Point-of-Use Measurement of Salivary Cortisol levels

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Abstract—The objective is to develop a novel methodology to analyze cortisol levels in saliva as an index of neuroendocrine response. In order to realize a portable, rapid and hand held biosensor of cortisol, we proposed an immuno–chromatographic test-strip based biosensor consisting of a disposable test-strip and a monitor. A fabricated disposable type test-strip has a size of $5 \times 1.5 \times 50$ mm$^3$. In order to realize an immuno–chromatographic test-strip, a glucose oxidase (GOD) –cortisol conjugate was newly synthesized, because the speed and selectivity of immunological reactions is particularly attractive as a strategy for molecular recognition of cortisol. The synthesized molecule was identified as the GOD-cortisol conjugate by both its molecular weight and specific detection of cortisol. Three min after addition of GOD enzyme assay solution, a color change reaction at a test line was observed. The $R^2$ value for the calibration curve of the cortisol test strip was 0.95, and the relationship between its reflectance, $r$, and the cortisol level was $r = 0.629 \times (1-0.232e^{-0.95 \times \text{cortisol}})$. The fabricated cortisol biosensor enabled us to analyze cortisol concentrations between 1 – 10 ng/ml within 25 min of dropping a cortisol solution on the test strip. Thus, it is suggested that the cortisol biosensor may possibly be used for point–of–use measurements in hospitals.

I. INTRODUCTION
As a key biomarker of an individual’s stress response, cortisol is the increasing focus of psychobiological stress research [1 – 3]. Advantages of salivary cortisol include (i) a simple relationship with corticotropin–releasing hormone (CRH); (ii) close correlation between plasma and saliva cortisol concentrations [4]; (iii) the concentration of the salivary cortisol ranges between 1.05 – 3.34 ng/ml (1 ng/ml = 0.1 μg/dl = 362 pmol/l) [5], which is comparatively high level for a salivary biomarker.

A biosensor for the analysis of salivary cortisol has been studied in order to develop a portable, rapid and hand held–type analytical device for use in hospitals. The speed and selectivity of immunological reactions is particularly attractive as a strategy for molecular recognition of salivary cortisol. Since cortisol is a hepten with a low molecular weight of 362.47, it is a monovalent antigen. Therefore, the sandwich immunoassay method cannot be applied. Thus, high–sensitivity immunoassays have been designed by synthesizing enzyme labeled antigen conjugates for use in competitive binding assays. Peroxidase (EC 1.11.1.7) – cortisol – conjugate [6], aequorin (EC 1.13.12.5) – cortisol – conjugate [7], and colloidal gold conjugate [8] are known labeled antigens. However, a salivary cortisol biosensor that realizes a clinical application has not yet been reported.

The most commonly used enzymes in biosensors contain redox groups that change state during the biochemical reaction. Enzymes of this type include glucose oxidase (GOD). GOD ($\beta$–D–glucose: oxygen 1–oxidoreductase, EC 1.1.3.4) catalyzes the oxidation of $\beta$–D–glucose to D–glucono–δ–lactone and hydrogen peroxide using molecular oxygen as an electron acceptor. We have synthesized the biochemical properties of an enzyme–labelled GOD–cortisol conjugate as the basis for an electrochemical biosensor for cortisol analysis. In this paper we report on the fabrication of a dry–chemistry based electrochemical biosensor to allow point-of-care assessment of the stress response biomarker – salivary cortisol.

The purpose of this research is to demonstrate a new design of a portable, rapid and hand held–type salivary cortisol biosensor. An enzyme–labelled cortisol conjugate was newly synthesized for the specific of antigen–antibody reactions. GOD was used an enzyme label because it is the most commonly used enzyme in biosensors [9] which shows comparatively high enzymatic activity. A dry–chemistry based colorimetric immuno–chromatographic test-strip was fabricated as a biosensor, which could be read with an optical analyzer, to allow point-of-use measurements of salivary cortisol.

II. MATERIAL AND METHODS
A. Synthesis of GOD-cortisol conjugate
GOD catalyzes the oxidation of $\beta$–D–glucose to D–glucono–δ–lactone and hydrogen peroxide. The GOD–cortisol conjugate was synthesized using adipic acid dihydrazide (ADH) as a linker [10]. Firstly, GOD was modified with an aldehyde in order to synthesize GOD–ADH. A solution consisting of 10 mg GOD, 0.1 M 10 μl sodium meta-periodate and 1 ml distilled water was incubated at room temperature for 30 min. The solution was equilibrated with 10 mM ammonium carbonate (pH 9.3) and filtered through a column (Sephadex G–25, GE Healthcare Ltd., UK). The

yellow colored fraction containing GOD–aldehyde was collected in a vial (Fig. 1a).

Next, GOD and ADH were joined by a forming hydrazone bond between them. A solution consisting of 1 ml yellow colored fraction and 100 mg ADH was incubated overnight at 4 ºC. The reaction mixture was added to 10 μl 5 M sodium cyanoborohydride in 1 mol/l NaOH and incubated at 4 ºC for 3 hours (Fig. 1b). The reaction mixture was equilibrated with 10 mM phosphate buffer solution and filtered through the Sephadex G–25column.

