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# Direct detection for concentration ratio of HbA1c to total hemoglobin by using potentiometric immunosensor with simple process of denaturing HbA1c

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ABSTRACT

A direct measurement method for HbA1c (%), which is the concentration ratio of HbA1c to total hemoglobin (Hb), was investigate with a potentiometric immunosensor. The direct measurement method is based on sandwich immunoassay that combines anti-Hb and enzyme-labeled anti-HbA1c antibodies. While the anti-Hb antibodies capture both Hb and HbA1c maintaining HbA1c (%) in blood sample, the enzyme-labeled anti-HbA1c antibodies bind only to HbA1c in the total captured Hb. Though we tried the direct detection of HbA1c (%) with a combination of four anti-Hb antibodies and two anti-HbA1c antibodies, the obtained result showed lower sensitivity and reproducibility. We presumed that the binding site of HbA1c for anti-HbA1c antibody hides inside the folding structure of HbA1c and anti-HbA1c antibody is not able to bind to HbA1c efficiently resulting in lower sensitivity. As a result of examination of both the type and concentration of the surfactant, it was found that the process of denaturing HbA1c with 0.2% dodecyltrimethylammonium bromide was added to enable it to be accessible to anti-HbA1c antibodies and could enhance the sensitivity. By adding the simple process of denaturing HbA1c, the direct measurement of HbA1c (%) was successfully performed with this immunosensor. The results showed a good correlation (correlation efficiency, $r^2 = 0.975$) between the certified and experimental values of HbA1c (%) in the clinically relevant range (from 5.6% to 10.6%).



## 1. Introduction

HbA1c, which is hemoglobin (Hb) glycated by glucose in blood, reflects the average blood glucose level of 1–2 months, so it is considered the gold standard for determining the treatment of diabetes. To prevent worsening of diabetes, monitoring of HbA1c levels for patients in the diabetes mellitus preliminary group is important. Point-of-care devices for HbA1c will help on-site disease management not only at large hospitals that have clinical laboratories but also at small hospitals. HbA1c (%), which is the concentration ratio of HbA1c to total Hb, is determined by a high-performance liquid chromatography (HPLC) using ion exchange or affinity columns to separate HbA1c from other Hb [1,2], and has been widely used as the HbA1c indicator. The HLPC used as a standard method can determine HbA1c (%) directly, but it requires large and expensive instruments.

To date, antibody-based immunoassay and enzyme-based assay methods that do not require large instruments have been increasingly developed. An electrochemical detection of HbA1c using flow immunoassay system was reported by Tanaka *et al.* [3]. In this method, HbA1c reacted with anti-HbA1c antibodies modified with ferrocene monocarboxylic acid, was separated by boronate-affinity chromatography, and then electrochemically detected. An enzyme-based method for detecting HbA1c using fructosyl peptide oxidase was reported by Hirokawa *et al.* [4]. These methods do not require large instruments and are also inexpensive. However, HbA1c (%) cannot be obtained using only these methods. To obtain HbA1c (%), a separate measurement of total Hb in blood and a calculation of the ratio of HbA1c to total Hb is required.

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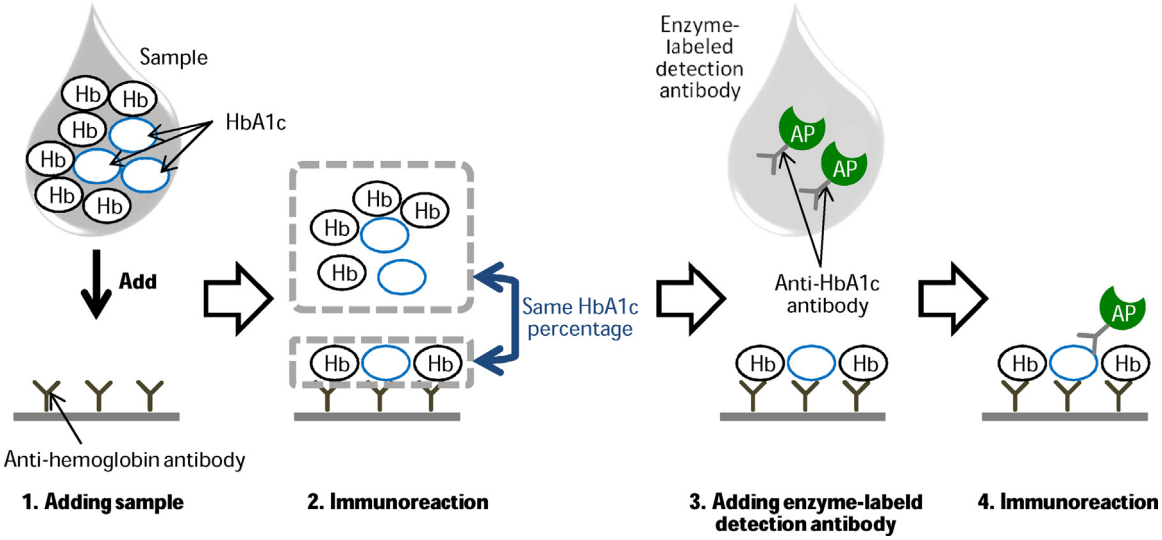


