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A quartz crystal microbalance-based biosensor for enzymatic detection of hemoglobin A1c in whole blood

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ABSTRACT

Here we present a quartz crystal microbalance (QCM) biosensor for enzymatic detection of hemoglobin A1c (HbA1c). It shows average plasma glucose concentration readings over the prior three months. HbA1c can be quantitatively measured based on changes of resonance frequency of QCM following mass changes on QCM sensor surface. These mass changes were caused by size enlargement of conjugated gold nanoparticle with thiol-terminated SAMs on the sensor surface due to gold staining by hydrogen peroxide (H_2O_2) generated from enzymatic HbA1c assay. Finally, we investigated sensor responses due to mass changes on various concentrations of applied H_2O_2. We also demonstrated its capability for analyzing HbA1c in whole blood sample with enzymatic assay. Our results showed that the proposed QCM biosensor could quantitatively analyze HbA1c with a detection limit of 0.147% HbA1c with respect to hemoglobin and a coefficient variation of less than 10%.

1. Introduction

Hemoglobin A1c (HbA1c) is generated by the binding of glucose to N-terminal valine residue of one or both hemoglobin β-chains [1]. HbA1c is primarily measured as average plasma glucose concentration in three-month because the lifespan of red blood cells in circulation is four months [2]. Thus, measurement of HbA1c level has become an important indicator for the diagnosis and treatment of diabetes [3–8]. Normal HbA1c levels fall within the range of 4–6% [9]. Various methods for measuring HbA1c have been reported, including ion-exchange high-performance liquid chromatography (HPLC), immunoassay, electrophoresis, boronate affinity chromatography, and enzymatic assays. These techniques have been recently reviewed [10–13]. In clinical laboratories, these techniques can provide accurate determination of HbA1c. Among these methods, enzymatic HbA1c assay using fructosyl amino acid oxidase (FAOD) has received increasing attention because this assay is rapid and reproducible [14–16]. Active research is being conducted to improve properties of this enzyme and develop novel FAOD-based detection techniques. FAOD is expected to become a major component of HbA1c detection. Usually, fructosyl amino acid reacts with H_2O and O_2 in the presence of FAOD to generate H_2O_2 as shown in Eq. (1). The concentration of H_2O_2 is proportional to that of HbA1c in the blood [17]. Enzyme peroxidase then catalyzes the reaction between H_2O_2 and chromogen to form an oxidized colored chromogen as shown in Eq. (2). The concentration of chromogen is proportional to the concentration of HbA1c in the sample [18].

$$\text{Fructosyl amino acid} + H_2O + O_2 \rightarrow \text{Amino acid} + \text{glucosone} + H_2O_2 \quad (1)$$

$$H_2O_2 + \text{Chromogen (colorless)} + \text{Peroxidase} \rightarrow H_2O + \text{Oxidized chromogen (color)} + \text{Peroxidase} \quad (2)$$

Colorimetric and electrochemical HbA1c sensing techniques are very popular in clinical laboratory. Although these detection techniques have proven their efficacy in a wide range of applications, they require the use of expensive equipment for measurement. They also require complex and extensive optimization. For these reasons, piezoelectric detection methods, especially quartz crystal microbalance (QCM) based detection, are attractive alternatives for detecting HbA1c. They have become versatile techniques, leading to the successful HbA1c biosensor [19,20]. QCM sensor is a typical piezoelectric sensor. It measures mass change per unit area by measuring changes in frequency of a quartz crystal resonator. QCM

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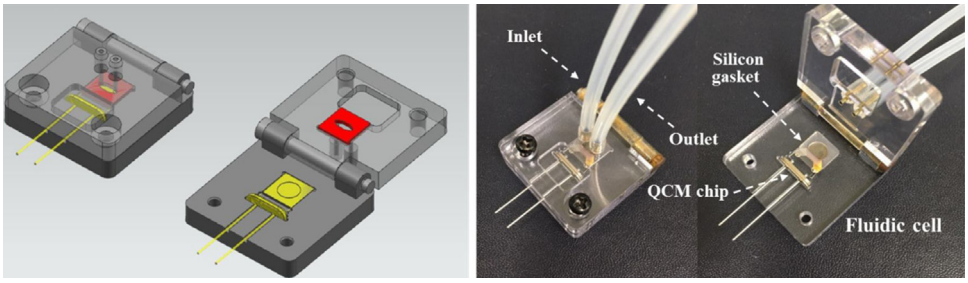

Fig. 1. The custom-made fluidic modules: schematic description (left) and practicality photograph (right).

biosensor has superb sensitivity, speed, and reliability [21–25]. QCM biosensors allow sensitive detection of target analytes in samples. In addition, their simple construction provides experimental simplicity and cost efficiency. Changes of resonance frequency are directly proportional to mass changes, thus enabling real-time detection of biochemical molecules on the sensor surface without labeling requirement. Moreover, QCM biosensors can be used to determine kinetic parameters of affinity interactions between target analytes and biochemical recognition molecules when such interactions are implemented on the sensor surface with selective sensing layer.

