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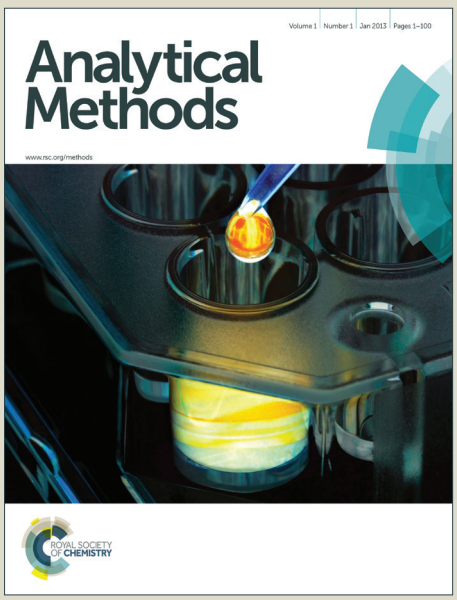

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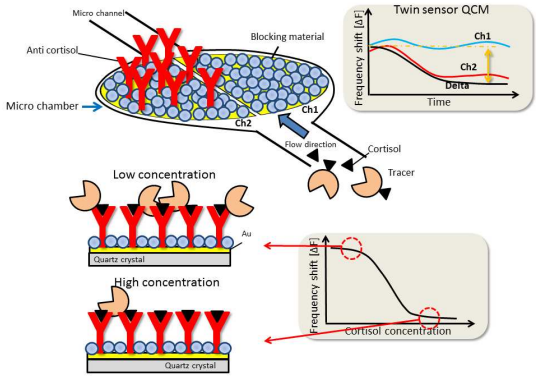
Research Highlights

- Cortisol, a stress marker, was detected using twin sensor QCM integrated with FIA.



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ARTICLE

High-sensitive and rapid sequential cortisol detection using twin sensor QCM

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We present a rapid cortisol detecting system with integration of flow injection analysis in order to adapt sequential analysis. The detecting system consists of a twin sensor chip on quartz crystal micro balance (QCM) method. One sensor monitored antigen–antibody interaction, together with the other sensor as a reference to remove environmental influences. Competitive assay was performed on monitoring antigen–antibody interaction using cortisol coupled with Bovine Serum Albumin (Crt–BSA) as a tracer. The binding between antigen and antibody could be uncoupled with glycine–NaOH solution to adapt sequential analysis. One detection cycle including regeneration step was performed within 10 minutes at the flow rate of 6 μL/min. The sensor system could detect the cortisol revel of 5 pg/mL to 100 pg/mL quantitatively. These results indicated the sensor system has the potential to be used for active stress–monitoring and in the diagnosis of disease.

Introduction

People these days are exposed to many stressors caused by life environments and physiological problems. Long term and strong stress might cause not only mental diseases such as depression but also heart disease and high–blood pressure. Cortisol is well known as a stress marker of endocrine system. Economic loss caused by mental stress recently causes considerable notice from national issues.[1] Psychological stress tests by monitoring cortisol levels in blood or saliva have been reported.[2-7] Cortisol levels in normal serum are in the range from 30 to 140 ng/ml in a day, and have a peak in early morning.[6] Non–invasive estimation of cortisol level is required since blood sampling can be responsible stressor. A sensing system for cortisol requires high sensitivity with short detection time for individual health care. Salivary cortisol levels in the morning range from 1 to 8 ng/ml in healthy adults.[7] For this reason, the targets of the detection range and measurement time are lower than 1 ng/mL and 10 minutes, respectively. When the above condition is provided, sample could be diluted to avoid the effect of foreign substances.

Radioimmunoassay (RIA) and Enzyme–linked Immunosorbent Assay (ELISA) are well known technique as high sensitive detection methods of cortisol,[8-11] and many ELISA–kits are commercially available. However, these techniques need time–consuming steps for secondary labeling and reactions. In addition, the handling of hazardous radioisotopes prevents its wide spread use on RIA. Immunochromatographic strip test is a user–friendly and rapid analysis, however, it lacks quantitatively due to low sensitivity.[12,13] Surface plasmon resonance (SPR) and electrochemical impedance spectroscopy (EIS) are reported as label–free detection methods.[14-17] Mitchell et al. reported that SPR could detect saliva cortisol from 10 pg/mL to 10 ng/mL.[14] It took longer detection time due to additional reaction of the secondary antibody. Arya et al. used EIS with interdigitated array (IDA) electrode to detect cortisol with high sensitivity.[16] This method was not for sequential measurement but for batch test with high cost equipment.

