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A colloidal gold-based lateral flow immunoassay for direct determination of haemoglobin A1c in whole blood

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We developed an immunosensor that operates based on the lateral flow principle designed to detect haemoglobin A1c (HbA1c), a biomarker for type 2 diabetes mellitus in human blood samples. Two different clones of antibodies were used to form a "sandwich" when HbA1c was present. Functionalization of colloidal gold with antibodies was carefully optimized to generate stable gold conjugates to amplify the signal from the formed "sandwich" for the immunoassay. The ideal blocking reagent to minimize background noises, the test line format on the strip, the selectivity of the assay towards HbA1c against HbA0 and glycated species of HbA0, and the potential interference contributed by the colour of the blood sample were investigated. Captured HbA1c on the lateral flow immunosensor can be distinguished based on the number and intensity of the test lines shown; visual detection of the lines shown then indicated the distinctive groups at normal, under control, and elevated levels of HbA1c. Also, a calibration curve that covered the detection range of 4% (20 mmol mol^{-1}) and 12% (108 mmol mol^{-1}) HbA1c was reported, indicating that the prototype can be used for future quantification utilizing a lateral flow reader. The resultant immunosensor was found to report results that were easier to be interpreted and relatively inexpensive compared to electrochemical biosensors developed for the detection of HbA1c.

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1. Introduction

Haemoglobin A1c (HbA1c) has long been used as a biomarker for diabetes management. Haemoglobin in the red blood cells interacts with blood glucose to form glycated haemoglobin (HbA1c), a stable Amadori adduct that serves as a reliable indicator of individual glycemic status over 120 days.[1] Because of its usefulness as a good gauge for diabetes care, HbA1c levels should be constantly monitored. In fact, the methodology for HbA1c detection differs between laboratory and point-of-care testing. Laboratory-based HbA1c detection methods, such as cation-exchange HPLC, affinity chromatography, and capillary electrophoresis,[2] involve bulky and expensive instruments and require a long turnaround time. In contrast, advances in technology that resulted in fast, easy-to-use, point-of-care (POC) HbA1c analyzers offer a better alternative for on-site facilitation to reduce patient inconvenience for better diabetes management.

To enhance the on-site facilitation for diabetes care, the attempt to develop POC devices for HbA1c detection never ceases. In general, HbA1c biosensors can be categorized into biosensors that directly detect HbA1c or fructosyl valine (FV, a peptide generated from HbA1c proteolysis)-based biosensors. With the advent of nanotechnology, more HbA1c biosensors were designed and reported to possess outstanding analytical performance. For example, a FV biosensor (which uses enzymes as the biorecognition element) utilizing a zinc-oxide nanoparticles-polypyrrole film for indirect detection of HbA1c was reported to have enhanced stability and sensitivity compared to previously reported FV biosensors.[3] On the other hand, biosensors that directly detect HbA1c can be further categorized as amperometric, potentiometric, and piezoelectric biosensors and biochips.[4] In fact, with the discovery of more biomarkers for different diseases, efforts to increase the specificity and sensitivity to detect the biomarkers have led immunoassay-based devices to become more clinically relevant.[5] Hence, most of the fabricated biosensors reported that operate based on the electrochemical principle (owing to the ease of miniaturizing the devices at high sensitivity) typically involve anti-HbA1c antibodies as the bio-recognition element. For example, a potentiometric HbA1c immunosensor using mixed SAM (Self Assembled Monolayer)-wrapped nanospheres was reported to possess good consistency in a clinical setting, in a miniaturized form.[6] Other than electrochemical immunosensors, SPR (surface plasmon resonance) biosensors[7] and antibody

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microarrays[8] were reported to detect HbA1c as well. While maintaining high sensitivity in HbA1c detection, the reported biosensors mostly involved expensive installation cost for instrumentation, professionals to decipher the results obtained, and a series of elaborated sampling procedures that could compromise the rapidity to generate results. In fact, the antibody-based microarrays reported involved a long incubation time of 2 hours,[8] rendering the approach of detection not suitable for routine diabetes management. For POC devices to be useful as a diabetes care tool where type 2 diabetes is prevalent, especially in resource-limited settings, relatively inexpensive technology with the ease of sampling, testing, and interpreting procedure is needed.

