



Dithiobis(succinimidyl propionate) modified gold microarray electrode based electrochemical immunosensor for ultrasensitive detection of cortisol

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ABSTRACT

Gold microelectrode arrays functionalized with dithiobis(succinimidyl propionate) self-assembled monolayer (SAM) have been used to fabricate an ultrasensitive, disposable, electrochemical cortisol immunosensor. Cortisol specific monoclonal antibody (C-Mab) was covalently immobilized on the surface of gold microelectrode array and the sensors were exposed to solutions with different cortisol concentration. After C-Mab binding, unreacted active groups of DTSP were blocked using ethanol amine (EA) and label-free electrochemical impedance (EIS) technique was used to determine cortisol concentration. EIS results confirmed that EA/C-Mab/DTSP/Au based biosensor can accurately detect cortisol in the range of 1 pM–100 nM. The biosensor was successfully used for the measurement of cortisol in interstitial fluid in vitro. This research establishes the feasibility of using impedance based biosensor architecture for disposable, wearable cortisol detector.

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1. Introduction

Cortisol, a steroid hormone, is a biomarker for numerous diseases and is important for the regulation of blood pressure, glucose levels, and carbohydrate metabolism, within the physiological limit (Zhou et al., 2004; Tai and Welch, 2004; Stevens et al., 2008). Abnormal increase in cortisol level inhibits inflammation, depresses immune system, increases fatty and amino acid levels in blood. 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 (Zhou et al., 2004; Stevens et al., 2008).

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 (Cook et al., 1997) and can also be found in bodily fluids like saliva, urine and interstitial fluids (ISF). Normal level of cortisol in serum is generally in the range of 100–500 nM. There is a good correlation in the amount of free cortisol present in the saliva and the total cortisol present in the blood (Gozansky et al., 2005), however, free cortisol levels in saliva

and urine are up to 100-fold lower than in serum (Morineau et al., 1997; Levine et al., 2007). There has been growing interest in measurement of cortisol to establish whether cortisol variation can be used as a precursor to medically and psychologically relevant events, the most recent affliction being post-traumatic stress disorder (PTSD) (Hauer et al., 2009; Lindley et al., 2004). Measurement of cortisol requires reliable and accurate collection of any body fluid, e.g., blood, saliva, or urine. Blood collection, however, requires trained medical personnel. Additionally, the trauma of venipuncture results in much reduced patient participation. Hence, researchers have been exploring non-invasive/minimally invasive techniques that ensure: (a) high fidelity samples (cortisol is a small molecule that diffuses rapidly) and (b) minimal patient participation in the sample handling process and trauma. These efforts have resulted in researchers exploring completely non-invasive sampling: (a) saliva or (b) urine for the free cortisol concentration estimation (Cook et al., 1997; Mitchell et al., 2009; Rowe et al., 2007; Gatti et al., 2005). 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 or transdermal body fluid (TDF) is a technique, which while minimally invasive, does not require the patient's compliance as a simple harvesting device is attached to the patients. These harvesting devices can harvest ISF continually over 3–4 days without any additional compliance from the patient (Nindl et al., 2006). The use of ISF collection approach necessitates development of a wearable biosensing technique that can accurately measure cortisol

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in ISF. This paper reports on the development of such a cortisol biosensor.

The ISF is an extra cellular fluid that surrounds the cells in the human body and consists of small and moderate sized molecules, including glucose, ethanol, and cortisol. The homeostatic feedback loop in the body ensures that these molecules have a direct correlation to the concentration of molecules in blood (Stout et al., 1999; Bantle and Thomas, 1997; Knoll et al., 2002). ISF is especially attractive for cortisol monitoring, as it can be drawn continuously from the dermis through an ablated stratum corneum by simply applying a small amount of vacuum.

