

Integrated Microfluidic Device for Glycated Hemoglobin (HbA1c) Testing Using Light-gas Generation Pump

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The incidence of diabetes mellitus is rapidly increasing in developed and developing countries. Point-of-care testing (POCT) is a useful tool in developing countries with a poorly developed infrastructure. Glycated hemoglobin (HbA1c) is a reliable indicator for diabetes mellitus testing, and POCT devices for HbA1c have been developed. In this study, we developed a microfluidic channel device for quantifying HbA1c that is small and easy to measure on the basis of the latex aggregation method. Microfluidic channels were integrated into the front and back surfaces of a polymethyl methacrylate substrate of 40 mm width × 60 mm length × 1 mm thickness. The front side was composed of a main channel, including a mixing channel. The reverse side featured a channel for funneling the gas (nitrogen) generated from the light-gas generation pumps. In these pumps, the azide compound was decomposed by light irradiation and nitrogen gas was generated. The generated nitrogen gas enabled liquid transfer. The optical system consisted of an LED (660 nm) and a photodetector. Its sensitivity was improved by optimizing the aperture size. This system can quantify HbA1c, and at HbA1c concentrations of 0, 4.4, 9.6, and 13.9%, the absorbance values were 0, 0.11, 0.28, and 0.39, respectively. A linear relationship between concentration and absorbance was observed with a determination coefficient of 0.99, a standard deviation (SD) of 0.009–0.015, and a coefficient of variation (CV) of 2.4–9.3%. The concentration of HbA1c in whole blood could also be determined on the basis of the calibration curve obtained. This system is expected to be useful for HbA1c testing in remote areas and clinics.

1. Introduction

Diabetes mellitus is a major public health problem, affecting people of all ages worldwide in developed and developing countries. It is associated with increased mortality and morbidity and a significant reduction in the quality of life, especially in industrialized countries, where risk factors such as obesity and unhealthy eating habits are more common.⁽¹⁾ Current treatment modalities include long-term blood glucose monitoring and control, special diabetic dietary management, and the use of exogenous insulin, metformin, and other drugs.^(2,3) Glycated

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hemoglobin (HbA1c) is widely used as an excellent indicator for monitoring long-term glycemic balance in diabetics. It is also used to identify patients with poor glycemic control and to accurately determine adjustments necessary in therapy.⁽⁴⁾

Methods for measuring HbA1c can be broadly divided into two types: separation methods and chemical methods. In separation methods, HbA1c and nonglycated Hb have different chemical properties, which enables the separation of their fractions and the quantification of HbA1c. This principle is applied in ion exchange chromatography,⁽⁵⁾ capillary electrophoresis,⁽⁶⁾ and affinity chromatography.⁽⁷⁾ In chemical methods, the HbA1c concentration is measured via a specific chemical reaction at the glycated N-terminal valine of the β -chain of HbA1c. This principle is applied in latex aggregation,⁽⁸⁾ chemical luminescence,⁽⁹⁾ and the chromogenic method.⁽¹⁰⁾ Latex aggregation has the advantage of being less affected by Hb types other than HbA1c owing to its use of highly specific antibodies. Furthermore, since the measurement is based on turbidity, the configuration of the device can be simplified.

Until recently, HbA1c testing has required a healthcare professional to draw a venous blood sample and send it to an accredited biochemistry laboratory for analysis. The equipment used to accurately measure HbA1c is expensive and requires specific training, limiting access to testing and reducing the speed of reporting. Cheaper point-of-care testing (POCT) devices for HbA1c measurement have become available, allowing the widespread and rapid assessment of this useful clinical marker to determine the progression and severity of diabetes.⁽¹¹⁾ Although POCT-HbA1c devices are sometimes considered to have inferior sensitivity and accuracy to automatic analyzers, they have met National Glycohemoglobin Standardization Program (NGSP) and National Academy of Clinical Biochemistry (NACB) standards.⁽¹²⁾

POCT-HbA1c testing devices have been developed and used in the clinic, such as Afinion HbA1c (Abbott, USA)⁽¹³⁾ and HbA1c I Gear S (Sanwa Kagaku Kenkyusho Co., Japan).⁽¹⁴⁾ Cartridges contain a sample-collecting slot and the necessary reagents, enabling the automation of rapid quantitative analysis using whole blood samples. HbA1c-measuring devices based on micro-total-analysis system technology include B-Analyst (Menarini Diagnostics, Italy)⁽¹⁵⁾ and COBAS 101 (Roche Diagnostics K.K., Switzerland).⁽¹⁶⁾ These devices use rotational force to transfer liquid and a microchannel structure to mix reagents and whole blood. However, they have moving parts, making them susceptible to failure. In general, fewer moving parts in a device reduce the risk of failure.

In a microfluidic device, a reagent and a blood sample are generally mixed using serpentine channels.⁽¹⁷⁾ In these channels, mixing is performed using the speed difference between the inside and outside of the corner, so several structures are required to achieve sufficient mixing, and the miniaturization of the device is an issue. In addition, it is difficult to mix droplets before and after using only the serpentine channels. Therefore, this device uses a semicircular repeating shape for the mixing channels to increase mixing efficiency and reduce size. In addition, we created a liquid pump using a method based on light-gas generation tape, which is made of a material that decomposes and generates gas upon light irradiation.⁽¹⁸⁾ The main compound of light was azide. Azide was decomposed by light and generated nitrogen gas. Furthermore, an adhesive film can be produced by mixing this compound with an acrylic polymer. On the other hand, microfluidic devices can be miniaturized by integrating functions, but are difficult to

miniaturize because liquid is pumped from outside. Our pump system is composed of light-gas generation tape and LEDs, and it is possible to miniaturize the device. This tape was not cytotoxic, and cell adhesion was possible on this surface. After the cells had grown sufficiently, light irradiation generated gas between the cells and the tape, allowing the cells to be detached.⁽¹⁹⁾ Then, it was possible to detach only one cell by narrowing down the irradiation light.^(20,21)

In this paper, we describe the development of an integrated microfluidic device for HbA1c measurement. We designed the device using the latex aggregation method, miniaturized it using a mixing channel and a gas-generating tape, and verified its quantitative performance. Furthermore, the obtained calibration curve was used to determine the concentration of HbA1c using whole blood.

2. Materials and Methods

2.1 Materials

Standard HbA1c solution (Metabolead calibrator HbA1c) and latex aggregation reagent (Metabolead HbA1c kit) were purchased from Minaris Medical Co., Ltd. (Japan). Pure water was purchased from FUJIFILM Wako Chemicals (Japan). The polymethyl methacrylate (PMMA, ACRYLITE) substrate of the device was purchased from Mitsubishi Chemical Group Corporation (Japan). The polycarbonate (PC, NOVAREXM) substrate of the device was purchased from Mitsubishi Gas Chemical Trading, Inc. (Japan). Red food coloring was purchased from Kyoritsu Foods, Inc. (Japan).

2.2 Microfluidic channel device and instrumentation

The microfluidic channel pattern was designed using CAD software (TurboCAD, Canon IT Solutions Inc., Japan) and fabricated by Adtec Engineering Co., Ltd. (Japan) on a PMMA substrate of 40 mm width × 60 mm length × 1 mm thickness. The main microfluidic channel was 1 mm wide and 0.8 mm deep. The mixed structure consisted of a repeating semicircular shape with a diameter of 5 mm and a depth of 0.8 mm. The detection part used a 0.8-mm-diameter hole that penetrated the top and bottom of the substrate. The microchannel was subjected to hydrophilic treatment using an atmospheric pressure corona (AGI-020, Kasuga Denki, Inc., Japan.). The corona surface treatment was performed at an output of 0.26 kW for 20 s. The front surface of the microchannel was covered with sealing tape (9793, 3M, USA), and the back surface was covered with gas-generating tape manufactured by Sekisui Chemical Co., Ltd. (Japan).⁽¹⁸⁾

The HbA1c detection system consisted of an optical system and a light irradiation system. The optical system consisted of a 660 nm LED (Hamamatsu Photonics K.K., Japan), a photodetector (C1339801-01, Hamamatsu), a control board circuit (C13390, Hamamatsu), and an anodized aluminum stage with a depression that matches the size of the device. A 660 nm LED was lighted at a forward current of 20 mA. The absorbance was calculated using Eq. (1), where I_0 is the value for water and I is the measured value for the reaction solution. I_0 and I were measured using the photodetector.

$$A = -\log_{10} \frac{I}{I_0} \quad (1)$$

The LED irradiation system consisted of UV-LEDs (NS395L-5RFS, Nitride Semiconductors Co., Ltd., Japan), an LED driver (AZ-50, Sekisui), and control software (LIMPS ver.1.0, Sekisui).

