Saliva) sample/base (B)</th>
<th>Saliva Cortisol Value after normalization (B/0.18)(nM) [Elisa value]</th>
</tr>
</thead>
<tbody>
<tr>
<td>8AM-2PM Sub 1-3,5</td>
<td>1</td>
<td>2.53</td>
<td>38.92 [39.64]</td>
<td>1.74</td>
<td>9.67 [9.06]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.95</td>
<td>45.38 [49.41]</td>
<td>2.37</td>
<td>13.17 [13.95]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2.27</td>
<td>34.92 [30.0]</td>
<td>1.56</td>
<td>8.67 [7.67]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>1.85</td>
<td>28.46 [28.37]</td>
<td>1.18</td>
<td>10.0 [10.3]</td>
<td></td>
</tr>
<tr>
<td>8AM-2PM Sub 1-3,5</td>
<td>7</td>
<td>1.67</td>
<td>24.61 [24.94]</td>
<td>1.12</td>
<td>8.44 [4.3]</td>
<td></td>
</tr>
<tr>
<td>2PM-8PM Sub 2,4,6</td>
<td>8</td>
<td>2.67</td>
<td>41.08 [-]</td>
<td>1.56</td>
<td>8.67 [6.21]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>2.29</td>
<td>35.23 [36.43]</td>
<td>1.69</td>
<td>9.39 [7.95]</td>
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<tr>
<td></td>
<td>11</td>
<td>1.97</td>
<td>30.31 [26.55]</td>
<td>1.39</td>
<td>7.72 [4.01]</td>
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</tr>
<tr>
<td>2PM-8PM Sub 2,4,6</td>
<td>10</td>
<td>2.88</td>
<td>44.31 [-]</td>
<td>1.96</td>
<td>10.89 [11.0]</td>
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<td></td>
<td>12</td>
<td>1.60</td>
<td>24.41 [24.94]</td>
<td>1.52</td>
<td>8.44 [4.3]</td>
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</table>

Conclusions:

Dithiobis(succinimidyl propionate) (DTSP) self-assembled monolayer (SAM) functionalized gold microelectrode array can be used to fabricate an ultrasensitive impedemetric cortisol immunosensor. Covalently immobilized monoclonal cortisol antibody based EA/C-Mab/DTDSP/Au electrode exhibits linear behaviour in the concentration range 1 pM–1μM, has low detection limit of 1 pM with high linear regression coefficient of 0.986. The bio-electrode was found selective against corticosterone. In addition, results show the possibility to verify the expected circadian rhythm both within saliva and ISF samples. This sensor, when used as part of a device, can allow a continuous readout of cortisol levels in the ambulatory setting, provide 24-hour diurnal data for the first time by allowing us to get the night time minimum value and can identify precise point in sleep cycle at which cortisol levels in the body begins its morning cycle.

References: