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Measurement of salivary cortisol by a chemiluminescent organic-based immunosensor

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Abstract. A highly sensitive chemiluminescent immunoassay (CLIA) using a sensitive organic photodetector was developed to detect human cortisol, an important biomarker for stress-related diseases. The developed CLIA was performed onto gold-coated glass chips, on which anti-cortisol antibodies were immobilised and chemiluminescent horseradish peroxidase-luminol-peroxide reactions were generated. Using cortisol-spiked artificial saliva samples, the CLIA biosensor showed a linear range of detection between 0.1 ng/mL and 175 ng/mL and a detection limit of 80 pg/mL. The sensor response was highly specific to cortisol and did not vary significantly between assays. The results indicate the potential clinical application of the CLIA sensor. Furthermore, the simple layered structure of the organic photodetector may encourage the realisation of integrated optical biosensors for point-of-use measurement of salivary cortisol levels.

Keywords: Thin-film organic electronics, optical biosensor, chemiluminescent immunoassay, salivary cortisol

1. Introduction

Cortisol, a steroid hormone, has drawn significant research attention for its potential use as a diagnostic marker of stress-related diseases [1, 2]. Abnormal increase in cortisol levels has been linked to chronic fatigue syndrome, irritable bowel syndrome and post-traumatic stress disorder. In addition, chronic elevations of cortisol secretion can lead to brain aging, bone fragility, immune dysfunction, or increased fatty and amino acid concentrations in blood [3, 4], whereas suppressed cortisol levels may cause weight loss and severe fatigue [5].

The development of highly sensitive immunoassays is crucial for the accurate measurement of cortisol levels. Various immunological methods have been developed for cortisol detection, including enzyme-linked immunosorbent assay (ELISA) [6], surface plasmon resonance immunoassay [7], and electrochemical-based immunoassay [1, 8]. However, despite the remarkable advances in electrochemical methods, optical detection is still the preferred technique for quantitative diagnostics and the method most widely employed in laboratory tests [9, 10]. Furthermore, optical detection based on chemiluminescence assays can be an ideal method for point-of-care biosensors because of its inherent sensitivity and simplicity [11].

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Chemiluminescence is conventionally measured by charge-coupled devices and photomultiplier tubes. These conventional optical detection devices that add substantial complexity to the detection system are difficult to miniaturise into a low-cost, portable and robust system [9]. Organic photodiodes (OPDs) can be an alternative approach to silicon photodiodes for miniaturised biosensors. Compared with silicon photodiodes which involve high-cost fabrication techniques, organic semiconductor devices may be fabricated at a low temperature by employing simple layer-by-layer deposition procedures that are fully compatible with flexible substrates. Furthermore, OPD technology can be easily integrated with microfluidics, allowing for sensitive detection measurements, even with no collection lenses [12]. Wang et al. [11] have developed an integrated OPD for the chemiluminescence detection of hydrogen peroxide and antioxidants. The device, fabricated with a heterojunction of poly (3-hexylthiophene) (P3HT) and [6, 6]-phenyl C_{61}-butyric acid methyl ester ($PC_{60}BM$), was reported to have a detection limit (LOD) in the tens of micromolar range. Using similar OPD design, Wojciechowski et al. [12] developed a detection system with a LOD of 0.5 ng/mL.

New classes of OPDs with enhanced optoelectronic characteristics have been developed recently [13]. Heterojunction devices of poly (2,7-carbazole) derivatives, especially poly [N-9′-heptadecanyl-2,7-carbazole-alt-5,5-(4′,7′-di-2-thienyl-2′,1′,3′-benzothiadiazole)] (PCDTBT), have exhibited lower dark currents and superior photon collection efficiencies compared with P3HT-based devices [13, 14]. These characteristics would make the PCDTBT-based OPDs promising for low-intensity light sensing applications. In the present study, a PCDTBT: $PC_{70}BM$ heterojunction OPD was developed for a highly sensitive chemiluminescent immunoassay (CLIA). The novel CLIA biosensor for cortisol detection was tested with artificial saliva, which is the preferred biofluid for in-field measurements of cortisol [15]. The results were compared with those of the standard ELISA.