We focused on quartz crystal microbalance (QCM) method, which is one of label free and real time measurement of antigen–antibody interaction with simplicity, convenience and low cost.[18-21] No study has been reported on cortisol detection using the QCM method. The major problem of the QCM method is reducing noise level caused by measurement environment, such as temperature and density of surrounding media. We introduce twin sensor chip on QCM method to detect cortisol. Since twin sensor removes environmental influences, noise level can be decreased drastically.[21] Twin sensor has two reaction regions on one AT–cut quartz crystal substrate. One channel was used as a reference (Ch1) and the other channel measured antigen–antibody interaction (Ch2) in this report. Difference of frequency shift between the Ch2 and the Ch1 was corresponding to the amount of analyte adsorbed on the Ch2 without environmental influence. Since molecular weight of cortisol is too small to detect directly on QCM and sandwich assay is costly with longer detection time by additional processes, competitive assay was applied for cortisol detection. Our sensing system is suitable to clinical laboratory use since the system is integrated with a flow injection analysis. Many samples were analyzed sequentially following sample injections and regenerations. We reported a rapid stress–monitoring method using twin sensor QCM combined with flow injection system.

Materials and methods

Materials

Monoclonal antibody to cortisol (anti–Crt) was obtained from HyTest Ltd. (Turku, Finland). Cortisol 3–BSA (Crt–BSA) was purchased from Fitzgerald Industries International (MA, USA). Cortisol standard solution (500 ng/mL) was obtained from Cayman

Chemical Company (MI, USA). Immunoassay stabilizer (IAS) was from Advanced Biotechnologies Inc. (MD, USA). Phosphate buffer saline (PBS, pH=7.4), sodium hydroxide and glycine hydrochloride were obtained from Wako pure Chemical Industries, Ltd. (Osaka, Japan). Bovine Serum Albumin was obtained from Sigma–Aldrich Co. LLC. (MO, USA).

Sensor system and measurement

Cortisol detection was carried out using a NAPiCOS QCM system consisting of thermostatic chamber and frequency counter (Nihon Dempa Kogyo Co., Ltd., Tokyo, Japan). Schematic figure of system setup with flow injection system was shown as Fig. 1. A micro syringe pump (Model 100, Kd Scientific, MA, USA) and a sample injector (injection volume: 20 µL, 7125, Rheodyne, WA, USA) were connected to a micro flow cell (inner volume: 5 µL) in the QCM system through PEEK tubes. We used a 30 MHz twin sensor chip (PSA–E–3002T, Nihon Dempa Kogyo Co., Ltd., Tokyo, Japan) just after opening packages. The sensor chip had two Au electrodes, and each surface area of them was 7.75 mm^2. Two Au electrodes were semicircle shape and symmetrical pattern. They located in parallel to the flow direction as shown in inset of Fig. 1. The electrodes were 1.5 mm apart from each other. The chip was mounted on a jig made of aluminium and the temperature was controllable. PBS was used as a carrier solution.

The surface of the electrodes was treated as follows and illustrated in Fig. 2. 10 µL of IAS was coated on the surface of the Ch1 for 10 minutes to avoid nonspecific adsorption using a preparation jig, while the other channel (Ch2) was kept as it was. IAS was used as a blocking buffer on ELISA.[22] By using the preparation jig, different treatment could be carried out for each electrode. After washing with PBS and drying the surface of the Ch1, the micro flow cell was set on the sensor chip and was inserted in the thermostatic chamber at 25 °C. Since there was no intervening wall on the flow cell, carrier buffer and sample solution were supplied to two electrodes at once. Firstly, the anti–Crt (68 µg/mL) was injected to be coated on the Ch2. Then, BSA solution (0.01 wt%) was injected to inhibit nonspecific adsorption on the Ch2. Finally, sample solution was injected and frequency shift of the twin sensor chip was monitored.

