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A lateral flow immunosensor for direct, sensitive, and highly selective detection of hemoglobin A1c in whole blood

Shu Hwang Ang[a], T. Malathi Thevarajah[b], Pei Meng Woi[a], Yatimah binti Alias[a], Sook Mei Khor[a,*]

[a] Chemistry Department, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia
[b] Department of Pathology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

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ABSTRACT

An immunosensor that operates based on the principles of lateral flow was developed for direct detection of hemoglobin A1c (HbA1c) in whole blood. We utilized colloidal gold-functionalized antibodies to transduce the specific signal generated when sandwich immuno-complexes were formed on the strip in the presence of HbA1c. The number and intensity of the test lines on the strips indicate normal, under control, and elevated levels of HbA1c. In addition, a linear relationship between HbA1c levels and immunosensor signal intensity was confirmed, with a dynamic range of 4–14% (20–130 mmol mol^{-1}) HbA1c. Using this linear relationship, we determined the HbA1c levels in blood as a function of the signal intensity on the strips. Measurements were validated using the Bio-Rad Variant II HPLC and DCA Vantage tests. Moreover, the immunosensor was verified to be highly selective for detection of HbA1c against HbA0, glycated species of HbA0, and HbA2. The limit of detection was found to be 42.5 μg mL^{-1} (1.35 mmol mol^{-1}) HbA1c, which is reasonably sensitive compared to the values reported for microarray immunoassays. The shelf life of the immunosensor was estimated to be 1.4 months when stored at ambient temperature, indicating that the immunoassay is stable. Thus, the lateral flow immunosensor developed here was shown to be capable of performing selective, accurate, rapid, and stable detection of HbA1c in human blood samples.
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1. Introduction

Slowing the progression of Type 2 diabetes requires rapid and constant monitoring of hemoglobin A1c (HbA1c) levels in patients. This need becomes a challenge in limited-resource settings lacking laboratory infrastructure, where the social burden of the disease is often overpowering [1]. Under these conditions, constant testing and monitoring can be facilitated by point-of-care devices. However, current point-of-care devices for HbA1c often require trained personnel and elaborate equipment, and are therefore not suited to these settings.

To better facilitate on-site Type 2 diabetes management, especially in limited-resources settings, significant effort has been invested in developing HbA1c biosensors for use as inexpensive point-of-care devices. As the number of biomarkers available for detection of chronic diseases increases, biosensing using the immunoreaction between target analytes and specific clones of antibodies has become more clinically relevant [2]. Immunoassays have been integrated with different sensing platforms that employ advanced technologies such as microarrays [3], electrodes [4], and biochips [5] for the detection of hemoglobin A1c, a biomarker important for Type 2 diabetes management. In addition to immunoassays, boronic acid, a biomimetic molecule, is commonly used as a bio-recognition element because of its ease of surface chemistry manipulation [6,7]. Sundrehagen described a lateral flow strip developed utilizing zinc and reporter-labeled boronic acid to bind and precipitate hemoglobin (both non-glycated and glycated species) in solution prior to testing [8]. However, because the sample pretreatment procedure is elaborate, it can be a barrier to unskilled end-users, and this design is therefore poorly suited to economically challenged settings. Another boronic acid-based lateral flow assay was reported by McCroskey and Melton, involving pH manipulation to induce preferential binding of boronic acid to glycated hemoglobin on a separation matrix on the strip [9]. However, because two types of buffers are required to adjust for preferential binding of HbA1c, this method is not well suited to unskilled users. Although previously reported lateral flow sensors showed improved capacity for miniaturization and reduced cost, little to none of the studies have investigated the selectivity for HbA1c versus all other glycated species of hemoglobin (because boronic acid cannot distinguish HbA1c from other glycated proteins present in whole blood). To improve selectivity while maintain-

* Corresponding author.
E-mail address: naomikhor@um.edu.my (S.M. Khor).

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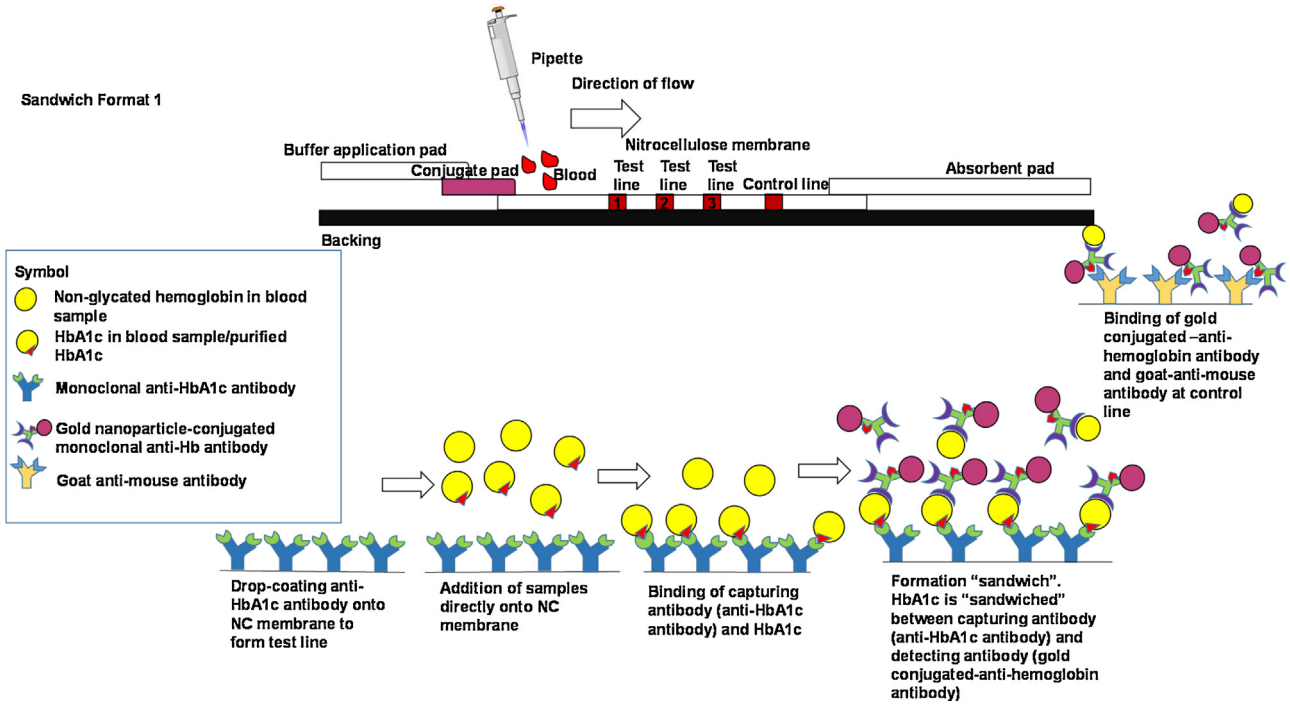

Scheme 1. Fabrication of HbA1c lateral flow immunosensor. Drop-coating of the capturing and monoclonal anti-HbA1c antibodies on the test lines; the secondary goat-anti-mouse antibody was applied to the control line using a pipette. Clinical samples were dispensed directly onto the nitrocellulose after manual dilution.