Currently, in clinical practice, total cortisol, which is the sum of free and protein bound fractions, is measured, however, free cortisol is the only biologically active fraction (Levine et al., 2007; Stevens et al., 2008). Hence, in order to diagnose and properly treat cortisol-related conditions, regular estimation of free cortisol is required. Current techniques for estimation of free cortisol, e.g. High Performance Liquid Chromatography (HPLC) (Oka et al., 1987); fluorometric assay (Appel et al., 2005); and reverse phase chromatography (Gatti et al., 2005); require long analysis time, are expensive, and cannot be implemented at point of care. Alternate techniques of cortisol detection including radioimmunoassay (RIA), flow immunoassay and enzyme-linked immunosorbent assay (ELISA) (Cook et al., 1997; Zhou et al., 2004; Schmalzing et al., 1995; Kaptein et al., 1997; Koutny et al., 1996; Sarkar et al., 2007) are reliable and accurate. However, these approaches are laborious, time-consuming, require large sample volume and involve the use of radioisotopes.

Free cortisol can be measured experimentally by equilibrium dialysis and ultrafiltration techniques (Jerkunica et al., 1980; Vogeser et al., 2007), or it can be derived by calculation from Coolens equations (Dorin et al., 2009). However, these methods are cumbersome as estimation requires assaying of both total cortisol and CBG on the same sample requiring 5 ml of blood per assay. Recently, various biosensors have been reported for cortisol estimation (Stevens et al., 2008; Mitchell et al., 2009; Kumar et al., 2007; Sun et al., 2008; Zhou et al., 2004). However, all of these involve complicated system, tedious fabrication, indirect cortisol estimation, measurement at very high voltages or modification of analyte itself.

Biosensor development using self-assembled monolayers (SAM) and electrochemical techniques have gained increased attention in recent years (Arya et al., 2007a,b; Valera et al., 2007; Geng et al., 2008; Chen et al., 2006; Maalouf et al., 2007; Khan and Dhayal, 2009; Loyprasert et al., 2008, 2010; Kim et al., 2010; Weng et al., in press; Ng et al., 2010). SAM formation allows the binding of biomolecules in the closest vicinity of electrode surface, and electrochemical detection technique results in enhanced sensitivity, fast response, low cost and portability. The use of electrochemical impedance spectroscopy (EIS) coupled with SAMs is promising as they allow for the possibility of recording of direct signatures of bio-recognition events, occurring at the electrode surfaces through changes in capacitance and resistance (Azcon et al., 2008).

In EIS, when a biological receptor binds to its counterpart, the measurement of changed impedance enables direct and label-free measurement. The data points are generated using a small perturbation in signal, that reduces the matrix interference (Katz and Willner, 2003; Barsoukov and MacDonald, 2005). The main drawback of EIS, however, is decreased sensitivity and lower detection limits. This can be overcome through the use of microelectrodes. Microelectrodes also lead to lower detection limits and minimization of an interference effect coming from non-target analytes (Laschi and Mascini, 2006; Hagelsieb et al., 2007; Thomas et al., 2004; Radke and Alocilja, 2005; Valera et al., 2007).

In this work, we report on a successful fabrication of a label-free impedance based immunosensor for ultrasensitive cortisol testing. SAM formation utilized the dithiobis(succinimidyl propionate) (DTSP), which was used for an antibody immobilization onto Au microarray electrode. The sensor was tested with ISF and the measurements were compared to ELISA. This biosensor enabled cortisol detection up to 1 pM within the 40 min analysis time

2. Materials and methods

2.1. Chemicals and reagents

Dithiobis(succinimidyl propionate) (DTSP) and sodium borohydride (NaBH_4) were purchased from ThermoFisher Scientific. Monoclonal cortisol antibody (anti-cortisol, C-Mab) 2330-4809 was procured from Abd serotec. Phosphate buffered saline and hydrocortisone (cortisol) were purchased from Sigma–Aldrich. SU-8 resist was purchased from Microchem Corp. All other chemicals were of analytical grade and were used without further purification. Working solutions of hydrocortisone were prepared by dilution in phosphate buffer saline (PBS) (10 mM, pH 7.4). Commercially available Cortisol Luminescence Competitive Immunoassay manufactured by IBL, Hamburg was used for the human ISF sample analysis which was performed by the Systems Laboratory (Dr. Clemens Kirschbaum) in Dresden, Germany.