Results and discussion

Sensor system and antibody coating

The basic principle of QCM sensor is depended on the frequency shift by analyte adsorbed on the QCM resonator. The amount of the analyte adsorbed on the QCM sensor is proportional to the frequency shift known as the Sauerbrey equation.[23] This equation is applicable to thin, rigid and uniform films. In a case of liquid loading, Kanazawa and Gordon expressed a relationship to account for the resonant frequency shift of quartz crystal.[24] Martin et al. derived an equation with mass loading and liquid loading,[25] and total frequency shift is described as the following equation at the fundamental resonance of quartz crystal (N=1).

$$\Delta F_S = \Delta F_M + \Delta F_L = -\frac{2{F_0}^2}{(\mu_q \rho_q)^{1/2}}\left[\frac{\Delta m}{S} + \left(\frac{\rho_L \eta_L}{4\pi F_0}\right)^{1/2}\right]$$

where, Δm is the mass change corresponding to adsorption on the active electrode surface. F_0 (30 MHz in this case) is the oscillation frequency. ΔF_M and ΔF_L are frequency shift due to the mass loading and liquid loading, respectively. ΔF_S is total frequency shift with mass loading and liquid loading. μ_q is the quartz shear module (2.95 × 10^{11} g/cm· sec^2). ρ_q (2.65 g/cm^3) and ρ_L are the density of the quartz crystal and liquid, respectively. η_L is the viscosity of the liquid. S is the surface area of the electrode. First term and second term denote the Sauerbrey equation and the Kanazawa–Gordon equation at the fundamental resonance of quartz crystal (N=1), respectively. Since twin sensor can monitor the frequency shift of two sensing area contacted with the same liquid, the second term is negligible on the differences between the frequency shifts on two sensor surface (delta = ΔF_D) described as below.

$$\Delta F_D = \Delta F_{S(Ch2)} - \Delta F_{S(Ch1)} = -\frac{2{F_0}^2}{S\,(\mu_q \rho_q)^{1/2}}\,[\Delta m_{Ch2} - \Delta m_{Ch1}]$$

where, $\Delta F_{S(Ch1)}$ and $\Delta F_{S(Ch2)}$ are the frequency shift corresponding to the mass and liquid loading on the each channel. Δm_{Ch1} and Δm_{Ch2} are the mass change of the Ch1 and Ch2. Using the above equation,

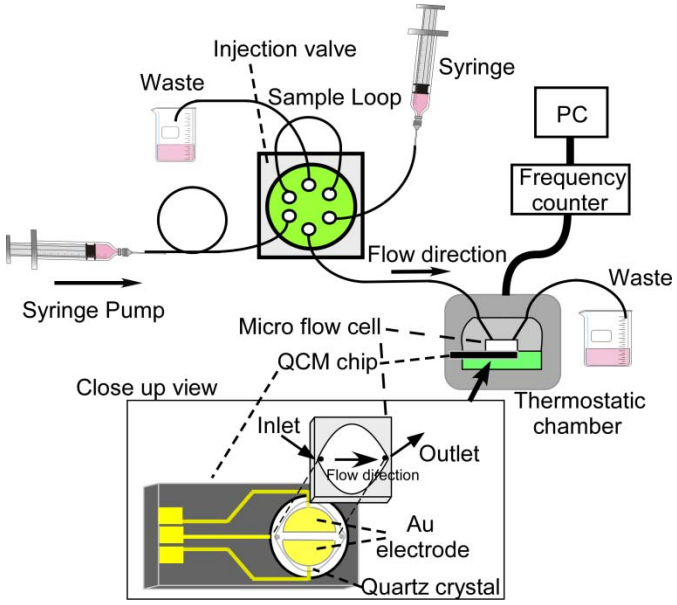

Fig. 1. Schematic figure of sequential cortisol detection system using a twin sensor QCM chip. Injection volume of the sample was 20 µL. The volume of a micro flow cell covered on the QCM chip was 5 µL. Carrier buffer, PBS, was pumped by a syringe pump.