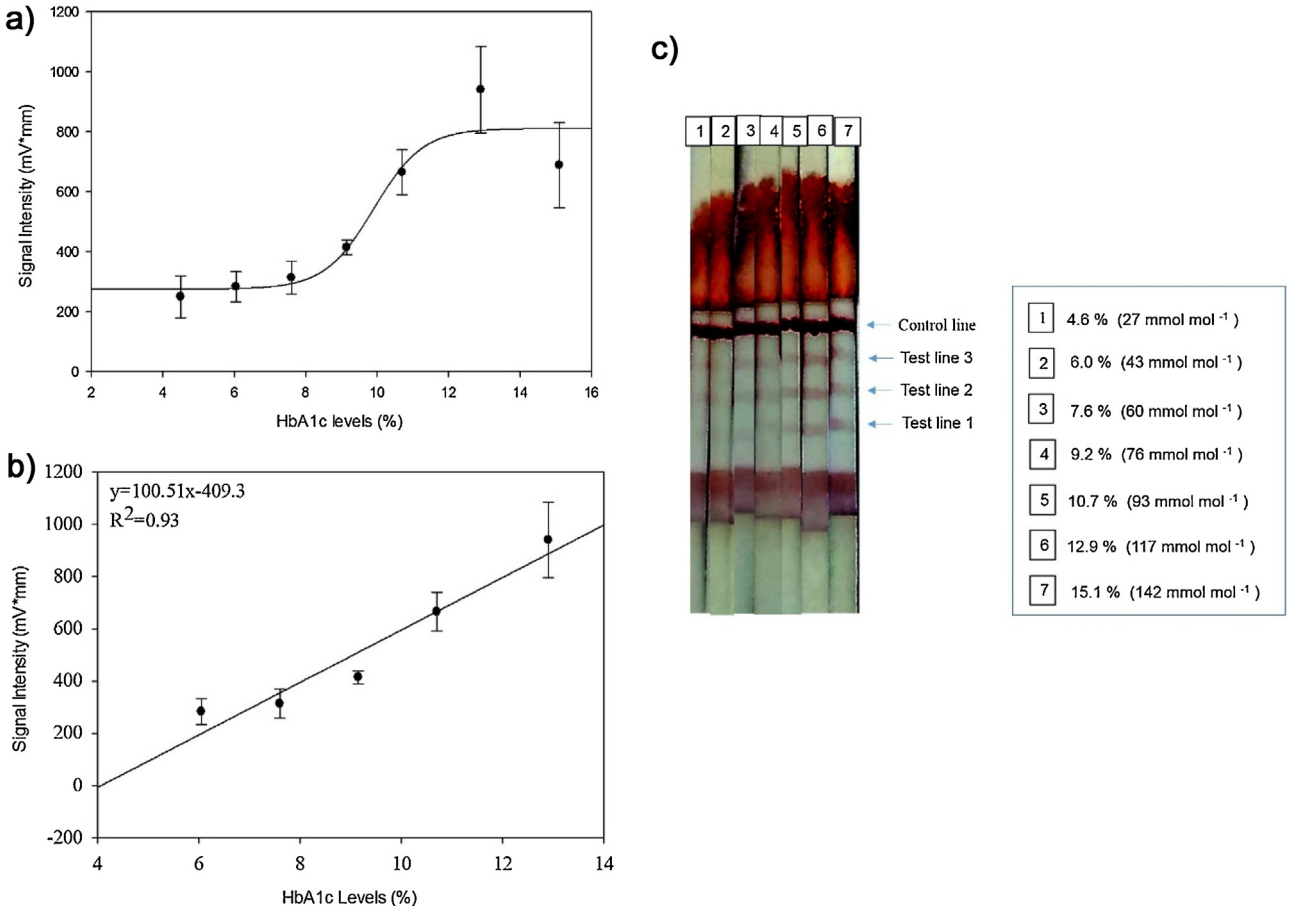

Fig. 1. (a) Signal intensity of test lines versus concentration of HbA1c (%), at 1:5 dilution, using calibrators. (b) The dynamic range of HbA1c detection fell between 4% and 14% (20–130 mmol mol^{-1}), with $R^2 = 0.93$. Error bars show the standard deviation of the signal intensity, determined from triplicate measurements of hemoglobin A1c immunosensors. (c) Visual representation of strips tested with calibrator diluted at 1:5. Lines 1–6 show increasing HbA1c levels (1: 4.6%; 2: 6.0%; 3: 7.6%; 4: 9.1%; 5: 10.7%, 6: 12.9%, and 7: 15.1%).