2.3 Evaluation of suppressed latex adsorption on substrate surface

The hydrophilicities of the corona-treated PC and PMMA substrates were evaluated with a contact angle measuring instrument (DMs-401, Kyowa Interface Science Co., Ltd., Japan) using pure water. A 1 μ L droplet was released onto the surface of a sample under atmospheric conditions at room temperature. After corona treatment, 3 μ L of latex solution was left on the corona-treated substrate for 5 min, the solution was collected, and absorbance was measured with a NanoDrop 2000c absorbance-measuring device (Thermo Fisher Scientific Inc., USA).

2.4 Standard curve of HbA1c for microfluidic device

The HbA1c used for the calibration curve utilized a standard HbA1c solution. The volume of HbA1c used was 1.2 ml, with HbA1c concentrations of 4.4, 9.6, and 13.9%. The volumes of water (R0), latex solution (R1), and antibody solution (R2) used in the measurement were 20, 30, and 15 μ L, respectively. After the HbA1c solution was in contact with the water, it was allowed to stand for 0.5 min. The diluted HbA1c and latex solution passed through the mixing channels in 0.5 min, and then was allowed to stand for 2 min. In addition, these solutions and the antibody solution passed through the mixing channels for 0.5 min, and absorbance was measured 2 min after the reaction solution had entered the detection area. The absorbance was calculated using Eq. (1). The measurement environment was at room temperature (25 °C).

3. Results and Discussion

3.1 HbA1c microfluidic device and system

The principle of HbA1c measurement using latex aggregation is shown in Fig. 1. The first reaction adsorbs all the hemoglobin in HbA1c onto the surfaces of the latex particles. In the second reaction, the HbA1c adsorbed onto the latex surface reacts with the monoclonal antibody to form a latex complex. The number of latex complexes is proportional to the amount of solid-phase HbA1c aggregation on the latex particle surface.

The design of our microfluidic device made specifically for HbA1c detection is presented in Fig. 2. This device was composed of sealing tapes, a microchannel plate, and a light-gas generation tape. The plastic substrate had microfluidic channels on the front and reverse sides and consisted of four reagent solution areas (sample, water, latex, and antibody solution), three mixing areas, a detection area on the front side, and flow paths for the gas on the reverse side. The device surface was made hydrophilic by corona treatment.

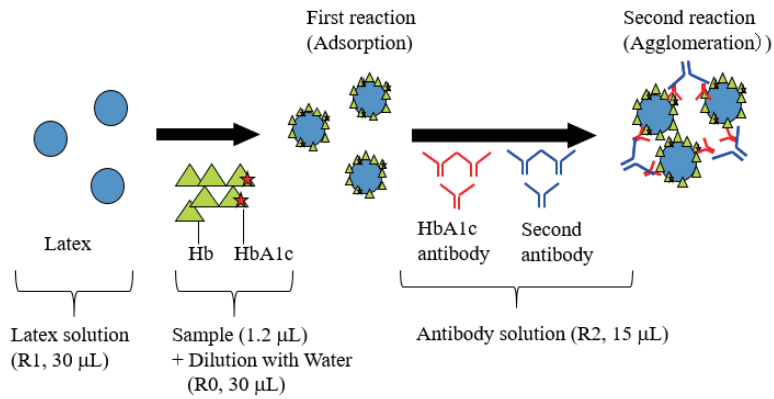


Fig. 1. (Color online) Principle of HbA1c measurement using latex aggregation.