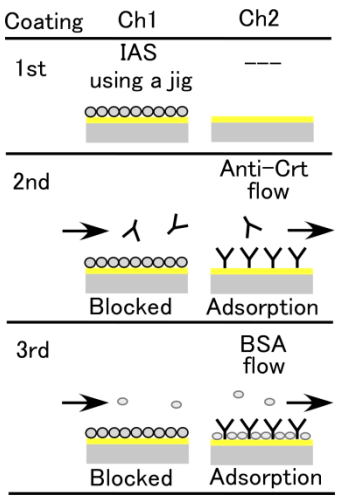

Fig. 2. Schematic figure of sensor preparation for cortisol detection. Firsrly, IAS was coated only on the Ch1 using a jig. Secondly, anti-Crt (68 µg/mL) was supplied by flow to the Ch1 and Ch2. At last, the BSA solution (0.01 wt%) was supplied by flow to the Ch1 and Ch2. Little amount of Anti-Crt and BSA attached to the sensor surface on the Ch1 since the surface was blocked with IAS.

theoretical mass change of the twin sensor system was estimated to be 37 pg of adsorption of analyte with the frequency shift of 1 Hz. Estimating the stability of our system, the delta (ΔF_D) was lower than 1 Hz for 7 minutes at the flow rate of 5 µL/min. The result indicated that the sensing system had low noise level even when the carrier buffer was flowing. To estimate the antigen–antibody interaction, we denote the magnitude of the ΔF_D as $|\Delta F_D|$ in following discussion.

On the antibody injection, significant frequency shift was observed on the Ch2 as shown in Fig. 3, since the anti–Crt was adsorbed on Au surface. Only small frequency shift occurred when the BSA solution was injected after the anti–Crt injection. In this case, adsorption of the antibody on the Ch2 was due to the covalent bond between Au surface and thiol group of the antibody because of no usage of surface treatment. Slight frequency shift was measured on the Ch1 among these injections. The result showed that very few antibodies and BSA adsorbed on the Ch1 because of its surface treatment. Since the Ch1 and Ch2 contacted with the same solution at the same time, the Ch1 was useful as a reference for cortisol detection. Average of the delta on the antibody adsorption was 1516 Hz with coefficient of variation (CV) of 3.3 % (N=8), indicating that the system had good reproducibility. The molecular weight of anti–Crt was approximated to be 150 kDa (the molecular weight of Mouse IgG), theoretical mass of injected anti-Crt ($M_{theoretical}$) was estimated as 1.36 µg (68 µg/ml × 20 µL). Frequency shift of 1516 Hz was equal to the mass adsorbed on the sensor surface ($M_{measure}$), 56.09 ng (1516 Hz × 37 pg/Hz). The ratio of antibody adsorption on the sensor chip was calculated as $M_{measure}/M_{theoretical} \times 100 = 4.1$ %.

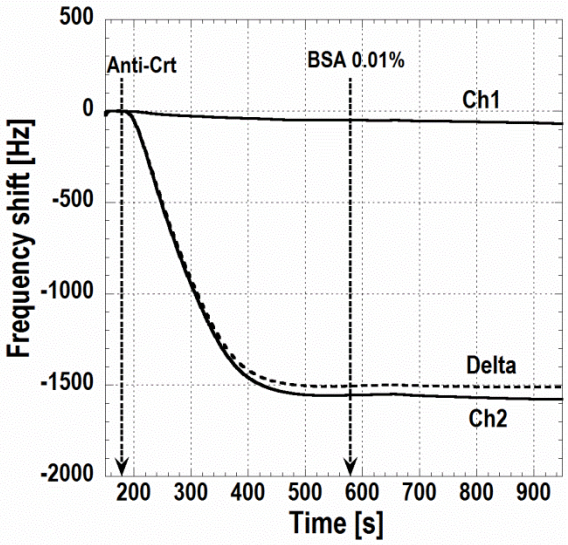

Fig. 3. QCM responces on coating the anti–Crt and 0.01% BSA solution. The delta means the differences between the Ch2 and the Ch1. IAS was coated on the Ch1 before measuring. The Ch2 was without any treatment. The delta means the differences between the Ch2 and the Ch1. PBS, a carrier buffer, was pumped with the flow rate of 5 µL/min. Injection volume of the sample was 20 µL.