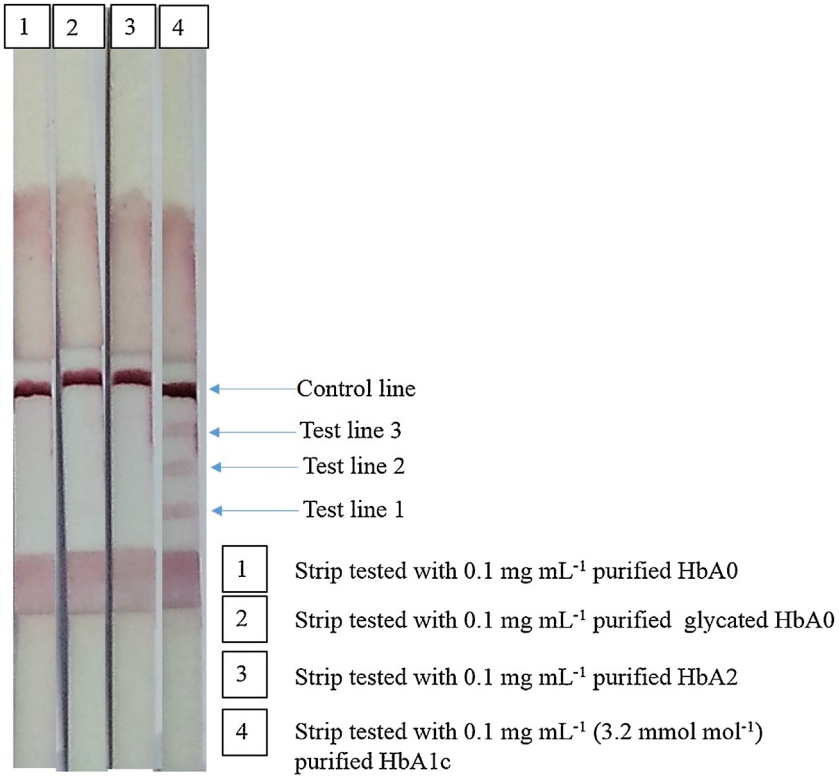

Fig. 2. Selectivity test. Synthetic reagents were used to test the cross-reactivity of the immunosensor. Little to no cross-reactivity was observed.

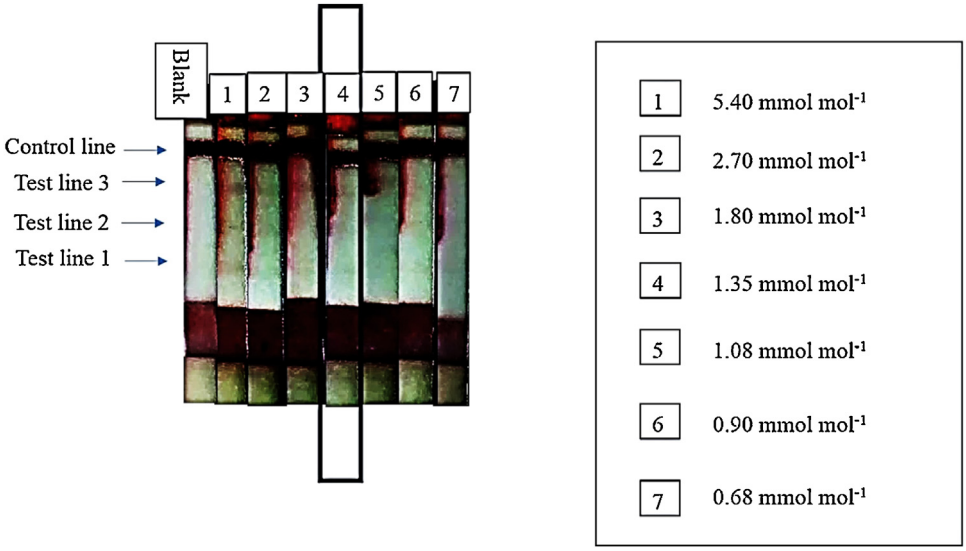

Fig. 3. Determination of the limit of detection (LOD) for the hemoglobin A1c immunosensor. Strip 4, showing 1.35 mmol mol^{-1} HbA1c, was determined to be the limit of detection for the immunosensor, determined by the degree of visibility of the test lines.

ing good sensitivity in the detection of HbA1c, we developed a lateral flow assay to detect HbA1c based on immunoreaction with a selective anti-HbA1c antibody. Considering the relatively small population of HbA1c in human blood (in healthy humans, HbA1c comprises 5% of the total hemoglobin [10]), our HbA1c immunosensor employs a "sandwich" format that specifically binds to HbA1c, and the binding reaction can then be transduced using anti-hemoglobin antibody-conjugated gold nanoparticles.

The concept of our design is simple. Using a 10-μL sample of whole blood (diluted 1:5 with water), HbA1c first encounters test line 1, which contains the capturing antibody (monoclonal anti-HbA1c antibody, IgG1) that binds specifically to the N-terminal valine at the β-chain of hemoglobin (HbA1c). Excess HbA1c will then move to the subsequent test lines. In contrast, anti-Hb-gold conjugates which can bind to any species of hemoglobin (glycated or non-glycated), would bind to the preformed HbA1c and capturing antibody complex to form the "sandwich," resulting in the formation of red lines on the lateral flow immunosensor (Scheme 1). Compare to the reported sandwich immunoassay on a microarray [3], we altered the design as using the previously reported microarray-based sandwich format, we detected no signal on our lateral flow immunosensor. We suspected that the

