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Automated-immunosensor with centrifugal fluid valves for salivary cortisol measurement

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ABSTRACT

Point-of-care measurement of the stress hormone cortisol will greatly facilitate the timely diagnosis and management of stress-related disorders. We describe an automated salivary cortisol immunosensor, incorporating centrifugal fluid valves and a disposable disc-chip that allows for truncated reporting of cortisol levels (<15 min). The performance characteristics of the immunosensor are optimized through select blocking agents to prevent the non-specific adsorption of proteins; immunoglobulin G (IgG) polymer for the pad and milk protein for the reservoirs and the flow channels. Incorporated centrifugal fluid valves allow for rapid and repeat washings to remove impurities from the saliva samples. An optical reader and laptop computer automate the immunoassay processes and provide easily accessible digital readouts of salivary cortisol measurements. Linear regression analysis of the calibration curve for the cortisol immunosensor showed 0.92 of coefficient of multiple determination, R^2, and 38.7% of coefficient of variation, CV, for a range of salivary cortisol concentrations between 0.4 and 11.3 ng/mL. The receiver operating characteristic (ROC) curve analysis of human saliva samples indicate potential utility for discriminating stress disorders and underscore potential application of the biosensor in stress disorders. The performance of our salivary cortisol immunosensor approaches laboratory based tests and allows noninvasive, quantitative, and automated analysis of human salivary cortisol levels with reporting times compatible with point-of-care applications.

1. Introduction

The intersection of the growing body of knowledge about mental health bioindicators with advances in proteomic technologies presents great potential in the early detection, diagnosis and management of a range of stress-related disorders. Of particular interest is the stress hormone cortisol which reflects the underlying neuroendocrine response to stressors and is commonly used as marker of stress reactivity [7,13]. On exposure to a stressor, the body's hypothalamic-pituitary-adrenal (HPA) axis responds by stimulating the production and secretion of cortisol from the adrenal glands. Consequently, cortisol is considered to be a useful measure of the HPA axis adaptation to stress and disease [12]. Altered cortisol levels have been linked to a range of stress-related disorders including depression [2], post-traumatic stress disorder [19], irritable bowel syndrome [6] and increased susceptibility to infections [22].

Although blood is the most commonly used biofluid for biomarker detection and measurement, saliva presents a very compelling alternate when it comes to cortisol estimation. For one, salivary cortisol correlates well with free and biologically-active cortisol in the blood (correlation coefficient, $R = 0.81–0.97$, [25,11]. Furthermore, the levels of the free cortisol in saliva are independent of transport mechanisms, flow rates or salivary enzymes [12]. The simple and nonintrusive nature of saliva collection offers multiple advantages. Unlike blood, saliva collections are readily acceptable to patients and do not provoke the needle-induced anxiety or distress that can artificially alter stress marker levels. Collections do not require trained healthcare professionals and can be accomplished by patients in naturalistic settings. Because saliva is produced continuously and does not clot, it can be sampled repeatedly at short intervals nor does it require special collection and processing equipment. All these qualities render saliva the preferred biofluid for assessing cortisol levels as a reflection of the neuroendocrine response to stressors.

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Actualizing the potential of salivary cortisol in the prevention and management of stress-related disorders requires corresponding developments in bioanalytical technologies to enable rapid analysis and results reporting. Immediate access to a patient's salivary cortisol levels would empower clinicians to make timely and appropriate decisions at the "point-of-care" and enable patient-centered healthcare. To address the need for near real-time results, various investigators have advanced a range of bioanalytical platforms and strategies for rapid cortisol analysis; immunosensors based on piezoelectric elements [5], labeled conjugates [8,16], ultrasound [9], colloidal gold conjugates [15], gold nanowires [14], immune-chromatography [28], graphite electrodes [10], micro-electrodes [4], carbon nanotubes [23], electrochemical impedance spectroscopy [24], and chemiluminescence [20]. Each of these approaches is involved and includes multiple sample processing steps such as passing the biofluid between reservoirs to achieve a micro total analysis system (μTAS) such a "lab-on-a-chip" or using repeated washing processes to remove impurities (e.g., proteins, other steroid hormones) from the sample so as to improve sensitivity and eliminate cross-reactivity. Previously, we had described a microfluidic device with a fluid control system incorporating a valve utilizing direct electro-wetting [17,18,27]. However, the multiple washing processes involved were time consuming and the flow speed constraints limited the practical utility of the system for point-of-care measurement of salivary cortisol levels. To overcome the limitations, we redesigned the immunosensor platform to integrate centrifugal fluid valves that reduce the washing cycles required for processing the saliva sample. The biosensor assembly comprises of a disposable disc-chip incorporating centrifugal fluid valves and a corresponding portable optical reader. To begin with, we optimized the performance of the disc-chip by evaluating the effects of different surface pre-treatments. Subsequently, we evaluated the performance characteristics of the optimized immunosensor using saliva samples as well as cortisol standards. Finally, we investigated the utility of the salivary cortisol immunosensor for discriminating mental health states.