Sequential detection of cortisol using competitive assay

Competitive assay was used for small molecular weight of cortisol (362 g). The desired detection range is lower than ng/mL level. In this method, sample solution including cortisol was injected with a constant concentration of weighted cortisol, called a tracer. Cortisol coupled with BSA (Crt–BSA) was used as the tracer because the anti –Crt has cross reactivity to Crt–BSA. Molecular weight of BSA is 66 kDa, which is sufficiently large compared to that of cortisol.

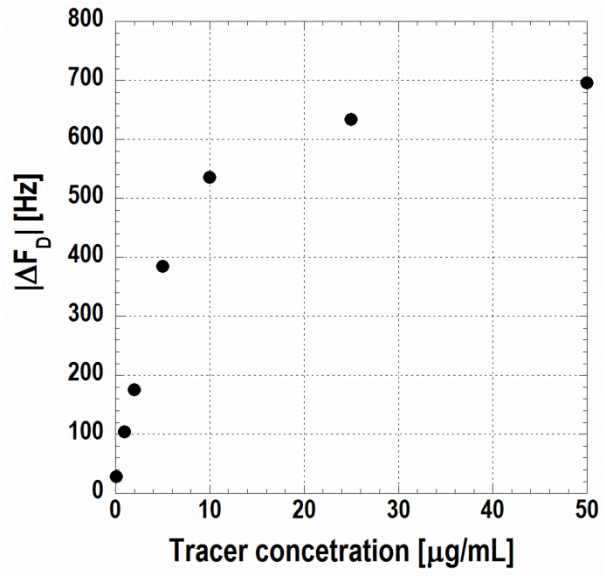

Fig. 4. Magnitude of the delta ($|\Delta F_D|$) plotted against the tracer concentration at the flow rate of 6 µL/min. Injection volume of the sample was 20 µL. The graph shows the characteristic of interaction between the tracer and the anti–Crt.

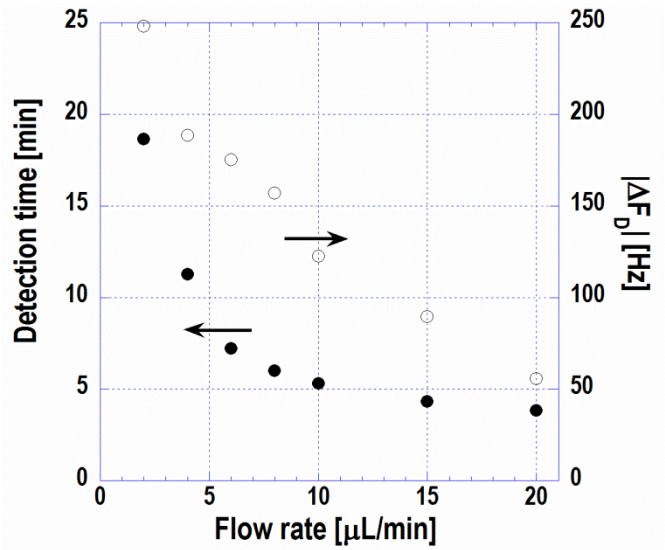

Fig. 5. Magnitude of the delta ($|\Delta F_D|$) and detection time were plotted against the flow rate. Open and closed circles show the $|\Delta F_D|$ and the detection time, respectively. Injection volume of the sample was 20 µL. The tracer concentration was set as 2 µg/mL. Detection time was calculated as T_{end}–T_{inj}, where, T_{end} was the time on the beginning of frequency–stabilized, and T_{inj} was the time on sample injection.

On the competitive assay, tracer concentration was constant but relative concentration of tracer to cortisol changed with the cortisol concentration. Relative concentration of the tracer to cortisol becomes high on the low cortisol level. In such conditions, the tracer mainly attaches to the anti–Crt, and large frequency shift was observed. On the other hands, relative concentration of the tracer to cortisol becomes low on the high cortisol level. So, small frequency shift was observed. Fig. 4 shows the interaction performance between the tracer and the anti–Crt at the flow rate of 6 µL/min.