As simple as the urine pregnancy test strip, our lateral flow HbA1c immunosensor is aimed to be user-friendly with relatively easy sampling, testing, and subsequent interpreting procedure. A defined volume of diluted blood sample was flowed through a lateral flow assay matrix where HbA1c was captured at the test line due to the immunoreaction with anti-HbA1c antibodies; subsequently colloidal gold-functionalized anti-haemoglobin antibodies will generate visible test line(s). Before the application of the approach, the ideal blocking reagents, the test line format, the selectivity of the assay and the potential interference from the colour of the blood sample were studied. With the calibration curve established between the test line intensity and HbA1c levels, the feasibility to perform measurements using the lateral flow immunosensor was demonstrated.

2. Materials and methods

2.1 Reagents and materials

The capturing antibody (monoclonal anti-haemoglobin A1c IgG1 antibody), the detecting antibody (monoclonal anti-haemoglobin IgG1 antibody), and the purified HbA1c, HbA0, and glycated HbA0 were purchased from Fitzgerald Industries International (Acton, MA, USA); the secondary antibody (polyclonal goat-anti-mouse IgG 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). The calibrators and haemolysis reagent were purchased from Kamiya Biomedical Company (Seattle, WA, USA). All other chemicals were purchased from Sigma-Aldrich (Selangor, Malaysia). Phosphate buffer (containing Na_2HPO_4 and NaH_2PO_4) was prepared at pH 7.4 by using Milli-Q water with a resistivity of 18.2 MΩ cm. NaCl, K_2CO_3, and HCl were diluted using Milli-Q water; all other reagents were diluted with the prepared phosphate buffer. The pH was adjusted to pH 7.4 to correspond to the normal physiological pH range between 7.35 and 7.45 in human blood.[9] Because of different matrices involved in optimization studies, distinctive reagents were needed to perform dilutions. For gold conjugates, blocking reagents and selectivity tests which involved purified proteins (*i.e.* antibodies, bovine serum albumin, and purified haemoglobin species *etc.*) that are sensitive to pH changes, the pH-adjusted phosphate buffer that resembles physiological pH was used. On the other hand, to lyse the erythrocytes completely, interference studies and real sample analysis that involved whole blood required a haemolysis reagent (distilled water with stabilizers). Also, in order to maintain the matrix similarity between whole blood and calibrators purchased, the haemolysis reagent was used to perform dilution to generate the calibration curve in the studies. Laminated nitrocellulose membrane card (ref. HF135MC100), cellulose fiber pads (ref. CFSP173000) and glass fiber pads (ref. GFCP083000) were purchased from Merck Millipore (Selangor, Malaysia). For the studies that involved blood testing, informed consent was obtained from patients recruited and the study protocol was implemented in accordance with the institutional medical ethics board committee.

2.2 Apparatus

The absorbance of the gold conjugates before and after treatment with 10% NaCl was measured at a wavelength of 530 nm using an Infinite® M200 PRO microplate reader (Tecan Group Ltd, Männedorf, Switzerland). Signals on the immunosensor were measured with an ESEQuant lateral flow reader (Qiagen Lake Constance GmbH, Stockach, Germany). Prior to manual lining of capture reagents on strips using a pipette, lateral flow strips were cut using a Matric 2360 programmable shear from Kinematic Automation (Twain Harte, USA).

2.3 Construction of lateral flow strips

2.3.1 Conjugation of gold nanoparticles to antibodies. Optimization of the gold nanoparticle-conjugated anti-haemoglobin-antibody was performed using a flocculation assay described by Thobhani *et al.*[10] In this assay, the optimum pH and the minimal antibody concentration required to stabilize the gold conjugates were determined. First, the stability of gold conjugates was tested by varying the pH of gold nanoparticles in the range of 5.0 to 9.0 (adjusted using K_2CO_3), at a constant antibody concentration of 30 μg mL^{-1}. The absorbance of the gold conjugates before and after treatment with 10% NaCl was measured at a wavelength of 530 nm using an Infinite® M200 PRO microplate reader (Tecan Group Ltd, Männedorf, Switzerland). To determine the ideal concentration of antibody that solvates the gold nanoparticles, gold nanoparticles with adjusted pH were titrated with different antibody concentrations, and the stability of the gold conjugates was assessed as described above. Once the ideal concentration of antibody and pH were determined, gold conjugates were produced on a large scale and diluted to OD 8 (to generate visually interpretable lines without compromising the release time from the conjugate pad) to be deposited onto the conjugate pad of the strips.