2. Experimental methods

2.1. Materials

Phosphate-buffered saline (PBS, pH 7.2), 1-ethyl-3-(3dimethylaminopropyl)carbodiimide hydrochloride (EDC), EZ-Link Sulfo-NHS-Biotin, StartingBlock™ blocking buffer in PBS with 0.05% Tween-20 and SuperSignal® ELISA Femto maximum sensitivity substrate were obtained from Thermo Fisher Scientific, Shanghai, China. Hydrocortisone (cortisol) and horseradish peroxidase (HRP)-labelled anti-cortisol antibody were purchased from MyBioSource Inc., San Diego, USA. Mouse monoclonal anti-cortisol antibody (Thermo Fisher Biochemical, Shanghai, China) was used as the capture antibody. The buffers and solutions were prepared in Milli-Q deionised water, whereas the dilutions of all cortisol assay components were made in 0.1 M PBS. Artificial saliva at pH 7.2 was prepared by dissolving 0.6 mg/mL Na_2HPO_4, 0.6 mg/mL anhydrous $CaCl_2$, 0.4 mg/mL KCl, 0.4 mg/mL NaCl, 4 mg/mL mucin and 4 mg/mL urea in deionised water according to the method described by Tlili et al. [16]. Commercially available cortisol ELISA kit, with all assay components, was procured from MyBioSource.

2.2. Photodetection system

The biosensing system is presented in Fig. 1a. This system consisted of a polycarbazole photodiode placed above the CLIA reaction chip. The reaction chip was fabricated by sputtering a 200-nm thick Au layer on top of the Pyrex 7740 glass wafer [17, 18]. The adhesion between the Au layer and glass

wafer was ensured by a 20 nm Cr film. The OPD comprised a layer of poly (3,4-ethylenedioxythiophene): polystyrene sulfonate (PEDOT:PSS) and PCDTBT: $PC_{70}BM$, the photoactive layer, sandwiched between the anode (ITO) and cathode (LiF/Al). All layer thicknesses are listed in Table 1. Spin-coating was used to deposit PEDOT: PSS film and PCDTBT: $PC_{70}BM$ film while thermal evaporation was employed for the top cathode electrode. The photocurrent induced by the chemiluminescent light emission was measured with a Keithley 236 source measure unit.

2.3. Cortisol detection procedure

The cortisol detection assays were performed on the surface of the CLIA reaction chip, with the Au layer also used as the reflective optical coating. Briefly, reservoirs made of poly (dimethylsiloxane) (PDMS) were attached between the reaction chip and the glass slide containing the OPD. The PDMS reservoirs that were held in place on the reaction chip using a poly (methylmethacrylate) holder [19] contained all assay components which were confined to the surface opposite to the OPD.

Prior to antibody immobilisation, the CLIA chip was pre-treated with 2% (w/v) thioctic acid (Fisher Scientific) followed by application of 1 mg/mL EDC and 0.8 mg/mL sulfo-NHS-biotin for 3 h [20, 21]. Subsequently, 30 µL of anti-cortisol (0.1 µg/mL) was added to the functionalised chip and incubated at room temperature for 1 h. The antibody-coated chip was blocked with StartingBlock™ blocking buffer and washed with PBS. The blocked detection system was then incubated with varying concentrations of cortisol for 30 min at room temperature and washed with PBS. Then, HRP-labelled anti-cortisol detection antibody (0.02 µg/mL) was added to the chip and the system was further incubated for 1 h. Subsequently, the system was rinsed again with PBS. Aliquots of 150 µL of the SuperSignal working solution which were prepared by mixing equal parts of luminol/enhancer and stable peroxide solution were transferred to the CLIA chip and reacted with HRP. Finally, chemiluminescent light was detected by the developed OPD. All the detection experiments were conducted in the dark.

A similar procedure was performed for the detection of cortisol spiked in artificial saliva samples. Commercial ELISA was performed according to the manufacturer's instructions. The chemiluminescence readings were obtained using a multi-mode microplate reader (BioTek).