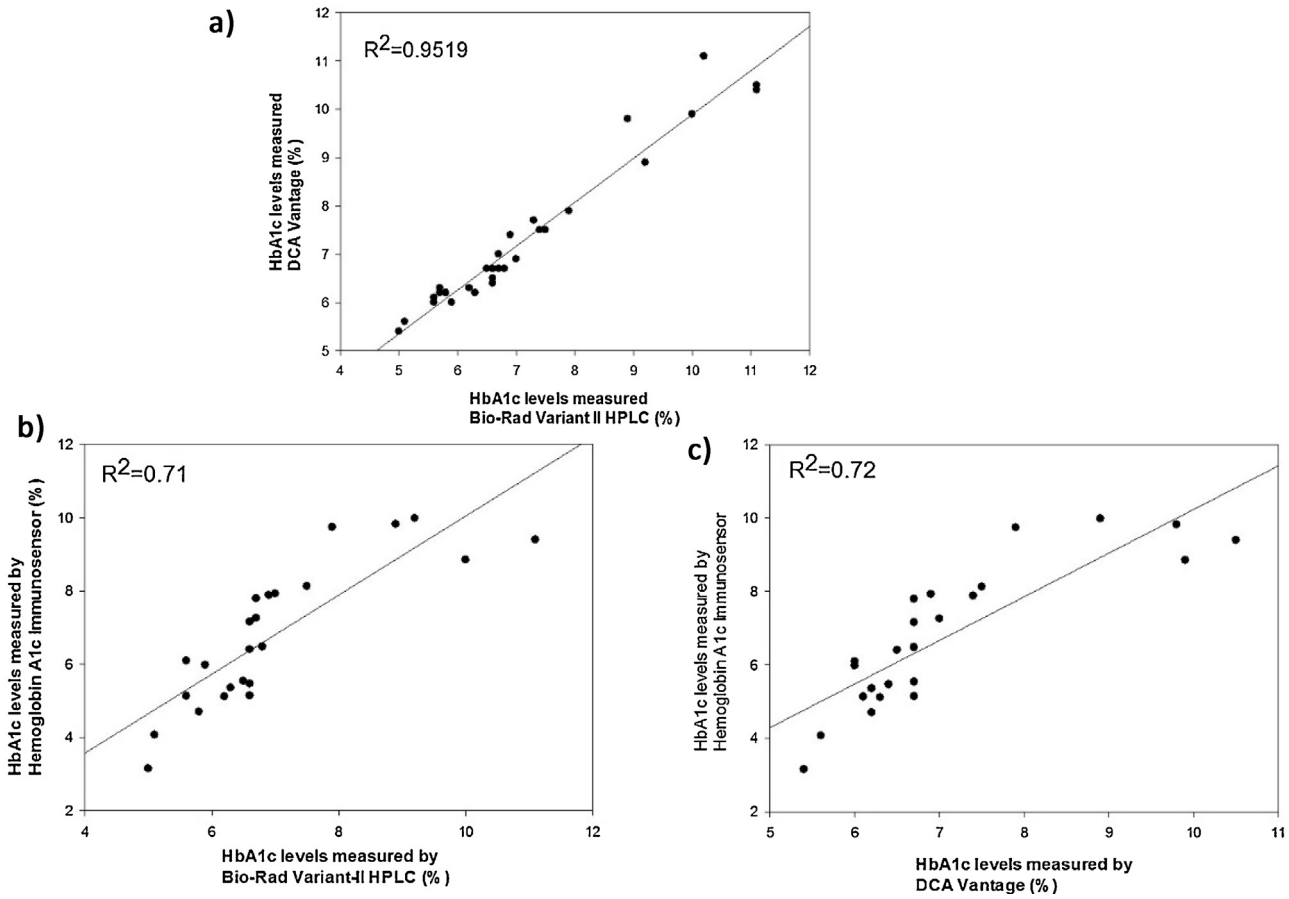

Fig. 4. (a) Regression plot. DCA Vantage versus Bio-Rad Variant II HPLC, across 24 clinical samples. (b) Regression plot. Hemoglobin A1c lateral flow immunosensor versus Bio-Rad Variant II HPLC, across all 24 blood samples. (c) Regression plot. Hemoglobin A1c lateral flow immunosensor versus DCA Vantage, 24 blood samples.

different orientations of HbA1c and steric hindrance from preformed immunocomplexes (between hemoglobin or HbA1c and the immobilized anti-hemoglobin antibody) on the strip impeded subsequent binding of the gold conjugated-anti-HbA1c antibody to the HbA1c bound to the immobilized anti-hemoglobin antibody on the strip (Supplementary materials, Fig. S1).

In present study, visual interpretation of the number and intensity of the test lines on the strip allowed us to perform semi-quantitative analysis on groups of HbA1c levels within distinctive ranges (normal range <6.5% HbA1c (48 mmol mol^{-1}), under control range 6.5–7.0% HbA1c (48–53 mmol mol^{-1}), and an elevated range requiring medical attention, >7.0% HbA1c (53 mmol mol^{-1})). Furthermore, the signal intensity of the test lines generated in the presence of HbA1c in human blood samples was assessed and recorded using an ESEQuant lateral flow reader. Following establishment of a calibration curve, the practical application of the immunosensor to quantitative measurement of HbA1c levels in blood samples was demonstrated and validated.

2. Experimental section

2.1. Chemicals and materials

The laminated nitrocellulose membrane card (ref. HF135MC100), cellulose fiber pad (ref. CFSP173000), and glass fiber pads (ref. GFCP083000) were purchased from Merck Millipore (Selangor, Malaysia). The calibrators and hemolysis reagents were purchased from Kamiya Biomedical Company (Seattle, WA, USA). The anti-hemoglobin A1c capturing antibody, the anti-hemoglobin detecting antibody, and purified HbA1c, HbA0, glycated HbA0, and HbA2 were purchased from Fitzgerald Industries International (Acton, MA, USA); the secondary goat-anti-mouse antibody was obtained from Thermo Fisher Scientific Inc. (Waltham, MA, USA); gold nanoparticles (40 nm) were purchased from Kestrel Biosciences Co., Ltd. (Pathumthani, Thailand); bovine serum albumin was purchased from Amresco LLC (Solon, OH, USA); and 10% Western Blocking Reagent was obtained from Roche Diagnostics (Selangor, Malaysia). All other chemicals were purchased from Sigma-Aldrich (Selangor, Malaysia). Phosphate buffer (containing Na_2HPO_4 and NaH_2PO_4), NaCl, K_2CO_3, and HCl were prepared using Milli-Q water with a resistivity of 18.2 MΩ cm. Except the NaCl, K_2CO_3, and HCl, all reagents were diluted with phosphate buffer. The pH of the phosphate buffer was adjusted to pH 7.4 to correspond to the normal physiological pH range of human blood (pH 7.35 to pH 7.45) [11].

2.2. Apparatus

Matric 2360 programmable shears from Kinematic Automation (Sonora, CA, USA) were used to cut the test strips. An Infinite® M200 PRO microplate reader (Tecan Group Ltd., Männedorf, Switzerland) was used to measure the absorbance of the gold conjugates at 530 nm. The signal from the immunosensor was measured with an ESEQuant lateral flow reader (Qiagen Lake Constance GmbH, Stockach, Germany).