$|\Delta F_D|$ increased with increasing the tracer concentration. The delta on the 10 μg/mL of Crt–BSA was about 535 Hz, which meant that the Ch2 adsorbed 18.7 ng of BSA–Crt. The delta value was about 10 times larger than that on the injection of 10 mg/mL BSA, which was corresponding to the nonspecific adsorption at the third treatment for sensor preparation as shown in Fig. 3. After the sensor preparation, $|\Delta F_D|$ was not affected by the BSA solution. These results indicated that the sensor chip had high selectivity for cortisol related materials to the other protein. The amount of injected Crt–BSA was 200 ng (10 μg/mL × 20 μL) on the 10 μg/mL of Crt–BSA. The reaction ratio of the antibody–antigen was about 9.4 % (18.7 ng/ 200 ng × 100 %). On the ELISA, Butler et al. reported that the ratio of functional antibody to adsorbed antibody on the cell is lower than 3 % on the monoclonal antibody.[26] Ratio of functional antibody on our system was about three times higher than that on ELISA. One of the reasons for high reaction ratio was due to the extremely small space of the chamber in our system. The delta linearly increased with the tracer concentration at lower than the tracer concentration of 5 μg/mL (R=0.999). Since lower concentration of the tracer with large frequency shift could perform high sensitivity, the tracer concentration was set to be 2 μg/mL in the later experiments.

$|\Delta F_D|$ increased with decreasing the flow rate on the same tracer concentration (2 μg/mL) as shown in Fig. 5. Detection time was calculated as $T_{end} - T_{inj}$, where, T_{end} and T_{inj} were the time at the beginning of frequency–stabilized and the time at sample injection, respectively. The antigen–antibody interaction was affected by diffusion and flow rate. From a qualitative point of view, small amount of antigen which existed on extremely–close to the sensor surface bound with antibody at the higher flow rate, since the injected sample flowed through the micro chamber rapidly. On the other hands, sample slowly flowed through the micro chamber at the lower flow rate. Antigen in a distance could diffuse to the antibody. As the results, the bonding amount increased at the lower flow rate. The sensitivity might increase at lower flow rate, however, it takes longer detection time. Our aim is that one detection cycle was less than 10 minutes including the regeneration of the sensor surface. The flow rate was set at 6 μl/min in the later experiments.

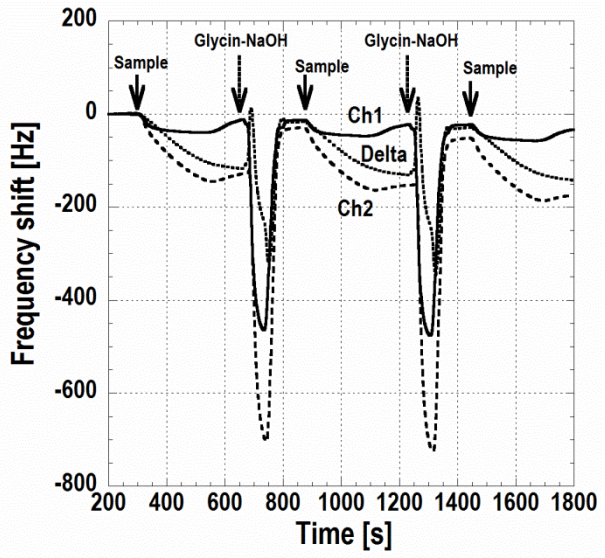

Fig. 6. QCM responces on sample injections and regeneration. The delta means the differences between the Ch2 and the Ch1. The Ch1 was used as a reference and the anti–Crt was coated on the Ch2. Sample solution contained 1 ng/mL of cortisol and 2 μg/mL of Crt–BSA. Samples and 4 mM glycine–NaOH were injected alternately. Injection volume of the sample and the flow rate were set to be 20 μL and 6 μL/min, respectively.