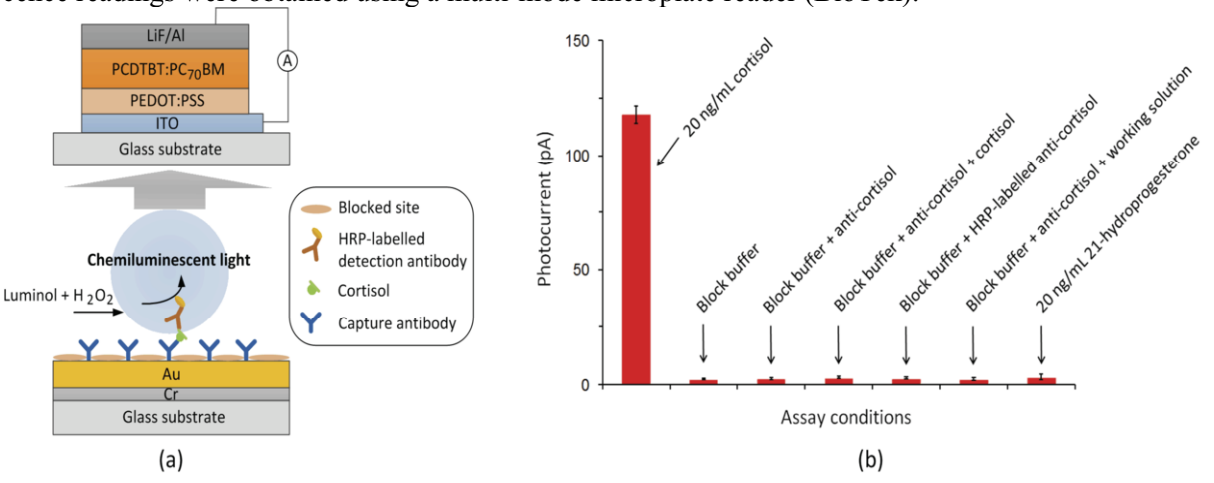

Fig. 1. (a) Schematic of the cortisol detection system comprising the organic photodiode and CLIA reaction chip. (b) Specific cortisol detection by the developed CLIA biosensor using various experimental controls. The error bars correspond to standard deviation for quintuplicate experiments.

Table 1

Thickness of different photodiode detector layers

Layer Designation	Thickness [μm]
Glass substrate	1000
ITO	0.1
PEDOT:PSS	0.04
PCDTBT:$PC_{70}BM$	0.12
LiF/Al	0.1.

3. Results and discussion

Fig. 1b demonstrates the detection of a cortisol positive control in PBS with the PCDTBT: $PC_{70}BM$ photodiode device. The photocurrent was obtained at the plateau region of the chemiluminescent signal (not shown) which occurred at 1 min after the addition of the working chemiluminescent solution. The currents measurements with the OPD were performed under conditions of zero bias voltage (short-circuit conditions), thus minimizing the background current [22]. Upon incubation of the CLIA chip with the cortisol control, the photocurrent changed significantly. The result showed that the current due to detection of 20 ng/mL cortisol was approximately 40-fold higher than that from 0 ng/mL cortisol concentration.

Specificity tests using various experimental controls were performed prior to the realization of the quantitative detection tests. No significant interference from the blocking buffer, antibodies or working solution on the photocurrent measurements was encountered (Fig. 1b). Moreover, the variation of photocurrent for 21-hydroprogesterone, a glucocorticoid hormone highly similar to cortisol, was negligible compared with cortisol.

The negative control CLIA chip/blocking buffer/anti-cortisol was further used to obtain the background level I_b, and the measured photocurrent was then normalised via Eq. (1).

$$I_r = \left[\left(I_{pl} - I_b \right) / I_b \right] \tag{1}$$

In this equation I_{pl} represents the plateau current.

The normalised data was plotted against various concentration of cortisol in PBS in order to obtain the dose-response plot (Fig. 2a). This calibration plot revealed linearity for cortisol in the range of 0.1 ng/mL to 180 ng/mL with a correlation coefficient of 0.994. The analytical sensitivity and LOD of the CLIA biosensor was 642 pg/mL and 65 pg/mL, respectively. The value of sensitivity was determined from the inverse slope of the linear region while the LOD was the concentration of cortisol corresponded to three times standard deviation of five blank measurements. Good reproducibility was also achieved for the analytical device as revealed by the low (1.9% to 3.2%) relative standard deviation of the data points obtained from five times repeated assays.