2.3. Conjugation of detecting antibody to gold nanoparticles

Gold conjugates were synthesized using gold nanoparticles (40 nm) and the monoclonal anti-hemoglobin antibody (IgG1)

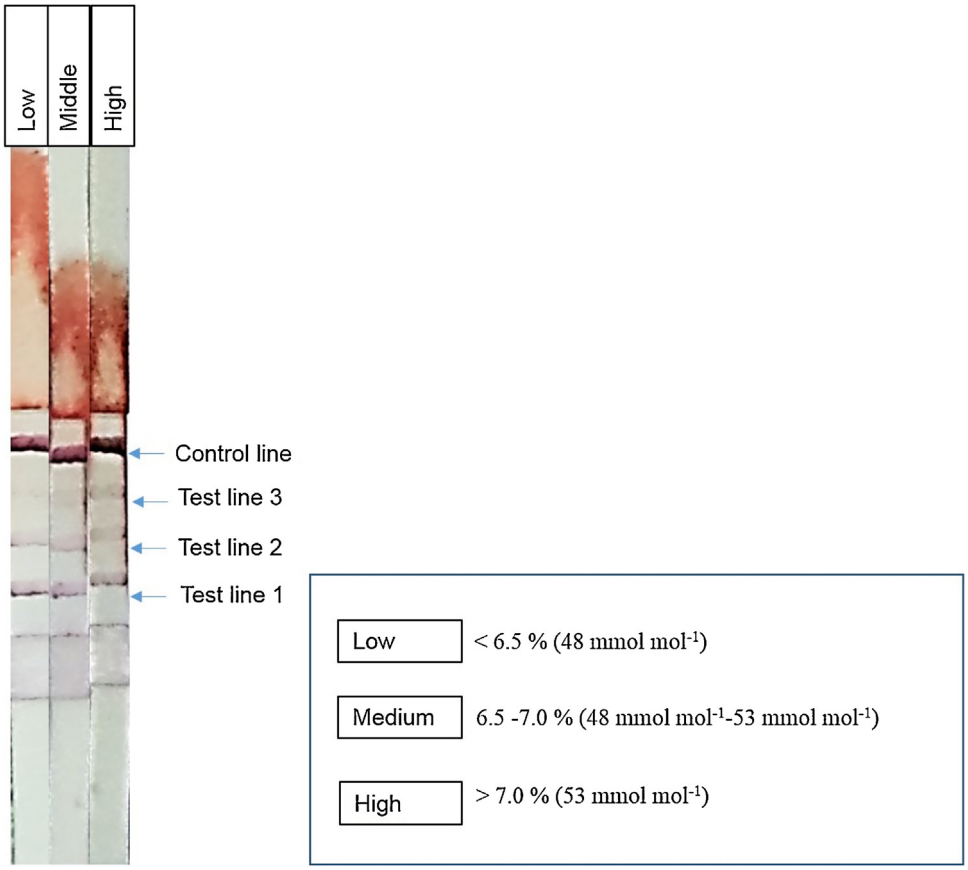

Fig. 5. Three representative strips from each category show responses in clinical testing (of 30 tests). In clinical testing, blood samples were divided into three distinct groups, the low (<6.5%, 48 mmol mol^{-1}), middle (6.5%–7.0%, 48–53 mmol mol^{-1}), and high (>7.0%, 53 mmol mol^{-1}) ranges. The signal intensity increased with the increase in HbA1c levels.

(detecting antibody), as described by Guo et al. [12], with modification of the centrifugation time, pH, and blocking buffers, to maximize the final yield of stable gold conjugates. For this study, 40-nm gold nanoparticles were chosen for their small size, allowing a high surface-packing density of antibodies on the gold conjugate pad [13]. This size is optimal for most diagnostic applications, providing a balance between visibility and steric hindrance [12]; in addition, they provide high stability [14] and are practical for use on most membranes (on which tests can be conducted within a reasonable run time). Thus, 40-nm gold nanoparticles are frequently used in fabrication of lateral flow assays [12,15–18]. The average size of the gold nanoparticles was verified using a spectrophotometer operating in the visible spectrum to determine the wavelength of maximum absorption (Supplementary materials, Fig. S2). A 10-mL volume of colloidal gold nanoparticles was adjusted to pH 8.0, and the monoclonal anti-hemoglobin antibody (IgG1) was added to a final concentration of 30.0 $\mu g\,mL^{-1}$. With gentle shaking at room temperature, the gold conjugates were incubated for 1 h. BSA (1% w/v) was then added to the gold conjugates and allowed to mix for 1 h with constant shaking. The mixture was centrifuged at 11,627 *g* for 35 min at 4 °C to precipitate the gold conjugates and the supernatant was discarded. The purified gold conjugate pellet was then resuspended in 10 mM of phosphate buffer containing 1% (w/v) BSA.

2.4. Fabrication of hemoglobin A1c lateral flow immunosensor

The hemoglobin A1c lateral flow immunosensor developed here consists of a buffer application pad, a conjugate pad, a laminated nitrocellulose membrane, and an absorbent pad (Scheme 1). The capturing antibody, a monoclonal anti-hemoglobin A1c antibody (IgG1), was coated on all test lines, and the secondary antibody, a polyclonal goat-anti-mouse antibody, was coated on the control line on the nitrocellulose membrane manually using a pipette. Following antibody coating, the immunosensor was dried in a desiccator for 30 min. All other protein-binding sites on the nitrocellulose membrane were blocked with 1% (w/v) western blocking reagent in 10 mM phosphate buffer. Gold conjugates at an optical density of 8 (measured at 530 nm) were prepared in 10 mM phosphate buffer supplemented with 10% (w/v) sucrose and deposited onto the glass fiber conjugate pad to dry overnight in a desiccator. The cut buffer application pad, dried conjugate pad, nitrocellulose membrane, and absorbent pad (all at 4 mm width) were then assembled, with 2 mm of overlap between each component, into the full hemoglobin A1c lateral flow immunosensor (Scheme 1).

2.5. Selectivity test

A selectivity test was performed using purified HbA0, glycated HbA0, HbA2, and HbA1c. All cross-reactants were diluted to 0.1 $mg\,mL^{-1}$ with 10 mM phosphate buffer. Samples (10 μL) of diluted cross-reactants were dispensed directly onto the nitrocellulose membrane of the hemoglobin A1c immunosensor. A 50 mM phosphate buffer containing Tween-20 was then used to mobilize the gold conjugates on the NC membrane and to eliminate background signal. Test strips were then read using an ESEQuant lateral flow reader.

2.6. Determination of the limit of detection

After diluting the 4.6% (27 mmol mol^{-1}) HbA1c calibrator to seven dilution factors (1:5, 1:10, 1:15, 1:20, 1:25, 1:30, and 1:40) with the hemolysis reagent (distilled water with blood stabilizers), eight hemoglobin A1c immunosensors were tested (with one negative control strip, tested with 10 mM phosphate buffer). Calibrator (10 μL) at different dilution factors was dispensed directly onto the NC membrane. For the negative control strip, 10 μL of 10 mM phosphate buffer was dispensed directly onto the NC membrane. All strips were washed with phosphate buffer containing Tween-20. The limit of detection was determined by the degree of visibility of test lines on the strips.