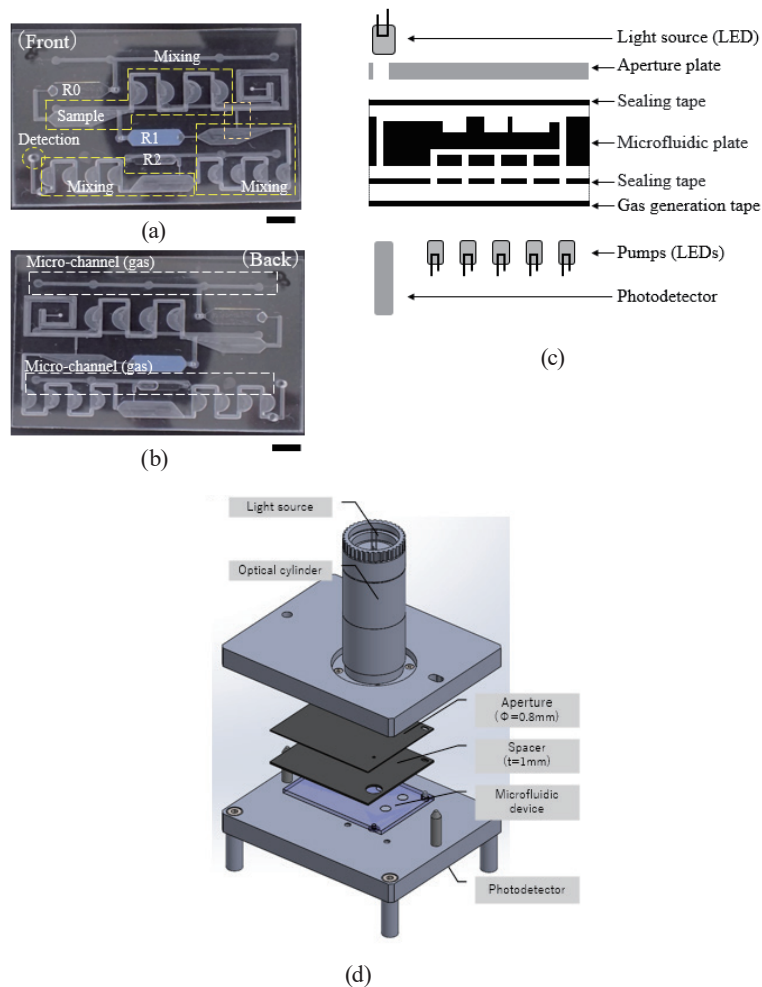


Fig. 2. (Color online) (a) Photograph of front side of the HbA1c device. (R0: water; R1: latex solution; R2: antibody solution). (b) Photograph of reverse side of the HbA1c device. (c) Schematic of the layers of the HbA1c system within the testing device, the pumps (gas generation tape and LEDs), and the optical system. (d) Schematic of the optical system. Scale bar: 5 mm.

3.2 Corona treatment of HbA1c device

Table 1 presents the water contact angles of PMMA and PC obtained before and after corona treatment. After corona treatment, PMMA and PC had similar contact angles. The water contact angles of PMMA and PC were $76 \pm 0.99^\circ$ and $100 \pm 1.1^\circ$ before corona treatment and $46 \pm 1.3^\circ$ and $53 \pm 0.92^\circ$ after the treatment, respectively. Corona treatment lowered the contact angle to around 50 degrees on the PMMA and PC substrates.

Next, the corona-treated substrates were evaluated for suppressed latex adsorption (Fig. 3). The absorbance of the reference latex solution was 0.073 ± 0.0001 . The absorbance values obtained before and after corona treatment on the PMMA substrate were 0.064 ± 0.0001 and 0.072 ± 0.0001 , respectively. From the absorbance of the latex solution, we found that 88% of the latex remained in the solution and 12% was adsorbed onto the PMMA substrate before the corona treatment of the PMMA substrate, whereas 97% of the latex remained in the solution and 3% was adsorbed onto the PMMA substrate after corona treatment. Then, the absorbance values obtained before and after the corona treatment of the PC substrate were 0.065 ± 0.0001 and 0.073 ± 0.0001 , respectively. The proportions of latex remaining in the solution and adsorbed on the PC substrate before and after corona treatment were the same as those for the PMMA substrate. Namely, the effect of corona treatment on suppressing latex adsorption was the same for both the PC and PMMA substrates. Since PMMA can be more easily milled than PC, we developed the device using PMMA.