Flow injection analysis is suitable to examine a lot of samples. Since antigen–antibody interaction was relatively–strong adhesive, uncoupling of the bound was required for sequential measurements. As an example of frequency shift on sample injection and the reduction was shown in Fig. 6. 1 ng/mL of cortisol was dissolved in the PBS with 2 μg/mL of the tracer. The sensor response on the Ch1 shifted downward corresponding to the sample injection. This characteristic was caused by the change of the viscosity of the sample solution with a little ethanol. The provided cortisol standard solution was dissolved in ethanol. After the frequency shift became stabilized, it turned back since the solution on the sensor surface was replaced with PBS. Large frequency shit was observed at the response of the Ch2. Then the frequency shift increased gently until the buffer was replaced similar with the Ch1. As the result, the delta stayed constant after the frequency shift on the Ch2 increased as described above. After the injection of 4 mM glycine–NaOH solution, the flow rate was changed to be 20 μL/min for 3 min for regeneration. Then, the flow rate was set as 6 μL/min again, the sensor response was reversed since Crt–BSA deviated from anti–Crt. As the results, the QCM sensor could be regenerated using glycine–NaOH solution.

Calibration curve

The condition for sequential cortisol detection using our system was optimized as discussed previous section. Lower concentration of tracer with large frequency shift could perform high sensitivity as described above. Sample solution was prepared as follows. After the cortisol standard solution was diluted with PBS, the tracer concentration was adjusted to be 2 μg/mL. Analytical curve was obtained as shown in Fig. 7. Error bars show the maximum and minimum values on each concentration (N=5). $|\Delta F_D|$ decreased with increasing the cortisol concentration from 5 to 300 pg/mL. Tracer concentration of 2 μg/mL was equal to 30.3 nM. Cortisol concentration of 30.3 nM was comparable mass concentration of 11.0 ng/mL. On the above condition, relative concentration of cortisol and the tracer was 1:1. However, $|\Delta F_D|$ reached constant on

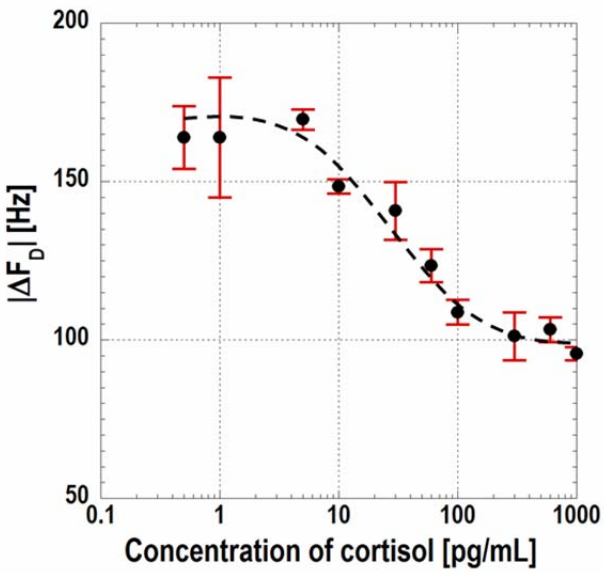

Fig. 7. Calibration curve of cortisol on sequential measurements. Injection volume of the sample and the flow rate were set to be 20 μL and 6 μL/min, respectively. The condition of the sequential measurements was the same as that on Fig. 5.

the cortisol level over 300 pg/mL. The result indicated that binding affinity of cortisol was higher than that of the tracer in this condition. Linear response was obtained from 5 to 100 pg/mL with correction coefficient of 0.94. Detection limit (S/N=3) of our cortisol sensing system was 11 pg/mL. The data was almost equivalent to the detection limit of cortisol detection by SPR.[14] From the view point of cost and process step, our system is superior to the above SPR methods, because our system does not need secondary antibody to achieve high sensitivity and the sensor chip was obtained at a low price. For an example the cost of the tracer per detection was estimated about 0.002 USD in this study.

Conclusions

We demonstrated a sequential label free detection methods for a low molecular weight stress marker, cortisol, by using twin sensor QCM. Anti–Crt was coated on the one channel (Ch2), and the surface preparation was treated on the other channel (Ch1) to avoid nonspecific adsorption. The Ch1 was used as a reference. One detection cycle including regeneration of antigen–antibody interaction can be completed in 10 minutes at the flow rate of 6 μL/min. Difference of the frequency shift between two channels decreased with cortisol concentration from 5 to 300 pg/mL. Linear response was obtained from 5 to 100 pg/mL with correction coefficient of 0.94. These results indicated that our system using twin sensor QCM combined with flow injection showed high sensitivity and high throughput with repeatability. An actual case of cortisol detection is now in action and reported in the near future.

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