2.7. Stability test

Fourteen aluminum pouches containing silica gel were prepared, each with three hemoglobin A1c lateral flow immunosensors. Seven of the pouches were stored in a 37 °C oven, and the rest were stored at 4 °C in the fridge for 14 days. Pouches were retrieved from the oven and the fridge on days 0, 1, 2, 5, 7, 9, and 14, at which time points the strips were tested with diluted HbA1c calibrator 2 (7.6%) (60 mmol mol^{-1}) (diluted 1:5 with hemolysis reagent). Diluted calibrator (10 μL) was dispensed directly onto the nitrocellulose membrane. A 50 mM phosphate buffer containing Tween-20 was used to mobilize the gold conjugates for reactions on the NC membrane and to eliminate background noise to improve signal measurement in the ESEQuant lateral flow reader.

3. Results and discussion

3.1. Establishing a calibration curve for quantitative analysis of HbA1c levels

For quantitative estimation of HbA1c levels in blood samples, a calibration curve was plotted for HbA1c level versus signal intensity measured by the ESEQuant lateral flow reader. The signal intensity was measured as reflectance in the ESEQuant lateral flow reader, which was designed specifically to detect colloidal gold reflectance on a lateral flow sensing platform. The reflectance signal intensity generated in test lines 1–3 was summed and plotted against the% HbA1c of each calibrator. In our previous experiments conducted for optimization of test lines (Supplementary materials, Fig. S3), the three-line format showed visual intensity gaps that best matched increasing HbA1c levels, compared to the one- and two-test line formats, and the three-test line format was used to conduct the test. The calibration curve was generated using HbA1c calibrators with assigned values, purchased from Kamiya Biomedical. Similar to Chen et al., who also used water for manual dilution of whole blood samples prior to testing [3], our HbA1c calibrators (lysed packed human erythrocytes) and whole blood samples were manually diluted before testing. Optimization of the dilution factor (Supplementary materials, Fig. S4) showed that undiluted calibrators generated a calibration curve with a weak correlation between immunosensor signal intensity and HbA1c levels; at a dilution of 1:2, the calibrators showed a good correlation, but the test required approximately 45 min to run. When the HbA1c calibrators were diluted at 1:5 using the hemolysis reagent (distilled water with blood stabilizers), we found that the assay time was reasonably short (approximately 20 min) and produced a good relationship between signal intensity and HbA1c levels ($R^2 = 0.93$).

The calibration curve showed a sigmoid shape (Fig. 1a). Signal intensity was shown to increase with HbA1c levels, and a plateau was reached when HbA1c exceeded 14%. A decrease in signal intensity was observed when HbA1c reached 15%, presumably owing to saturation of the membrane with HbA1c. Using the sigmoidal curve, a linear dynamic range was established between 4–14% (20–130 mmol mol^{-1}) of HbA1c (Fig. 1b); the coefficient of determination was $R^2 = 0.93$, indicating a good linear relationship between HbA1c levels and immunosensor signal. We found that the dynamic range had a high slope value, suggesting that the lateral flow immunosensor is sensitive to small increments in HbA1c levels. The signal would show a wide intensity "gap" between distinct HbA1c levels, allowing semi-quantitative visual interpretation of results and preventing confusion for end-users. In addition to visual interpretation, to improve the capability of the end-users differentiate between HbA1c levels, a portable lateral flow reader can be used to perform quantitative measurement. The critical treatment goal values of 6.5–7% HbA1c (48–53 mmol mol^{-1}) fell within the linear dynamic range of the immunosensor (4–14% of HbA1c) (20–130 mmol mol^{-1}) making the device useful for diabetes care. For diabetic patients with poorly controlled glycemic status, especially those with HbAlc levels >6.5% (48 mmol mol^{-1}) [19] (or >7% (53 mmol mol^{-1}) in the United States) [20], the immunosensor will show an intense dark red color in all test lines, indicating that medical attention is needed. The results of the quantitative analysis (using an ESEQuant lateral flow reader) used to generate the calibration curve are presented in Table S1, Supplementary materials.

3.2. Selectivity of the hemoglobin A1c immunosensor

To determine the selectivity of the lateral flow immunosensor towards HbA1c, a cross-reactivity test was conducted. Because the concept of our hemoglobin A1c immunosensor took advantage of the amplification power of the anti-hemoglobin antibody-gold nanoparticle conjugates, it was important to ensure that amplification of signal only occurs when HbA1c is bound to the capturing antibody (monoclonal anti-HbA1c antibody, IgG1) and subsequently forms a "sandwich" with the anti-hemoglobin antibody-gold conjugates. HbA0, glycated species of HbA0, and HbA2 were chosen as the cross-reactants in this test. Because anti-hemoglobin antibody binds to all types of hemoglobin, it is thus critical to ensure that non-HbA1c species that form immuno-complexes with free-flowing anti-hemoglobin antibody-functionalized colloidal gold did not form a "sandwich" with the anti-HbA1c antibody on the test lines. Additionally, to justify that the chosen anti-HbA1c antibody was selective for the glycan moiety at the β-chain N-terminal valine, a glycated species of HbA0 (with glycation sites other than β-chain N-terminal valine) was tested. Although HbA2 is a minor form of hemoglobin that does not contain a β subunit, glycan moieties could be available on the α chain. Therefore, it was critical to test the cross-reactivity of the anti-HbA1c antibody with HbA2.

The cross-reactants and purified HbA1c (human hemoglobin β subunits with glycation at the N-terminal valine) were diluted to 0.1 mg mL^{-1} prior to testing. Little to no significant interference was observed for all cross-reactants tested (Fig. 2). The results indicated that the "sandwich" can only be formed, and signal observed, when HbA1c is present in the sample. The anti-HbA1c antibody was found to be highly selective for the N-terminal glycation site at the valine in the β chain. Other hemoglobin glycation sites (not the β-subunit N-terminal valine) will not bind to produce a signal. As such, when the gold conjugated anti-hemoglobin antibody (detecting antibody) binds to cross-reactants, the complexes formed will not interact with the capturing antibody and will be rinsed off the NC membrane.