Table 1
Water contact angles of PMMA and PC ($n = 3$).

Substrate	PMMA	PC
Pre-corona	$76^\circ \pm 0.99^\circ$	$100^\circ \pm 1.1^\circ$
Corona	$46^\circ \pm 1.3^\circ$	$53^\circ \pm 0.92^\circ$

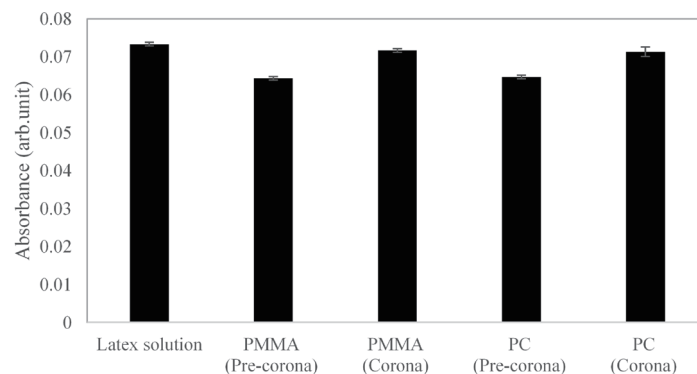


Fig. 3. Absorbance of latex that did not adsorb onto the substrate before and after corona treatment. The measurements were performed using a NanoDrop 2000c absorbance-measuring device.

3.3 Design of the mixing structure of the HbA1c device

A schematic diagram of the mixing channel is shown in Fig. 4. The basic mixing structure was a straight channel of 0.8 mm width and 5 mm length combined with a semicircular structure of 5 mm diameter, which was repeated to form this mixing channel. The volume of the solution to be mixed should be equal to or greater than that of the basic mixing structure. For example, the least amount of solution used was in the HbA1c dilution process, where 20 ml of water was added to dilute 1.2 μ L of HbA1c. The volume of the basic mixing structure was designed to be about 20 ml.

Figure 5 shows photographs of the mixing process resulting from liquid feeding using the light-gas generation tape, which facilitated the pumping of liquid into the mixing structure. Figure 6 shows the absorbance of the red dye as it passed through several mixing channels. The absorbance values for two, four, and eight basic mixing structures were 0.053, 1.1, and 1.2, respectively. Since 30 ml of water and 1.2 ml of red dye were mixed, a higher absorbance indicates a higher mixing effect. Therefore, the mixing effect was low for the two basic mixing structures, and the same level of mixing effect was obtained for the four and eight basic mixing structures. Because of device size constraints (width \times length = 40 \times 60 mm), the design with four semicircular structures was chosen.

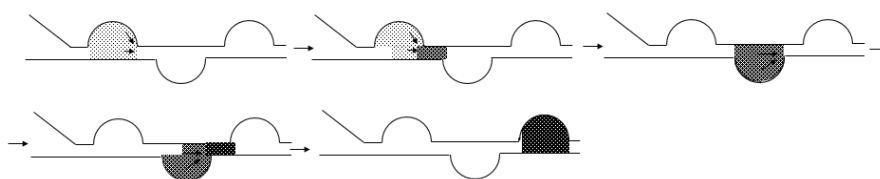


Fig. 4. Schematic of the mixing mechanism in the asymmetric microfluidic channel.