2.2. Measurement and apparatus

Electrochemical impedance (EIS) was utilized to characterize the EA/C-Mab/DTSP/Au bio-electrodes and to estimate cortisol as a function of its concentration. EIS measurements were carried out at equilibrium potential called open circuit potential (OCP) without bias voltage in the frequency range of 0.1– 10^5 Hz with a 5 mV amplitude using Autolab Potentiostat/Galvanostat (Eco Chemie, Netherlands). Three-electrode cell configuration with Ag/AgCl as reference electrode and the gold electrode as a counter electrode was used for measurements. EIS measurements were carried out using 30 μl of phosphate buffer saline (PBS) solution (10 mM, pH 7.4) containing mixture of 5 mM $\text{Fe}(\text{CN})_6^{4-}$ (Ferrocyanide) and 5 mM of $\text{Fe}(\text{CN})_6^{3-}$ (Ferricyanide) i.e. 5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ as a redox probe. Using the redox probe (5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$), change in charge transfer resistance at electrode/electrolyte interface has been investigated in electrochemical impedance.

2.3. Fabrication of test chip

The biosensor chips were fabricated on an oxidized 4" silicon wafer using standard photolithography techniques (Sun et al., 2008). Briefly, Cr/Au (200/2000 Å) and Cr/Ag (200/3000 Å) layers deposited using evaporation and were patterned, through liftoff. Gold microelectrode arrays with 5 μm 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 (Fig. 1a). Further SU8 was hard baked at 200 °C to improve its resistance against hard solvents like acetone.

2.4. SAM preparation and antibody immobilization

The gold microelectrode array chips were pre-cleaned with acetone, isopropyl alcohol, and de-ionized water. Next they were exposed to 2 mg ml⁻¹ solution of DTSP in acetone for 1 h for SAM formation. DTSP solution was first reduced using NaBH_4 and then dispensed on the pre-cleaned chips at room temperature for SAM formation. The DTSP SAM modified electrodes were then rinsed with acetone to remove any unbound DTSP followed by

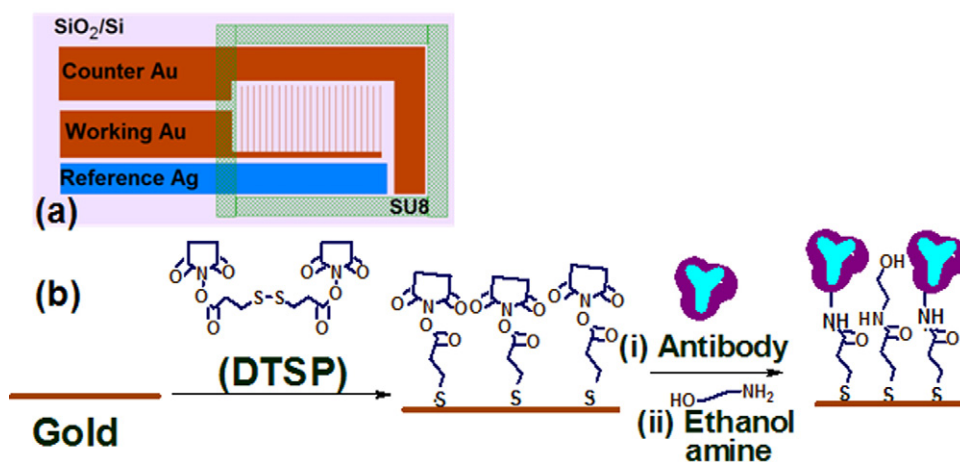


Fig. 1. Schematic for (a) gold microarray chip and (b) EA/C-Mab/DTSP/Au bio-electrode fabrication.