3.3. Reproducibility study for the hemoglobin A1c immunosensor

To determine the reproducibility of the results of the hemoglobin A1c immunosensor, nine replicates were prepared and

Table 1
Comparison with other patented HbA1c lateral flow devices.

Patents	Similarities	Differences	Linear Range	Limit of Detection (LOD)
Device for measuring reflective absorbance and integrated device for measuring reflective absorbance and lateral flow analysis [25]	• Sandwich immunoassay was utilized to detect Hb and HbA1c	• The devices are comprised of different components (multiple devices are physically connected); Untrained users would not be able to conduct the test • Device measures reflective absorbance	• 4–14% (20–130 mmol mol^{-1}) HbA1c	• NA*
System for quantitative measurement of glycohemoglobin and method for measuring glycohemoglobin [26]	• Anti-HbA1c antibody was used to detect HbA1c	• Laser-induced epifluorescence detection • LED device is used to measure the signal • Fluorophore is used as signal transducer • Competitive assay (interpretation not user-friendly)	• 5–12% (31–108 mmol mol^{-1}) HbA1c	• NA*
Glycated hemoglobin assay method [8]	• Lateral flow strip is used as the sensing platform	• Boronic acid conjugate is used to detect HbA1c • Zinc is used to precipitate and immobilize hemoglobin on the membrane (pretreatment is needed) • Fluorescence is measured at two wavelengths, not user-friendly	• NA*	• NA*
Systems and methods for determining the percentage of glycated hemoglobin [27]	• Anti-Hb antibody was immobilized on the test line to collectively bind to all types of Hb species (inclusive of HbA1c)	• Dual-fluorescence measurements are used (not user-friendly) • Boronic acid is used to bind with HbA1c	• NA*	• NA*
Ratiometric determination of glycated protein [9]	• Reflectance is measured	• pH-induced binding of glycated proteins (not limited to HbA1c) • Negatively-charged carboxyl or carboxylate groups and hydroxyl groups are used to capture different kinds of glycated proteins	• 5–13% (31–119 mmol mol^{-1}) HbA1c	• NA*
Our lateral flow immunosensor	• Reflectance is measured • Sandwich immunoassay is used to detect HbA1c • Does not require pretreatment of whole blood • User-friendly owing to its simple visual interpretation, suitable for unskilled end-users • Portable lateral flow reader available to perform quantitative measurements		• 4–14% (20–130 mmol mol^{-1}) HbA1c	• 42.5 μg mL^{-1} (1.35 mmol mol^{-1})

*NA-Not Available.

tested on a diluted calibrator (consisting of 7.6% HbA1c) (Fig. S5). The coefficient of variation (CV) of the lateral flow immunosensor was found to be 13.45%, indicating reasonably good reproducibility. Therefore, the immunosensor should generate consistent signal intensity for samples containing the same HbA1c level.

3.4. Limit of detection of the sandwich immunoassay

When the HbA1c level is too low to form a sufficient number of "sandwiches," the signal generated can be too faint to be detected by the naked eye. Similar to Parolo et al. [21], the limit of detection

for our device was determined using the degree of visibility of the test lines with naked eye.

A range of dilution factors (1:5, 1:10, 1:15, 1:20, 1:25, 1:30, and 1:40) was performed using the 4.6% HbA1c calibrator (27 mmol mol^{-1}). Fig. 3 shows that the signal intensity on the test strips slowly diminished as the HbA1c level decreased (from left to right). Strip 4, at a dilution of 1:20 (1.35 mmol mol^{-1}, 42.5 μg mL^{-1}), was chosen as the limit of detection for the immunosensor because the visibility of the test lines was beyond visual interpretation when the HbA1c level was <1.35 mmol mol^{-1}. The red color "trail" as observed along the side of the strips is not due to any leakage or incomplete wash, but the effects of cutting edges of the nitrocellulose membrane, which can be a common phenomenon for lateral flow strips, and it is especially obvious for test strips tested with biological samples (human whole blood) [15,22–24].

3.5. Performance of the hemoglobin A1c immunosensor in real sample analysis

Because whole blood contains various bulky proteins and nutrients, viscosity can be an issue for immunosensing, as it creates high levels of background signal, shielding the generated signal and prolonging the assay. To resolve the viscosity problem, manual blood dilution was performed for optimization of the dilution factor. A dilution factor of 1:5 (with distilled water and stabilizers) was found to be ideal for generating an HbA1c level-dependent signal. Whole blood samples (n = 24) were collected from the University of Malaya Medical Center (UMMC). To validate the performance of our immunosensor for quantification of HbA1c levels, all blood samples were subjected to testing with a Bio-Rad Variant II-HPLC, a DCA Vantage, and our hemoglobin A1c lateral flow immunosensor.