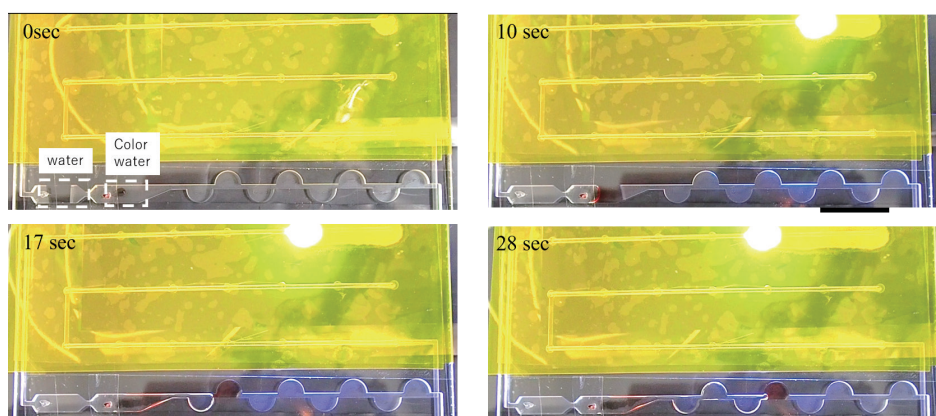


Fig. 5. (Color online) Photographs illustrating mixing over time using the mixing channel. Scale bar: 10 mm. The mixing was evaluated using 3% food coloring and water in a microfluidic channel.

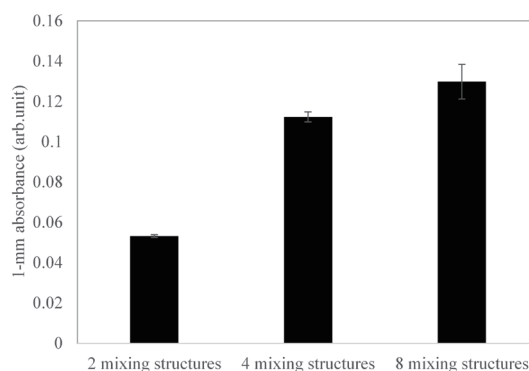


Fig. 6. Comparison of the mixing effects for different numbers of semicircular structures.

3.4 Evaluation of detection equipment to improve absorbance

The absorbance is defined by the Lambert–Beer law and increases in proportion to the optical path length. However, there is a limit to the optical path length because the thickness of the device is used as the optical path length in design. Therefore, we attempted to optimize the absorbance by optimizing the irradiation area (aperture size). Figure 7 shows the results of optimizing the aperture size of the light source. The absorbance values at the aperture diameters of 0.6, 0.8, 1.6, and 2.6 mm were 1.2, 1.4, 0.78, and 0.31, respectively. The highest absorbance was observed when the diameters of the aperture and detection area were the same (0.8 mm). On the other hand, as the diameter of the aperture increased from 0.8 mm, the absorbance decreased. This was because the increase in aperture caused scattering and stray or ambient light effects, which did not provide an adequate signal.

3.5 Evaluation of HbA1c microfluidic device

The HbA1c detection system was evaluated using the HbA1c reagent. Figure 8 shows that for HbA1c concentrations of 0, 4.4, 9.6, and 13.9%, the absorbance values were 0, 0.11, 0.28, and 0.39, respectively. A linear relationship between the concentration and the absorbance was observed with a determination coefficient of 0.99, a slope of 0.029, a standard deviation (SD) of 0.009–0.015, and a coefficient of variation (CV) of 2.4–9.3%. Generally, the HbA1c indices are as follows: 4.6–5.7% for normal, 5.8–6.4% for borderline diabetes, and 6.5% or higher for diabetes.⁽²²⁾ The HbA1c microfluidic device can be used for quantification within the HbA1c index range.

Next, the HbA1c concentration was then determined from the obtained calibration curve using whole blood. The absorbance obtained with this device was 0.133; the HbA1c concentration was calculated to be 4.9% from the standard curve from Fig. 8. On the other hand, the HbA1c concentration of this blood was determined to be 4.7% by HPLC. This method resulted in a 0.2% higher HbA1c concentration than that determined by HPLC and a variation of 3.6% when the HbA1c concentration was 4.7%. The variability of the gold standard method (HPLC) was 1%,