water rinsing and then utilized for antibody immobilization. Cortisol antibodies were covalently attached to DTSP self-assembled monolayer by incubating the electrode in 30 μl of 1 $\mu\text{g ml}^{-1}$ antibody in phosphate buffer saline (PBS) solution (10 mM, pH 7.4) for 1 h. Covalent binding (amide bond formation) results from the facile reaction between amino group of antibody and reactive succinimidyl group of the DTSP on the SAM surface. The sensor (C-Mab/DTSP/Au) was washed thoroughly with phosphate buffer (10 mM, pH 7.4) to remove any unbound biomolecules followed by a 10 min washing with 10% ethanolamine solution in PBS. Ethanol amine was used to block unreacted succinimidyl group on DTSP SAM and to remove extra unbound antibodies onto the electrode surface. Fig. 1 schematically illustrates (a) gold microarray chip and (b) 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. Electrochemical impedance studies

Electrochemical impedance spectroscopy (EIS) is a technique that utilizes an application of periodic small amplitude AC signal over wide frequency range to get electrical response. It is very sensitive and can be used as a characterization tool for studying the charge transfer processes occurring at the sensor-sample interface. Nyquist plots of impedance spectra in present studies have been exploited to study (i) charge transfer change at sensor-solution interfaces after DTSP SAM formation, C-Mab binding and ethanol amine blocking, (ii) 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 (10 mM, pH 7.4) containing 5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ as a redox probe.

3.1.1. EA/C-Mab/DTSP/Au bio-electrode fabrication

Fig. 2 shows the Nyquist plots of impedance spectra measured on the gold microelectrode arrays after each modification processes. Diameter of semicircle represents the charge transfer resistance (R_{ct}) on the electrode surface. In Fig. 2(ii), increase in R_{ct} from 670 Ω for curve 2(i) to 4008 Ω reveals DTSP SAM formation. Increasing R_{ct} is an indication that SAM formation retards the charge-transfer process. Assuming the current was due to defects in monolayer, the change in charge transfer value used in equation $\Theta = 1 - R_{\text{ct}}/R'_{\text{ct}}$ to estimate the surface coverage. R_{ct} and R'_{ct} indicate charge transfer resistance before and after SAM formation. The above suggests 85% electrode coverage indicating the presence

of some pin holes in the SAM. Covalent binding of C-Mab (curve (iii)) onto the DTSP SAM result in increase of R_{ct} to 8648 Ω due to non-conducting nature of C-Mab. After the treatment with EA slight decrease in R_{ct} to 7813 Ω (curve (iv)) was observed. The decrease in R_{ct} can be attributed to the removal of unbound C-Mab molecules.

3.1.2. Cortisol response studies of EA/C-Mab/DTSP/Au bio-electrode

Fig. 3 shows the EIS spectra obtained on the EA/C-Mab/DTSP/Au bio-electrode for cortisol concentrations 1 pM–100 nM. For each concentration, the bio-electrode was incubated in cortisol solution for 30 min, followed by PBS washing and EIS spectra recording using PBS (10 mM, pH 7.4) containing 5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ as a redox probe. With increasing cortisol concentration, diameter of the Nyquist plots was found to increase regularly, indicating that cortisol binds to immobilized C-Mab on bio-electrode, producing a packed layer that decreases the electron transfer for redox probe. A linear relationship between the change in R_{ct} values and the logarithm of cortisol concentrations was observed for the cortisol concentrations in the range of 1 pM–100 nM. The relationship could be characterized using the following linear equation: $\Delta R_{\text{ct}} (\text{k}\Omega) = 30.1 + 2.296 \log C_{\text{cortisol}} (\text{M})$. This biosensing electrode reveals the sensitivity of 2.296 $\text{k}\Omega \text{ M}^{-1}$ with standard deviation of 0.399 $\text{k}\Omega$ and correlation coefficient of 0.997.