The CLIA biosensor was further tested with artificial saliva samples. Various cortisol concentrations were detected in 10× diluted samples of artificial saliva. A new calibration plot was obtained (Fig. 2b) following the normalisation indicated in Eq. (1). The analytical performance results are summarised in Table 2. Comparison with the commercially available cortisol ELISA was conducted to assess the suitability of the novel sensor for clinical diagnostic applications. The obtained LOD was more than threefold better than that of the commercial ELISA and below the 1 ng/mL lowest limit of the cortisol reference range for healthy adults [5]. However, comparable analytical sensitivity was

achieved by both detection methods. Compared with the results for PBS, the CLIA LOD and the analytical sensitivity results showed no significant variation with the background matrix. This result is in contrast to that of electrochemical-based sensors [15]. An intra- and inter-assay variability inferior to 7% and 8%, respectively, also represents an important advantage of the CLIA biosensor over the ELISA method. The intra-assay variability in this study, obtained from the relative standard deviation, was calculated from the five assay repetitions for each cortisol concentration on a single day. The inter-assay variability was determined from the five assay repetitions for each concentration on three different days. Notably, the developed sensor may offer a miniaturised solution for cortisol detection with twofold decreased immunoassay duration (see Table 2).

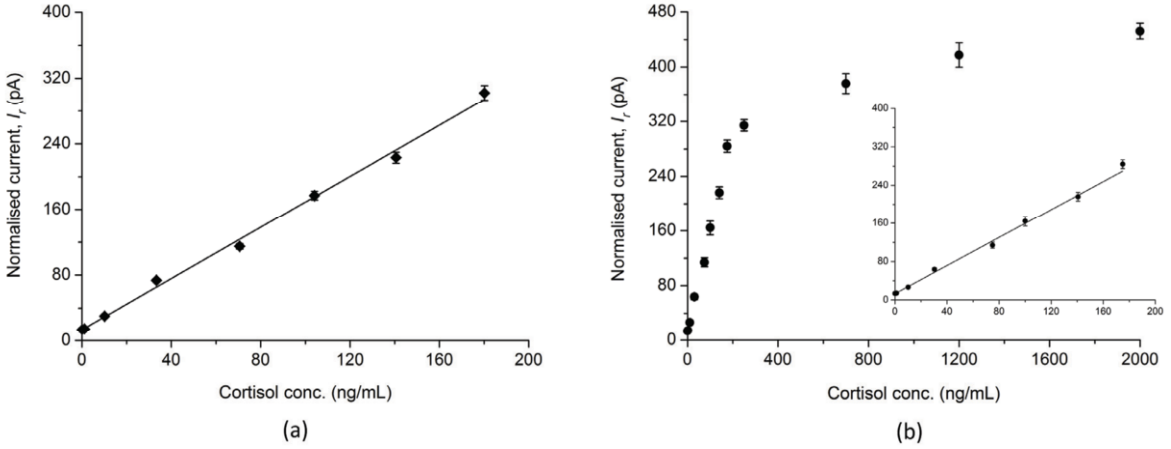

Fig. 2. Dose-response curves obtained from the normalized photocurrent for cortisol detection in (a) PBS and (b) artificial saliva. The inset in (b) depicts the linear range for the measurements performed in artificial saliva. The error bars correspond to standard deviation for quintuplicate experiments.

Table 2

Analytical performance of the CLIA biosensor in comparison with ELISA method

	Developed CLIA with OPD detection	**Conventional ELISA**
Detection range 10^3(pg/mL)	0.1 – 175	1 – 500
Analytical sensitivity (pg/mL)	685	628
Detection limit (pg/mL)	80	315
% Variability		
Intra-assay	3.3 – 6.7	4.1 – 12.3
Inter-assay	3.2 – 7.2	6.9 – 16.7
Total assay duration (h)	~6	~14
Biosensor miniaturisation	Yes	**No**

4. Conclusions

A novel CLIA cortisol biosensor was developed employing a highly sensitive OPD. The OPD was used as the optical detector for chemiluminescent immunossays that were performed on top of functionalised Au-glass chips. The analytical performance of this cortisol biosensor was thoroughly characterised in this study. The sensor exhibited a linear response of four orders of magnitude, with a LOD in the ~tens of pg/mL and analytical sensitivity in the ~hundreds of pg/mL in both the PBS buffer and

artificial saliva. Furthermore, the data also indicated high detection specificity and reproducibility of the developed CLIA biosensor. These results suggest that the analytical system is promising for monitoring cortisol levels in clinical settings. This concept may be suitable for general biomarker detection in real patient samples.

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