We found that the correlation between the DCA Vantage and the Bio-Rad Variant II HPLC tests was 0.95 across all HbA1c levels (Fig. 4a), confirming that the DCA Vantage yields a precise and accurate estimation of HbA1c values, similar to the Bio-Rad Variant II- HPLC method. At the same time, using the calibration curve (Fig. 1), HbA1c levels in the blood samples were estimated based on the signal intensity of the test lines, as measured by the ESEQuant lateral flow reader. Regression analysis of the results from the Bio-Rad Variant II-HPLC and the DCA Vantage with those from our lateral flow immunosensor was conducted. From the regression analysis, the correlation between the results from the hemoglobin A1c lateral flow immunosensor and the Bio-Rad Variant was found to be 0.71; for the DCA Vantage, the correlation was 0.72 (Fig. 4b and c). The correlation between the results of the DCA Vantage and the Bio-Rad Variant II HPLC was high, whereas the correlation between the immunosensor and these standard methods was only general. The DCA Vantage analyzer is a commercially available point-of-care (POC) device with clinically proven results, whereas our lateral flow immunosensor is a newly developed prototype that requires further optimization before it is ready for commercialization. One possible way to improve the correlation between the results of our new immunosensor and these standard methods is by using a dispensing machine to carefully and accurately align the test lines on the NC membrane. Another possible explanation for the lower correlation with standard methods as compared DCV Vantage Analyzer is that our lateral flow matrix was saturated, therefore increasing steric hindrance of immunoreactions, when HbA1c levels were >7.0%. Instead of showing a linear response within the 4–14% (20–130 mmol mol^{-1}) HbA1c range, as shown in the calibration curve (Fig. 1b), the linear range of HbA1c detection covered by the lateral flow immunosensor was reduced to 4.0–7.0% (20–53 mmol mol^{-1}) HbA1c range in real blood samples. With manual dilution, we presumed that the whole blood samples remained comparatively more viscous than the calibrators, which contained lysed packed human red blood cells, without other proteins that are likely to contribute to the viscosity of whole blood. The decreased viscosity of the diluted whole blood facilitated longer retention, enhancing the interaction with the capturing antibody (monoclonal anti-HbA1c antibody, IgG1) on the test lines, yielding a high-intensity signal. Although a longer retention time enhances the interaction of HbA1c in the samples with the capturing antibody and gold conjugates to generate a more intense signal, with the elevated level of HbA1c present in the blood samples (>7.0%) (53 mmol mol^{-1}), the dynamic range of detection was saturated due to steric hindrance and the limited number of binding sites available. The surplus HbA1c was then washed off the sensing platform and flowed to the absorbent pad. Nevertheless, saturation of our immunosensor did not affect the visual interpretation. Fig. 5 shows three representative immunosensors tested with whole blood samples from different categories. The whole blood samples with low HbA1c levels (<6.5%) (48 mmol mol^{-1}) showed two distinct lines on the immunosensor. The whole blood samples with HbA1c levels from 6.5–7.0% (48–53 mmol mol^{-1}) showed three test lines, but the third line was faint. Immunosensors tested with blood samples containing high HbA1c levels (>7.0%) (53 mmol mol^{-1}) showed three intense test lines. No false positive results (>6.5% HbA1c) were observed for blood samples in the normal HbA1c levels category (<6.5% HbA1c), indicating that the immunosensor shows potential for use in both diagnosis and diabetes care. Because of the intensity gaps observed in response to the increasing HbA1c levels in blood samples and the consistency of the signal pattern generated on the immunosensors for the different groups of HbA1c levels, end users without a lateral flow reader can also interpret the results generated on our developed immunosensor.

Compared to the results of Chen et al. [3], the correlation values obtained in the present study were lower. The differences in correlation values can be attributed to the different HPLC method (TOSOH) and ELISA kit used by Chen et al. for the comparison. A higher correlation value has been demonstrated between a separation matrix boronic acid-based lateral flow assay and a boronate affinity HPLC method [9]. Furthermore, the saturation of our immunosensor with whole blood samples containing HbA1c levels >7.0% (53 mmol mol^{-1}) lowered the correlation values with the results of the Bio-Rad Variant II HPLC and DCA Vantage.

To determine the relationship between the results of the hemoglobin A1c immunosensor, the Bio-Rad Variant II HPLC, and the DCA Vantage, a two-tailed Student's *t*-test was performed. No significant difference was observed between the measurements performed by the Bio-Rad Variant II HPLC and those of the immunosensor ($p = 0.6997$, Table S2a, Supplementary materials), or between the results of the DCA Vantage and our immunosensor ($p = 0.5183$, Table S2b, Supplementary materials). In summary, there was no significant difference between our immunosensor and the Bio-Rad Variant II HPLC or the DCA Vantage.

Unlike the zinc-boronic acid conjugate-based lateral flow strip [8], our assay does not require much sample pretreatment, and direct detection of HbA1c can be performed using whole blood diluted with distilled water. Moreover, in our assay, neutral buffer was used only for rinsing; unlike the previously reported separation matrix boronic acid-based lateral flow assay that utilized two types of buffers for important induction of preferential binding, the device's analytical performance is potentially at risk if either one of the buffer was introduced out of sequence [9]. With its direct and simple procedure for sampling and testing, other than showing no significant difference from the standard method and existing point-of-care device in measuring HbA1c level, the lateral flow immunosensor developed here shows promise as a portable device for diabetes care. Compared to several recently patented lateral flow sensing platforms (e.g., similarities, differences, linear range and limit of detection (LOD)) that detect HbA1c (Table 1), our lateral flow immunosensor not only shows good selectivity and sta-

bility, but also, with a direct and simple design that is well-suited for unskilled end users, it facilitates ease of application in economically challenged areas.

3.6. Stability study of the hemoglobin A1c immunosensor

For the stability test, the lateral flow test strips were stored in aluminum pouches supplemented with silica gel at either 37 °C or 4 °C for 14 days. Over the course of 14 days, triplicate samples were obtained from each storage condition and were tested using a diluted calibrator (1:5 dilutions) with a known HbA1c value of 7.6%. The stability of the immunosensor was assessed by measuring the loss of signal over the course of 14 days (Supplementary materials, Fig. S6a and b). The hemoglobin A1c immunosensors generated consistent signals during the 14 days of storage under both storage conditions. Overall, the signal generated by the immunosensors remained consistent compared to the mean value (Supplementary materials, Fig. S6e). This observation indicated that the developed immunosensors are reasonably stable for 14 days of storage at higher temperatures. An accelerated stability test at 37 °C was conducted, as described by Chua et al. [28], and we estimated the shelf life of the hemoglobin A1c lateral flow immunosensor to be at least 1.4 months when it is stored at ambient temperature (25 °C).

4. Conclusion

Hemoglobin A1c (HbA1c) is a useful biomarker for diabetes management. Tested in a clinical setting and validated as an effective device by comparison with the Bio-Rad Variant II-HPLC and DCA Vantage, the hemoglobin A1c lateral flow immunosensor was proven to be useful for individual diabetic care, requiring only manual blood dilution. Owing to the stable analytical performance and the ease of performing the diagnostic test without professional personnel, the lateral flow immunosensor offers an alternative to commercialized point-of-care devices or HPLC methods that involved patient compliance to routine visit to clinics and hospitals. Our immunosensor was designed to perform both semi-quantitative (line number and intensity of test lines) and quantitative measurements. For semi-quantitative measurements, the concept of a pH paper will be presented, and representative scales and categories of HbA1c levels will be provided with the test kit. Patients can utilize the scales provided to perform visual interpretation of the number and the intensity of test lines shown. In addition, we anticipate that the prototype developed in this study can be integrated with inexpensive detection technologies, such as smartphone-based imaging [29], to enable rapid point-of-care evaluation of numerical results for HbA1c levels, better facilitating on-site diabetes care in resource-limited settings. Overall, the hemoglobin A1c immunosensor will be useful for delaying the progress of Type 2 diabetes by providing efficient personalized diabetes care for patients.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2016.01.059.

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