Though the impedance of all electrodes fabricated in the same batch can be expected to be the same, lack of industrial

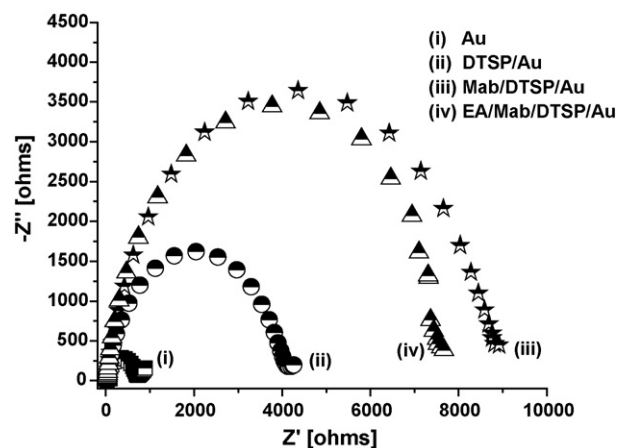


Fig. 2. Nyquist plots of (i) blank Au microelectrodes array, (ii) DTSP/Au electrode, (iii) C-Mab/DTSP/Au bio-electrode and (iv) EA/C-Mab/DTSP/Au bioelectrode.

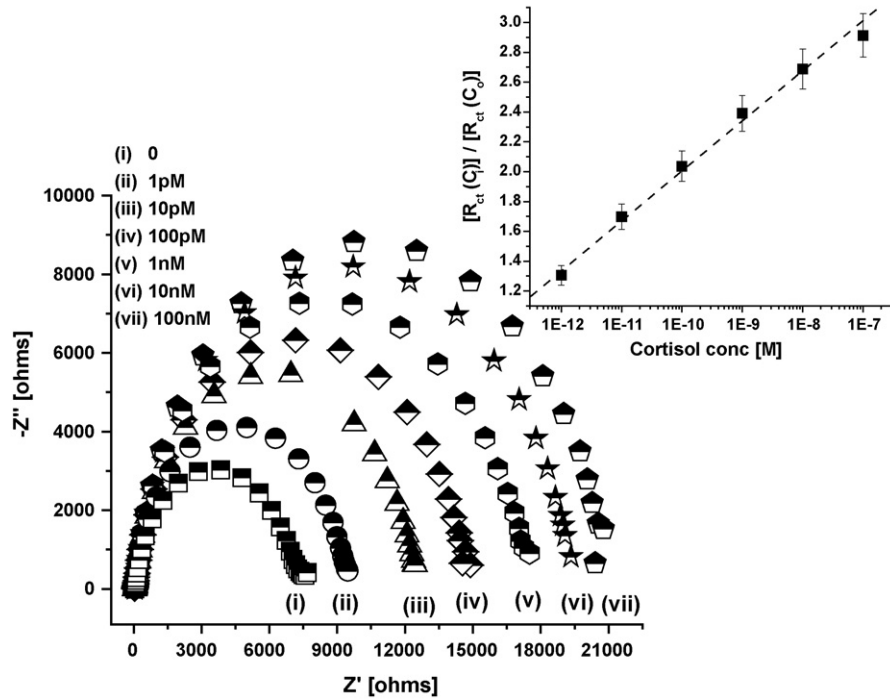


Fig. 3. EIS spectra of EA/C-Mab/DTSP/Au for cortisol concentration (i) buffer, (ii) 1 pM, (iii) 10 pM, (iv) 100 pM, (v) 1 nM, (vi) 10 nM and (vii) 100 nM. Linearity curve for normalized data obtained from EIS studies for different cortisol concentration (inset).

scale process control in the lab leads to variations in impedance of individual electrode and C-Mab modified electrode. In order to confirm that observed change in impedance was due to surface modification and not due to superimposed effects, all data were normalized. For linear range detection normalization was achieved by plotting [charge transfer resistance for desired concentration ($R_{ct}(C_i)$)]/[charge transfer resistance of blank EA/C-Mab/DTSP/Au bio-electrode ($R_{ct}(C_o)$)] versus logarithm of cortisol concentration (Fig. 3, inset). After normalization, all electrodes with different base impedance for EA/C-Mab/DTSP/Au bio-electrode exhibited similar response within the 5% error for each concentration. Fig 3 inset, shows curve for normalized data and reveals the linear range of 1 pM–100 nM. It follows linear equation $[R_{ct}(C_i)]/[R_{ct}(C_o)] = 5.362 + 0.336 \log C_{\text{cortisol}} \text{ (M)}$ and exhibit correlation coefficient of 0.997 and standard deviation of 0.507.