2. Materials and methods

2.1. Chemicals

A monoclonal anti-cortisol antibody (10R-C145A, host: Mouse, Cosmo Bio Co., Ltd., Japan) was used for the immunoassay. An alkaline phosphatase-labeled anti-cortisol antibody (ALP-labeled antibody) conjugate was synthesized using an alkaline phosphatase labeling kit (Kit-NH2, Wako Pure Chemical Industries, Ltd., Japan). Cortisol-3-bovine serum albumin (Coltisol-3-CMO-BSA, Cosmo Bio Co., Ltd., Japan) was immobilized on a polystyrene pad (MS-92302, Sumitomo Bakelite Co., Ltd., Japan). A chemiluminescent substrate (chemiluminescent AP microwell, wavelength: 540 nm, BioFX Laboratories Inc., MD) was used for the ALP, and a phosphate buffer solution (PBS; pH 7.3, 1 mM, Dulbecco A, Oxoid Ltd., UK) was used as a washing buffer for a reaction reservoir. A bovine serum albumin (BSA; Cas no. 9048-46-8, Wako Pure Chemical Industries, Ltd., Japan), a PBS (pH 7.3, 1 mM), and a surfactant (Tween 20, Cas no. 9005-64-5, Sigma–Aldrich Co. LLC., MO) were used to synthesize a blocking agent, BSA-PBS-T. The BSA-PBS-T, a milk protein (UK-B80, DS Pharma Biomedical Co., Ltd., Japan), and an IgG polymer (MAB-IgG/Fab(polymer), SQ Poly MAB 33, Roche Diagnostics GmbH, Germany) were used as blocking agents. Salivary cortisol levels were measured using a cortisol enzyme-linked immunosorbent assay (ELISA) kit (1-3002, monoclonal antibody to cortisol, 450 nm measurement wavelength, Salimetrics LLC, State College, PA). A cortisol standard solution in the ELISA kit was used to determine the calibration curve.

2.2. Centrifugal immunosensor

The disc-chip with a diameter of 120 mm was fabricated using an acrylic resin and an incorporated centrifugal fluid valve provided the fluid control mechanism (Fig. 1). The disposable disc-chip comprised of the following surface elements: a buffer reservoir (1455 mm^3 volume, 2.5 mm depth), an injection reservoir (35.6 mm^3 volume, 1.5 mm depth), a substrate reservoir (35.6 mm^3 volume, 1.5 mm depth), a reaction reservoir (32.0 mm^3 volume, 1.0 mm depth), and a waste fluid reservoir (556.9 mm^3 volume, 2.5 mm depth), all of which were interconnected by miniature flow channels. The flow channels also functioned as valves, and the disc-chip has two different types, labeled A and B. A polystyrene pad integrated into the disc-chip was sealed with a transparent upper sealing layer (P96T01S, polyethylene terephthalate, Stem Co., Japan). The pad with cortisol-3-BSA was placed in the reaction reservoir. The upper layer contained an inlet for the buffer as well as an inlet for the sample and conjugate. Additionally, there was an inlet for the substrate and an air hole.

Fig. 2A describes the mode of operation for the centrifugal fluid valves which control the flow of the biofluid. Fig. 2B shows the principles of the immunoreaction underlying the molecular recognition of cortisol. The automated analytical process is accomplished through the following steps:

i. 10 μL of whole saliva sample and 10 μL of conjugate solution, including the ALP-labeled antibody conjugate, are dropped simultaneously into the injection reservoir through the inlet in the upper layer and allowed to react for 1 min to initiate the immunoreaction.
ii. Immediately afterwards, the disc-chip rotates at 800 rpm for 8 s to blend the solution and propel it through valve A (0.6 mm width, 0.8 mm depth, and 3 mm length). This step ends with the mixed sample transferring from the injection reservoir to the reaction reservoir.
iii. Once in the reaction reservoir, a competitive reaction takes place between the cortisol in the saliva sample and the cortisol-3-BSA on the pad for the duration of 1 min.

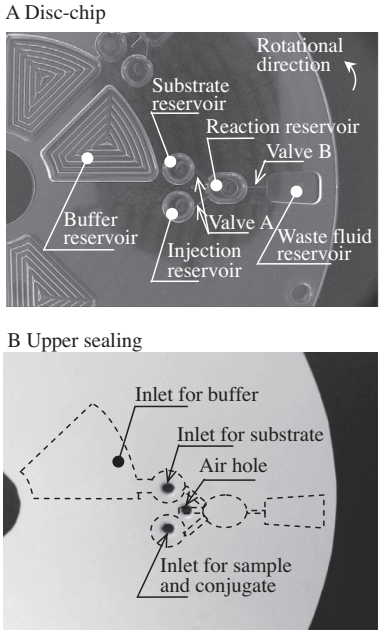

Fig. 1. External view of disc-chip incorporating centrifugal fluid valves for a salivary cortisol immunosensor.