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Development of a screen-printed carbon electrode based disposable enzyme sensor strip for the measurement of glycated albumin

Mika Hatada [a], Wakako Tsugawa [a,b], Eri Kamio [a], Noya Loew [b], David C. Klonoff [b,c], Koji Sode [a,b,*]

[a] Department of Biotechnology and Life Science, Graduate School of Engineering, Tokyo University of Agriculture and Technology, 2-24-16 Naka-cho, Koganei, Tokyo, 184-8588 Japan
[b] Institute of Global Innovation Research, Tokyo University of Agriculture and Technology, 2-24-16 Naka-cho, Koganei, Tokyo, 184-8588 Japan
[c] Diabetes Research Institute, Mills-Peninsula Health Services, 100 South San Mateo Drive, San Mateo, CA, 94401 USA

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ABSTRACT

Glycated proteins, such as glycated hemoglobin (HbA1c) or glycated albumin (GA) in the blood, are essential indicators of glycemic control for diabetes mellitus. Since GA, compared to HbA1c, is more sensitive to short term changes in glycemic levels, GA is expected to be used as an alternative or together with HbA1c as a surrogate marker indicator for glycemic control. In this paper we report the development of a sensing system for measuring GA by combining an enzyme analysis method, which is already used in clinical practice, with electrochemical principles. We used fructosyl amino acid oxidase, hexaammineruthenium(III) chloride as the electron mediator, and an inexpensive and economically attractive screen-printed carbon electrode. We used chronoamperometry to measure protease-digested GA samples. The developed sensor strips were able to measure protease-digested samples containing GA in very small sample volumes (1.3 μL) within about 1 min. We also prepared enzyme sensor strips suitable for clinical use in which the enzyme and the mediator were deposited and dried on. This sensor system showed a clear correlation between the GA concentration and the resulting current. The strips were stable following 3 months of storage at 37 °C. We conclude that this disposable enzyme sensor strip system for measuring GA is suitable for point-of-care test (POCT) applications.

1. Introduction

Diabetes mellitus is a common disease. It is very important to maintain the blood glucose level of patients with diabetes at a normal level to avoid long-term complications. Circulating glycated proteins, such as glycated hemoglobin, which is also known as hemoglobin A1c (HbA1c) and glycated albumin (GA), are important indicators of mean glycemic control for diabetes mellitus. Glycated proteins are the product of a non-enzymatic reaction between glucose and various circulating blood proteins. HbA1c originates from the glycation of the β subunit N-terminal valine of hemoglobin (which resides intracellularly within erythrocytes) and reflects the mean blood glucose level over the prior 2–3 months (Bunn et al., 1978; Goldstein et al., 2004; Franco, 2012). GA primarily originates from the glycation of the ε-amino group of internal lysine residues of circulating albumin in plasma and reflects the average blood glucose level over the prior 2–3 weeks (Guthrow et al., 1979; Andersen et al., 2014). GA reflects a shorter timeframe than HbA1c because erythrocytes have a lifespan of over three months whereas albumin has a half-life of three weeks. Just as HbA1c is expressed as a percentage of the total hemoglobin molecules that are glycated, GA is usually quantified as the proportion of the GA concentration divided by the total albumin concentration. GA values for normal individuals range from 11 to 16% (Japan Diabetes Society, 2014).

HbA1c is the standard accepted indicator of glycemic control because sufficient evidence has been established to support the relationship of HbA1c and diabetes-associated complications (The Diabetes Control and Complications Trial Research Group, 1993; Stratton et al., 2000). Although HbA1c is the world's most widely used marker of mean glycemia, GA has two advantages over HbA1c. First, GA reflects glycemic status over a shorter period than HbA1c which makes it a better metric for monitoring mean glycemia when a patient's condition is rapidly changing. Second, HbA1c may not accurately reflect glycemic status in the presence

* Corresponding author at: Department of Biotechnology and Life Science, Graduate School of Engineering, Tokyo University of Agriculture and Technology, 2-24-16 Naka-cho, Koganei, Tokyo 184-8588, Japan.
E-mail address: sode@cc.tuat.ac.jp (K. Sode).