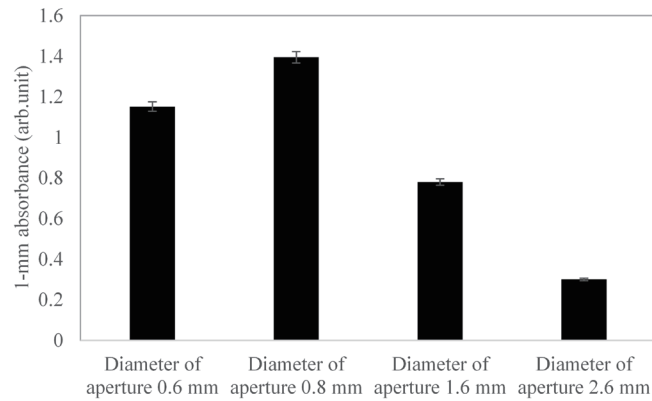


Fig. 7. Aperture optimization for improved sensitivity. A latex solution (0.25%) was added to the detection area of the microfluidic device, and measurements were taken with a photodetector. The absorbance was calculated using Eq. (1).

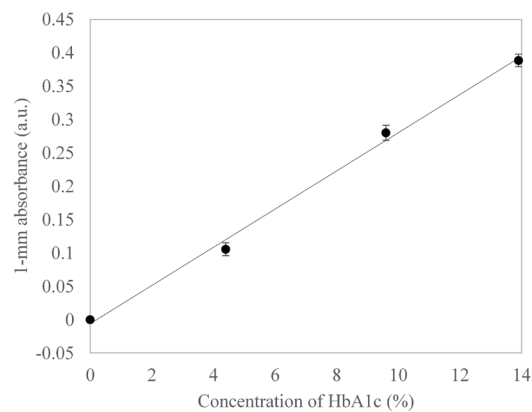


Fig. 8. Standard curve of HbA1c by microfluidic device.

and that of the commercial POCT (Afinion) was 3%.⁽¹²⁾ The variability when HbA1c was 4.7% was similar to that determined by the Afinion method, although not as small as that determined by HPLC. These results indicate that this method can be used to measure the HbA1c concentration.

3.6 Comparison of HbA1c measurement systems

Table 2 shows systems for HbA1c measurement. For comparison, Tosoh's HLC725 was selected for HPLC and Abbott's Afinion was selected for the disposable cartridge method.⁽¹²⁾ The volume of the HbA1c sample required for the measurement is not significantly different among the three methods, but HPLC requires a 3 to 5 ml tube to be set in HPLC. In the measurement of HbA1c, it is necessary to accurately measure around the diabetes reference

Table 2
Comparison of systems for HbA1c measurement.

	This report	HLC-723 (TOSO)	Afinion (ABBOT)
Principle	Latex aggregation	HPLC	Latex aggregation
Pump	Gas generation tape + LEDs	High pressure liquid pump	Dispensing device
Sample volume	1.2 μl	3 μl (Sample tube requires 3 to 51 mL)	1.5 μl
Range of HbA1c	4.6–6.2%	4.6–6.2%	4.6–6.2%
CV of HbA1c	3.6%	1%	3%
Test number	1 test	100 test	1 test
Time	5 min	1 min	3 min
r	$60 \times 40 \times 1 \text{ mm}^3$	—	$50 \times 10 \times 60 \text{ mm}^3$
Instrument size	$100 \times 100 \times 200 \text{ cm}^3$	$530 \times 515 \times 482 \text{ cm}^3$	$200 \times 190 \times 330 \text{ cm}^3$

value, and all the methods were able to quantify within this range. On the other hand, HPLC was the best method in terms of CV. As for time, the measurement was completed in a few minutes for both methods. However, our method and Afinion require more reaction time than HPLC because of the aggregation reaction time. With respect to system size, our method did not use a pipetting device or a high-pressure pump, which made it possible to reduce the system size.

4. Conclusions

In this report, we describe our design of a microfluidic device for quantifying HbA1c that is based on the latex aggregation method and fabricated using microfluidic channels on PMMA. The microfluidic channels were made hydrophilic by corona treatment to suppress latex adsorption. Furthermore, the semicircular mixing structure increased the mixing efficiency and its sensitivity was improved by adjusting the aperture diameter. The HbA1c standard allowed us to obtain a calibration curve in a range that included the criteria for diabetes mellitus. The concentration of blood could also be calculated from the obtained calibration curve. This HbA1c device is expected to simplify and accelerate diabetes testing in remote areas and clinics.

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