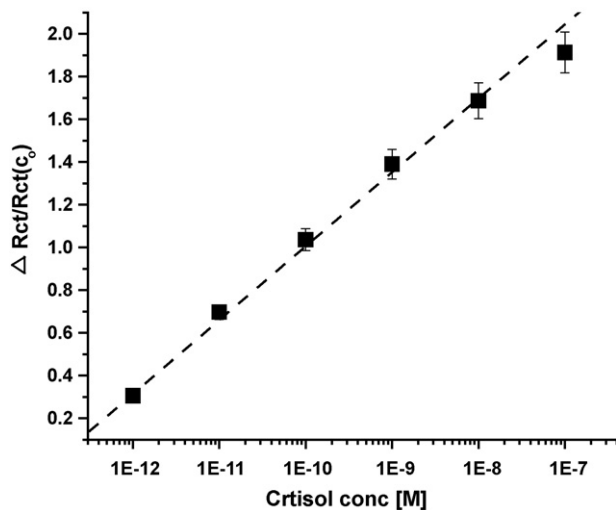


Fig. 4. Curve between $\Delta R_{ct}(C_i)/R_{ct}(C_o)$ and concentration of cortisol for measurement of K_a .

Further, to account for the variation in initial impedance values for individual electrodes, all experiments were carried out using a step-by-step approach to increasing cortisol concentration. Similar step-by-step concentration studies have been reported by other researchers and help in avoiding superimposed effects of multi-electrode measurement (Stevens et al., 2008; Maalouf et al., 2007; Su and Li, 2004; Huang et al., 2008; Lee and Chang, 2005; Lien et al., 2010; Ionescu et al., 2007).

3.2. Estimation of association constant between the covalently bound C-Mab and cortisol

Association constant for binding interaction was determined using Langmuir isotherm approach, where isotherm assumes equal binding energy for all binding sites. This EIS based approach has already been reported by Szymanska et al. (2007) and Li et al. (2008). Briefly, in binding experiments, change in R_{ct} may be related to the binding of cortisol with immobilized C-Mab and can be represented by Eq. (1), where M , $R_{ct}(C_o)$ and $R_{ct}(C_i)$ indicate occupied binding sites, charge transfer resistance without cortisol and with desired concentration of cortisol respectively.

$$M = 1 - \frac{R_{ct}(C_o)}{R_{ct}(C_i)} \quad (1)$$

In Langmuir isotherm, M can be related to association constant using Eq. (2) (Atkins, 2001)

$$M = \frac{K_a c}{1 + K_a c} \quad (2)$$

where K_a is association constant and c is concentration of molecule in solution. On rearrangement Eq. (2) we get

$$K_a c = \frac{M}{1 - M} \quad (3)$$

From Eqs. (1) and (3)

$$K_a c = \frac{R_{ct}(C_i) - R_{ct}(C_o)}{R_{ct}(C_o)} = \frac{\Delta R_{ct}}{R_{ct}(C_o)} \quad (4)$$

Table 1
Estimation of cortisol concentration in real ISF sample.

Subject	ISF sample	R_{ct} base ($10^3 \Omega$)	R_{ct} sample ($100\times$ dilution) ($10^4 \Omega$)	R_{ct} sample/base (A)	Cortisol value after normalization [$(A \times 10)/0.65$] (nM)	Cortisol values from ELISA (nM)	% Deviation in measured value from ELISA value
Sub. 1	ISF1	5.73	1.45	2.53	38.92	39.64	1.8
Sub. 2	ISF2	9.65	2.09	2.16	33.23	32.19	3.1
Sub. 3	ISF3	6.58	1.94	2.95	45.38	49.41	8.1
Sub. 4	ISF4	8.79	2.24	2.55	39.23	40.38	2.8
Sub. 5	ISF5	5.82	1.32	2.27	34.92	30.0	14.1
Sub. 6	ISF6	1.37	2.53	1.85	28.46	28.37	0.3