Subsequently, the cortisol was activated. A solution consisting of 5 mg cortisol–21–hemisuccinate (086–05583, Wako Pure Chemical Industries, Ltd., Japan), 200 μl dimethyl formaldehyde, 200 μl 1,4–dioxane, 100 μl distilled water, 10 mg N–hydroxysuccinimide and 20 mg 1–ethyl–3–(3–dimethyl–amino–propyl) carbodiimide–HCl was incubated overnight at 4 ºC. The GOD– (ADH)–cortisol conjugate was synthesized by mixing 1 ml GOD-ADH with 500 μl of activated cortisol, incubated overnight at 4 ºC, and then filtered through the Sephadex G-25column ( Fig. 1c).

B. Characteristics of GOD-cortisol conjugate

In order to verify the characteristics of GOD–cortisol conjugate, the molecular weight of the resultant product was measured by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Unmodified GOD was used as a molecular weight standard. A solution consisting of 4.5 μl GOD–cortisol conjugate, 0.5 μl 2–mercaptoethanol, 5 μl 2 × sample buffer consisting of 50 % tris-HCl (0.25 M, pH 6.8), 4 % SDS, 11 % sucrose, 0.4 % bromophenol blue and distilled water was incubated at 95 ºC for 3 min. This solution was subjected to 10 % SDS – PAGE (ready Gels J, 161-J320, Bio-Rad Laboratories, Inc., CA) at 20 mA for 90 min. Gels were stained with coomassie brilliant blue.

Next, the concentration of the GOD-cortisol conjugate was measured by an antigen–antibody reaction using cortisol antigen. The antigen–antibody reaction of GOD–cortisol conjugate and cortisol antibody were measured by using a cortisol antibody coated ELISA plate (1-3002, Salimetrics LLC, PA). A 25 μl GOD–cortisol conjugate was added to each well in the plate. Immediately after that, in order to bind the GOD–cortisol conjugate (antigen) to cortisol antibody, the plate was incubated at 37 ºC for 1 hour. After the plate was washed with wash buffer, 300 μl GOD enzyme assay solution was added to each well and incubated at 37 ºC for 1 hour. The GOD enzyme activity of the GOD–cortisol conjugate was measured by the absorbance for each concentration. The optical density was measured at 490 nm using a fluorescence plate reader (Wallac 1420 ARVO MX / Light, PerkinElmer Inc., MA). A plate without cortisol antibody was used as the control.

C. Test-strip

A biosensor for salivary cortisol analysis (cortisol biosensor) consists of a disposable immuno–chromatographic test-strip (test-strip) and a monitor. A fabricated test-strip (5 × 1.5 × 50 mm³) is made from a sample pad (5 × 0.83 × 10 mm³, CFSP203000, Millipore Co., MA), a conjugate pad containing 20 μl GOD–cortisol conjugate (5 × 0.41 × 5 mm³, GFCP103000, Millipore Co.), a nitrocellulose membrane (5 × 0.24 × 50 mm³, HF240MC100, Millipore Co.) with cortisol antibody (Murine Monoclonal Anti–Cortisol; MIC0202PG,
Serady Inc., IN) and absorption pad (5 × 0.83 × 5 mm³, CFSP203000, Millipore Co.).

When a 100 μl sample solution is dropped on the sample pad (0 min), the impurities with large molecular weights are filtered by the sample pad (Fig. 2a). Immediately after, the sample solution dissolves GOD–cortisol conjugate in it from the conjugate pad. The sample solution moves in a vertical direction on the membrane by capillary action and reaches the test–line (Fig. 2b). The target molecule (cortisol, antigen) in the sample solution is immobilized with cortisol antibody by the antigen–antibody reaction. So, the concentration of trapped GOD cortisol conjugate is inversely proportional to the concentration of cortisol in the sample. Five min after dropping the sample solution, 40 μl GOD enzyme assay solution is dropped on the strip. A red colored band appears on the test–line. The GOD activity is measured by the amount of light received according to the following reactions:

GOD

\[
\text{C}_6\text{H}_{12}\text{O}_6 + \text{O}_2 \rightarrow \text{C}_6\text{H}_{10}\text{O}_6 + \text{H}_2\text{O}_2 \quad (1)
\]

Peroxidase

\[
2\text{H}_2\text{O}_2 + 4\text{aminoantipyrine} + \text{Phenol} \\
\rightarrow \text{Quinoneimine dye} + 4\text{H}_2\text{O} \quad (2)
\]

The amount of light received is inversely proportional to the concentration of cortisol in the sample. Finally, the change in the amount of light received is measured (Fig. 2c).