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of states with altered erythrocyte life span or genetic variations in the structure of the hemoglobin molecule. Specifically, disease processes associated with reduced red blood cell formation, such as iron and vitamin B12 deficiencies, can elevate HbA1c levels, whereas conditions with increased hemolysis and replacement of older erythrocytes with young erythrocytes can lower HbA1c levels (Hare and Shaw, 2016). GA levels, however, are unaffected by all these conditions affecting hemoglobin production (Koga, 2014). Recent studies have demonstrated that a GA assay can be used in evaluating mean glycemic status and predicting a diabetic complication similar to an HbA1c assay (Ueda and Matsumoto, 2015). GA is expected to be increasingly used as an adjunct or alternative to HbA1c for estimating glycemic control.

Clinical analyses of GA have been conducted using boronate affinity methods, ion-exchange high-performance liquid-chromatography (HPLC), or immunoassay. However, within the current principles of GA clinical analyses, only the enzymatic method is used, which is the enzyme reagent for autoanalyzers commercialized by Asahi-Kasei Pharma (Tokyo, Japan) as Lucica® GA-L used during central laboratory testing (Kohzuma and Koga, 2010). The principle of enzymatic analysis for GA measurement is detailed below. GA is digested by protease, and ε-fructocyl lysine (ε-FK) is released. Ketoamine oxidase, also known as fructosyl amino acid oxidase (FAOx), which catalyzes the oxidation of glycated amino acid, is then used, and hydrogen peroxide is produced. Subsequently, the liberated hydrogen peroxide is measured in the presence of peroxidase, *N, N*-Bis(4-sulfobutyl)-3-methylaniline, disodium salt (TODB), and 4-aminoantipyrine (Kouzuma et al., 2002). This enzymatic assay is optimized to formulate reagents which are ready to use in conventional autoanalyzers. FAOxs- or fructosyl peptide oxidase (FPOxs)-based enzymatic assay systems are now commercially available for both GA and HbA1c clinical analyses (Ferri et al., 2009; Sakurabayashi et al., 2003; Hirokawa et al., 2004; Kohzuma and Koga, 2010; Abidin et al., 2013; Yonehara et al., 2015). There is an expectation that GA measurement will be increasingly used to assess glycemic control in: 1) hospitalized patients; 2) outpatients whose control is rapidly changing; or 3) patients whose hemoglobin formation is altered (Koga, 2014). Therefore, the development of accurate GA assays, which are suitable for point-of-care testing (POCT), is expected.

Electrochemical sensing systems are widely applied to enzyme-based POCT. A representative example is the multitude of commercial meter/strip systems for self-monitoring of blood glucose (SMBG) that are currently available in the global market. They are generally accurate and easy to use. We expect that electrochemical principles and enzymatic methods can be integrated into well designed monitoring systems for measuring GA, particularly those employing disposable sensor strips. Several studies on the development of electrochemical enzyme sensors using FAOx focusing on the measurement of glycated protein have been reported (Tsugawa et al., 2000, 2001; Ogawa et al., 2002; Sakaguchi et al., 2003; Nanjo et al., 2007; Fang et al., 2009; Chawla and Pundir, 2011, 2012; Jain and Chauhan, 2016). However, to the best of our knowledge, none of these studies have reported on disposable enzyme sensor strips for measuring GA in a POCT platform.