Using Eq. (4), the curve was plotted between $\Delta R_{ct}/R_{ct}(C_o)$ and concentration of cortisol (Fig. 4). Fig. 4 reveals that $\Delta R_{ct}(C_i)/(R_{ct}(C_o))$ varies linearly with concentration and follow linear equation $[\Delta R_{ct}(C_i)/(R_{ct}(C_o))] = 4.468 + 0.346 \log C_{\text{cortisol}} (M)$ with correlation coefficient of 0.998 and standard deviation of 0.997. K_a was estimated from the slope of regression equation and found to be $0.346 M^{-1}$.

3.3. Non-specificity and selectivity studies

The non-specific adsorption was determined by testing the EA/DTSP/Au electrode against various cortisol concentrations (Supplement Fig. 1), and the selectivity test was conducted using EA/C-Mab/DTSP/Au electrode against corticosterone which is very similar in structure to cortisol in a steroid family. There was no significant change in R_{ct} with changing cortisol concentration in non-specific studies, which reveals the specificity of the electrode. In selectivity studies, EA/C-Mab/DTSP/Au bio-electrode was investigated in relation to corticosterone. Fig. 5 shows the selectivity study results which include the EIS electrode spectra of a buffer only (i), after incubation with corticosterone (ii), and after incubation with cortisol (iii). As shown in Fig. 5, the change in R_{ct} for corticosterone was minuscule 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.

3.4. ISF sample studies

Impedance-based biosensor, described above, was used to analyze the human ISF samples. ISF samples of six subjects, collected between 8 AM and 2 PM, were analyzed and compared with the commercially available competitive ELISA results obtained for the same samples (Table 1). For each EA/C-Mab/DTSP/Au bio-electrode,

EIS spectra were first recorded in PBS buffer to get the base value. For each ISF sample, the bio-electrode previously analyzed was then incubated with the sample diluted 100 times in PBS buffer for 30 min, followed by PBS washings, and EIS spectra recording in PBS (10 mM, pH 7.4) containing 5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ as a redox probe. For each bio-electrode, diameter of the Nyquist plots was found to increase after incubation with an ISF sample. The $R_{ct}(C_i)/(R_{ct}(C_o))$ was calculated to determine the cortisol concentration. These values were compared with the values obtained with the commercial ELISA and it was found that the $R_{ct}(C_i)/(R_{ct}(C_o))$ value projected a similar trend. It was observed that the values obtained can be used to get the cortisol concentration if normalized with the factor of 10/0.65 (Table 1).

4. 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/DTSP/Au electrode exhibits linear behavior in the concentration range 1 pM–100 nM, has low detection limit of 1 pM with high linear regression coefficient of 0.997. The bio-electrode was found selective against corticosterone and was validated using the human ISF samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.03.016.

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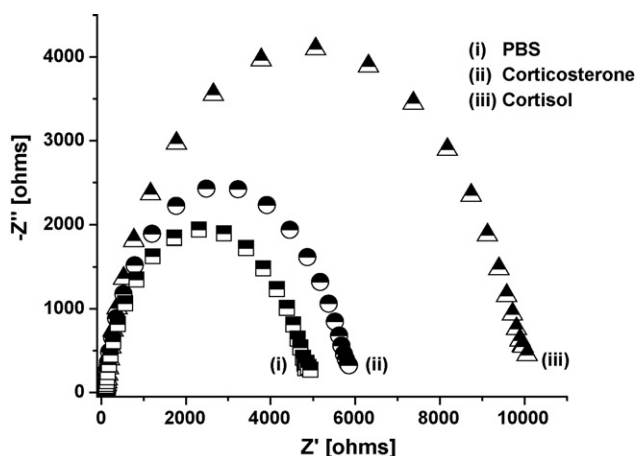


Fig. 5. EIS spectra of EA/C-Mab/DTSP/Au for (i) buffer, (ii) corticosterone, and (iii) cortisol.

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