D. Optical Reader

The appearance of quinoneimine dye formed by coupling of 4-aminoantipyrine and phenol was measured at 520 nm using an optical analyzer (26.5 × 25.5 × 14.5 cm³, 11 kg) (Fig. 3a). The fabricated optical analyzer consisted of a holder of the test–strip, a light emitting diode (LED), a charged coupled device image sensor (CCD) unit for the measurement of the optical density, a temperature control unit for the test–strip and a display (Fig. 3b). The optical analyzer connected to a personal computer (Dimension 2400, DELL Inc., TX) in order to control it. When the cortisol test–strip is put in place, the LED illuminates the strip and the CCD detects the test line (Fig. 3c). The density of the test line is measured by the CCD at a wavelength of 520 nm. Twenty five min after dropping the sample solution, the reflectance \(r\) of the test line is calculated using the following equation:

\[
r = S / S_0
\]

Where, \(s\) : the amount of light received after reaction,

\(S_0\) : the amount of light received before reaction.

The signal (reflectance \(r\)) detected by the optical analyzer is not only proportion to the concentration of the cortisol, but also inversely proportional to the concentration of GOD–cortisol conjugate. Thus, salivary cortisol concentrations can be calculated from a calibration curve between the reflectance and the concentration of cortisol. The analytical accuracy of the cortisol biosensor was evaluated using standard cortisol solution (sample solution, Salimetrics LLC, PA) between 1 – 10 ng/ml.

III. RESULTS AND DISCUSSIONS

A. Characteristics of GOD-cortisol conjugate

The GOD–cortisol conjugate and GOD migrated in polyacrylamide gel electrophoresis (SDS–PAGE) with a molecular mass 200,000 and 75,000 Da, respectively. The molecular weights of each material were GOD 75,000 Da, cortisol 414 g/mol (= 414 Da) and ADH 174.2 g/mol (= 174.2 Da). Thus, the molecular weight of GOD-cortisol conjugate was more than twice as large as the predicted molecular weight (75,560 Da) which was the sum molecular weight of the GOD, cortisol and ADH. Multiple cortisols seem to have combined on to one GOD molecule and there might well be cross-coupling of GOD molecules. It was considered that this chemical phenomenon may help improve the sensitivity of cortisol analysis.

The absorbances of the enzymatic reactions (\(A_{bs}\)) were 2.34, 1.38, 1.15, 0.76, 0.42 and 0.31, when the concentrations of GOD–cortisol conjugate were 1.06, 0.53, 0.21, 0.16, 0.053 and 0.021 mg/ml, respectively. The absorbances of the controls (\(C_{ont}\)) were 1.42, 0.78, 0.88, 0.40, 0.24 and 0.17. Thus, \(A_{bs} / C_{ont}\) (\(A_{bs} – C_{ont}\)) were 0.93, 0.60, 0.27, 0.36, 0.17 and 0.14, respectively (Fig. 3). The specificity of the antigen–antibody reaction induced was in proportion to the concentration of GOD-cortisol conjugate. These results indicated that synthesized molecule was identified as the GOD–cortisol conjugate by both the molecular weight and the specific detection of cortisol.

B. Test-strip

When 0, 1, 5 and 10 ng/ml standard cortisol solutions were dropped into the sample pad, the reflectances (mean ±SD) of the test line were 0.466 ± 0.018, 0.538 ± 0.016,
0.551 ± 0.018 and 0.635 ± 0.015, respectively. The correlation coefficients (CV) were 3.8, 2.9, 3.3 and 3.1 %, respectively (Fig. 4). With regard to the calibration curve for the cortisol test-strip, the R2 value was 0.95; the relationship between the concentration of cortisol and reflectance, \( r = 0.629 \left(1 - 0.232 e^{-1.78 \times \text{cortisol}}\right) \). Therefore, it was indicated that analysis of cortisol concentrations in a range of 1 – 10ng/ml is possible in a total of 25 min from dropping cortisol solution on the test strip through to the analysis.

C. Cortisol biosensor

When 0, 1, 5 and 10 ng/ml standard cortisol solutions were dropped into the sample pad, the reflectances (mean ± SD) of the test line were 0.466 ± 0.018, 0.538 ± 0.016, 0.551 ± 0.018 and 0.635 ± 0.015, respectively. The correlation coefficients (CV) were 3.8, 2.9, 3.3 and 3.1 %, respectively (Fig. 4). With regard to the calibration curve for the cortisol test-strip, the R2 value was 0.95; the relationship between the concentration of cortisol and reflectance, \( r = 0.629 \left(1 - 0.232 e^{-1.78 \times \text{cortisol}}\right) \). Therefore, it was indicated that analysis of cortisol concentrations in a range of 1 – 10 ng/ml is possible in a total of 25 min from dropping cortisol solution on the test strip through to the analysis.

IV. CONCLUSIONS

To replace time-consuming laboratory analysis and facilitate point-of-care measurement of salivary cortisol, we have developed an immuno-chromatographic biosensor system utilizing small disposable test-strips (5 × 1.5 × 50 mm3) and a desk-top based reader (26.5 × 25.5 × 14.5 cm3). Capitalizing on the speed and selectivity of immunodetection approaches, we successfully synthesized a glucose oxidase (GOD)-cortisol conjugate and verified its characteristics through molecular weight determination and its specific binding to cortisol. The cortisol biosensor system prototype allowed accurate measurement of salivary cortisol concentrations in the range of 1 – 10 ng/ml within a time span of 25 minutes. Our findings indicate the potential utility of our immuno-chromatographic system for developing point-of-care biosensors for measuring salivary cortisol.

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