In this study, we developed a screen-printed carbon electrode-(SPCE-) based disposable electrochemical enzyme sensor strip for the measurement of GA using FAOx, and hexaammineruthenium (III) chloride (Ru complex) as the electron mediator. The measurement principle in the present study is based on the commercially available GA measurement kit (Lucica® GA-L), as detailed below. GA in the blood is digested by a protease to release ε-fructosyl lysine (ε-FK), following which ε-FK is oxidized by FAOx and the Ru-complex is simultaneously reduced. The amount of reduced Ru-complex formed is measured by chronoamperometry with a SPCE, where the enzyme and electron mediator are deposited. The ε-FK concentration contained in protease-digested GA is determined by the concentration of generated reduced mediator (Fig. 1). We evaluated the performance of this new enzymatic electrochemical-based GA sensor, which can be developed in a POCT platform.

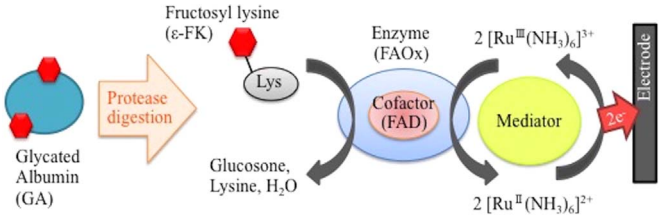

Fig. 1. The principle of GA measurement. The principle of GA measurement was based on the commercially available GA measurement kit: GA in the blood is digested by protease to release ε-fructosyl lysine (ε-FK), following which ε-FK is oxidized by FAOx and Ru-complex is simultaneously reduced. The amount of reduced Ru-complex formed is measured by chronoamperometry with SPCE.

2. Materials and methods

2.1. Materials

FAOx was prepared following the method by Kouzuma (Kouzuma et al., 2002). Recombinant human serum albumin was purchased from Merck (Darmstadt, Germany) and its glycated form was prepared as below. Twenty-five grams of recombinant human serum albumin and 80 g of D-glucose (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were dissolved in 500 mL of pure water and incubated for 24 h at 37 °C. After that, the mixture was dialyzed against pure water for 3 h for 5 times and then ultrafiltrated (4 °C, 3500 rpm, 20 min). The glycated albumin (GA) concentration and the albumin concentration of the prepared sample were determined using Lucica® GA-L (Asahi-Kasei Pharma, Tokyo, Japan), as a reference method. Then, two samples of GA with a ratio of GA to total albumin of either 15% or 30% and absolute GA concentrations of 0.741 g/dL and 1.442 g/dL, respectively, were prepared. Proteinase (protease type X X VUU) was obtained from Sigma-Aldrich (St. Louis, MO). N^{α}-carbobenzyloxy-N^{ε}-fructosyllysine (*Z*-FK) was prepared following the method by Hashiba (Hashiba, 1976). Hexaammineruthenium(III) chloride (Ru complex) was purchased from Sigma-Aldrich. Screen-printed carbon electrodes (SPCEs, WE: Carbon, 2.4 mm^2, RE: Ag/AgCl, CE: Carbon) were kindly supplied by i-SENS (Seoul, Republic of Korea). All electrochemical experiments were performed with a HSV-100 potentiostat (Hokuto Denko Co., Tokyo, Japan).

2.2. Electrochemical measurement of fructosyl lysine or glycated albumin

A solution of 1.3 μL containing various Z-FK concentrations, 60 U/mL of FAOx, and 300 mM of Ru complex in 100 mM potassium phosphate buffer (P.P.B.) (pH 8.0) was absorbed into the spacer layer (120 μm thick) of the SPCE. After incubation for 1 min at room temperature, a potential of +100 mV vs. Ag/AgCl was applied and the current was monitored. The Ru complex concentration of 300 mM was sufficiently high not to limit the sequential enzyme reaction of oxidation of the substrate by FAOx, and subsequent reduction of the mediator (Supplementary material S1). We used +100 mV vs. Ag/AgCl as the suitable potential for the oxidation of the reduced Ru complex (Supplementary material S2).

The measurement of GA was conducted as detailed below. The mixture of prepared GA samples with ratios of GA to total albumin