**Fig. 1.** Procedure for direct detection of ratio of HbA1c to total hemoglobin.

An immunoassay using the direct adsorption method without any capture molecule was developed for directly measuring HbA1c (%) by Engbaeck *et al.* [5]. The direct adsorption method is susceptible to other protein in blood and can causes lower reproducibility and sensitivity. To solve this problem, an immunosensor using membrane-immobilized haptoglobin as an affinity matrix to capture Hb and HbA1c was developed to bind Hb selectively from blood samples [6]. The haptoglobin-based sandwich immunoassay demonstrated a better reproducibility and higher sensitivity than the anti-Hb antibody-based assay. The main reason is that the complex formation of HbA1c with haptoglobin will cause a partial unfolding of the HbA1c molecule. This strongly suggests that it is necessary to denature HbA1c to enhance the interaction between anti-HbA1c antibodies and HbA1c.

In this study, we focused on a simple sample pretreatment of a blood sample to enhance the sensitivity of HbA1c (%) detection using a conventional sandwich immunoassay with both anti-Hb and anti-HbA1c antibodies. Direct detection of HbA1c (%) in the clinically relevant range was performed by this immunoassay method with a disposable potentiometric immunosensor.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Screen-printing carbon electrodes on a polyethylene terephthalate (PET) film was made by Advanced U-corporation (Gunma, Japan). Pasting graphite powder was purchased from BAS (Tokyo, Japan). Lyophilized streptavidin powder was purchased from Sigma-Aldrich (MO, USA). Monoclonal anti-Hb capture antibodies (Clone M1709Hg1, M1709Hg2, M1202099, M1202100) were purchased from Fitzgerald (MA, USA), and monoclonal anti-HbA1c detection antibodies (Clone B842 M, 75C9) were purchased from Abcam (Cambridge, UK). Biotin labeling kit and alkaline phosphatase labeling kit were purchased from Dojindo Molecular Technologies (Kumamoto, Japan). Certified reference material (JCCRM423) of HbA1c was obtained from the Health Care Technology Foundation (Kanagawa, Japan). Dodecyltrimethylammonium bromide (DTAB) was obtained from Wako Pure Chemical Industries (Osaka, Japan). Milk protein solution was purchased from Kirkegaard & Perry Laboratories (MD, USA).

### 2.2. Fabrication of disposable immunosensor

The disposable cartridge potentiometric immunosensor was made of a PET film with screen-printed carbon electrodes and PET-EVA films. Antibodies capturing target molecules were immobilized on the surface of the flow channel as follows. The flow channel was incubated with 1 mg/ml streptavidin solution in carbonate buffer at room temperature for 1 h, and washed with 1 ml Tris-buffered Saline with Tween 20 (TBST). Twenty μg/ml of biotinylated anti-Hb capture antibodies were added to the flow channel and incubated at room temperature for 1 h. The number of molecules of the added biotin-labeled anti-Hb antibody is sufficiently smaller than the number of molecules of streptavidin adsorbed on the flow path. Since the affinity of streptavidin and biotin is high ($K_D = 10^{-15}$ mol/l), all of the added biotin-labeled anti-Hb antibody is captured on the surface of the flow path. From the above, it is possible to control the number of molecules of the anti-Hb antibody immobilized on the channel surface by controlling the concentration of the biotin-labeled anti-Hb antibody to be added. The flow channel was washed with 1 ml TBST again. The flow channel was then blocked with milk protein solution at room temperature for 1 h, and washed with 1 ml TBST.

### 2.3. Immunoassay (Measurement procedure)

The proposed HbA1c (%) detection method is based on a sandwich immunoassay combining anti-Hb and anti-HbA1c antibodies (Fig. 1). First, the certified reference material (JCCRM 423) used as a sample is diluted 50 times with 0.2% DTAB. The pretreated solution is added to capture antibodies immobilized on the surface. The sample contains an excess number of Hb and HbA1c against the number of anti-Hb antibodies. The anti-Hb antibodies can capture a certain portion of Hb maintaining HbA1c (%) in the sample. Next, 20 μg/ml enzyme-labeled anti-HbA1c antibodies are added. Finally, enzyme activity is measured by potentiometric measurement after adding 2.5 mM ascorbic acid phosphate (AsA-P) into a borate buffer (100 mM $H_3BO_3$, 200 mM NaCl, 1 mM $MgCl_2$, 4.95 mM ferricyanide, 0.05 mM ferrocyanide, pH9.5). The potentiometric measurement system was previously described [7,8]. Calibration of the experimental value (HbA1c (%)) is performed using the output potential data in volts of one sample with certified HbA1c (%) of 5.61%.