In this contribution, we present an innovative quantitative enzymatic assay to detect HbA1c using gold nanoparticle-based QCM biosensor. In this assay, H_2O_2 was generated from proteolytic digestion of glycated hemoglobin with subsequent addition of fructosyl amino acid oxidase (FAOD). It used the same process used for Direct Enzymatic HbA1c Assay™ developed by Diazyme Laboratory. To detect H_2O_2, Direct Enzymatic HbA1c Assay™ uses enzymatic process (horseradish peroxidase catalyzed reaction of a suitable chromogen to an oxidized colored chromogen). However, we used colloidal gold staining process in which H_2O_2 catalyzed the reduction of $HAuCl_4$ to Au (0) which was stacked on the surface of colloidal gold added in advance. (Eq. (3))

$$2HAuCl_4 + 3\,H_2O_2 + AuNP \rightarrow 8HCl + 3O_2 + \text{Size-enhanced AuNP (by produced 2Au)} \quad (3)$$

For H_2O_2 detection, our process might be no less simple than Direct Enzymatic HbA1c Assay™ because we don't use enzyme or unique long wavelength chromogen (>700 nm) to avoid absorption interferences from hemoglobin. We only use $HAuCl_4$ and homemade gold nanoparticles. In the aspect of detection novelty and sensitivity, the size enhancement approach described in this study is novel method for the detection of HbA1c. In addition, the high sensitivity of our QCM biosensor for detecting HbA1c comes from the signal amplification strategy through size enhancement of gold nanoparticles by colloidal gold staining chemistry.

Meanwhile, we measured changes of frequency by mass loading effect from size enlargement of gold nanoparticles. This reaction was run on QCM sensor surface. In this study, gold nanoparticles were captured on the sensor surface through direct binding with gold-thiol self-assembled monolayers (SAMs) formation. Formation of thiol SAMs and conjugation of gold nanoparticle on the sensor surface was discussed. We demonstrated the dependence of sensor response on H_2O_2 concentration and quantitative detection of HbA1c in whole blood sample.

2. Materials and methods

2.1. Reagents and materials

All chemicals used in the synthesis of gold nanoparticles, including gold (III) chloride trihydrate (chloroauric acid, $HAuCl_4$) and sodium citrate, were of analytical grade. They were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). (3-Mercaptopropyl)-trimethoxysilane (3-MPTMS), protease from *Aspergillus oryzae*, and fructosyl-amino acid oxidase (FAOD) from *Corynebacterium* sp. were also purchased from Sigma-Aldrich. An HbA1c linearity set was purchased from Bio-Rad Co. (Hercules, CA, USA). PBS buffer was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Ethyl alcohol (99.5%) and other organic solvents were purchased from Samchun Pure Chemical Co. Ltd. (Seoul, South Korea). All aqueous solutions were prepared in deionized (DI) water which was obtained from a Milli-Q water-purifying system (18 MΩ cm).

2.2. Apparatus and measurement system

A QCM sensing system was comprised of fluidic and detection modules to obtain real-time sensor information during assay. Mass loading on silicon dioxide coated QCM resonators (5 mm in diameter; 9 MHz resonance frequency) was monitored by tracking resonance change of bulk acoustic waves at central frequency of 9 MHz with a Princeton Applied Research QCM922A (SEIKO EG&G, Tokyo, Japan). Flow cell was constructed with a peristaltic pump (ISM597; ISMATEC, Glattbrugg, Switzerland), a custom-made fluidic block, and a silicone rubber gasket. Accurate fluidic control is one of important factors for reproducible and user-friendly detection of biomolecules in liquid media. Therefore, we developed fluidic modules for our QCM sensor as dipicted in Fig. 1. The top piece contained recessed regions for reaction chambers and silicone rubber gaskets to prevent liquid leakage due to hydrodynamic pressure. It had fluidic connectors to permit flow across QCM devices. Sample flow and buffer solution flow to reaction chambers in the fluidic block were actuated by peristaltic pump. The flow rate was kept at 1.0 ml/min. The volume of each reaction chamber was 20 μL. After each run, reaction chambers and silicone rubber gaskets were thoroughly rinsed with DI water and 0.05% Tween 20 (Sigma-Aldrich, MO, USA) in PBS solution. Teflon® tubing (0.032 in. I.D., The Lee Company, CT, USA) was used to connect fluidic and detection modules together.

2.3. Sensor surface preparation

Silicon dioxide (SiO_2)-coated QCM sensor chip (SEIKO EG&G, Tokyo, Japan) was sequentially rinsed with deionized water and absolute ethanol followed by drying under nitrogen gas. In this QCM sensor chip, silicon dioxide layer was coated on the top of gold electrodes to prevent non-specific gold staining that might occur at the surface of gold electrodes. The QCM sensor chip was then placed in a UV/ozone chamber (144AX-220; Jelight Company, Inc., Irvine, CA) for 10 min. It was then incubated in a solution of 5% (vol./vol.) freshly prepared 3-MPTMS in methanol for 1 h followed by rinsing with methanol for 2 min and drying under nitrogen gas. The silanized sensor was then baked in an oven at 110 °C for 1 h followed by rinsing with methanol for 2 min and drying under nitrogen gas.