2.3.2 Optimization of blocking reagents. Blocking reagents are important to eliminate the non-specific binding of protein on the NC membrane and to minimize the interference of the background noise from nonspecific binding on the test strip. In search of the ideal blocking reagent for our immunosensor, industrial polymers such as polyvinylpyrrolidone (PVP) and

polyvinyl alcohol (PVA), carrier proteins such as casein in the western blocking reagent (WBR), and bovine serum albumin (BSA) were tested. Ten microliters of purified HbA1c (0.1 mg mL^{-1}) (Fitzgerald Industries Inc., Acton, MA, USA) was dispensed onto fully assembled lateral flow test strips (positive test strips), and to determine the background generated, a corresponding negative control strip (blocked with the same blocking reagent) was tested with 10 μL of phosphate buffer substituted for the purified HbA1c. The signals generated from positive test strips and corresponding negative control strips (blocked with different blocking reagents) were then measured using an ESEQuant lateral flow reader (Qiagen Lake Constance GmbH, Stockach, Germany). The ratio of the signal to background was calculated by dividing the background (signal generated on negative control strips) using the signal generated on positive test strips.

2.3.3 Preparation of lateral flow strips. The lateral flow strip consists of four overlaying components (the buffer application pad, the conjugate pad, the laminated nitrocellulose membrane, and the absorbent pad). It allows the test to be run by capillary flow along the strip. The nitrocellulose membrane contains three test lines, where the capturing antibody (monoclonal anti-HbA1c IgG1 antibody) was deposited (Fig. 1). A control line, which contains polyclonal goat-anti-mouse IgG antibody, was created as an internal control of the lateral flow strip. The capturing antibody lined on the strips was dried for at least 30 min in a desiccator before blocking was performed. With four components overlaying each other at 2 mm in length, the lateral flow strip at 4 mm in width was fully assembled after the strip was lined with the capture antibodies and blocked with the optimized blocking reagent (please see Section 2.3.2 for optimization of blocking reagents). A defined amount of diluted samples (calibrators or whole blood) of 10 μL was first dispensed directly onto the laminated nitrocellulose membrane. Following application of the washing buffer (50 mM phosphate buffer, pH 7.4, with 5% Tween-20), the gold conjugates on the conjugate pad were mobilized to react with the antigen (HbA1c) and capture antibodies on the nitrocellulose membrane. The non-reacted gold conjugates and antigens (HbA1c) will then be mobilized to the absorbent pad.

2.3.4 Selectivity and interference test. In the selectivity test, glycated species of HbA0, HbA0 and HbA1c were diluted to 0.1 mg mL^{-1} with phosphate buffer (0.01 M, pH 7.4) prior to the test. Then, 10 μL of HbA1c, HbA0, and glycated species of HbA0 were dispensed directly onto the NC membrane. Washing buffer (50 mM phosphate buffer, pH 7.4, with 5% Tween-20) was applied to mobilize the gold conjugates and antigens (glycated species of HbA0, HbA0, or HbA1c) to complete the assay.

To assess the specificity of the signal generated on the nitrocellulose (NC) membrane, an interference test was conducted. A control strip with a conjugate pad dried with 1 mg mL^{-1} monoclonal anti-haemoglobin antibody (IgG1, without conjugation to gold nanoparticles) was prepared. For comparison, the control strip and test strip (with the conjugate pad containing gold-conjugated-monoclonal anti-haemoglobin antibody) were tested with 10 μL of a 1 : 5 dilution of whole blood. After direct dispensing of the diluted whole blood onto the strips, the strips were washed with washing buffer.

2.3.5 Optimization on test lines and dilution factors. The amount of test lines was optimized for ideal identification of different HbA1c levels in blood samples. Strips were either lined with one, two, or three lines of monoclonal anti-HbA1c antibody

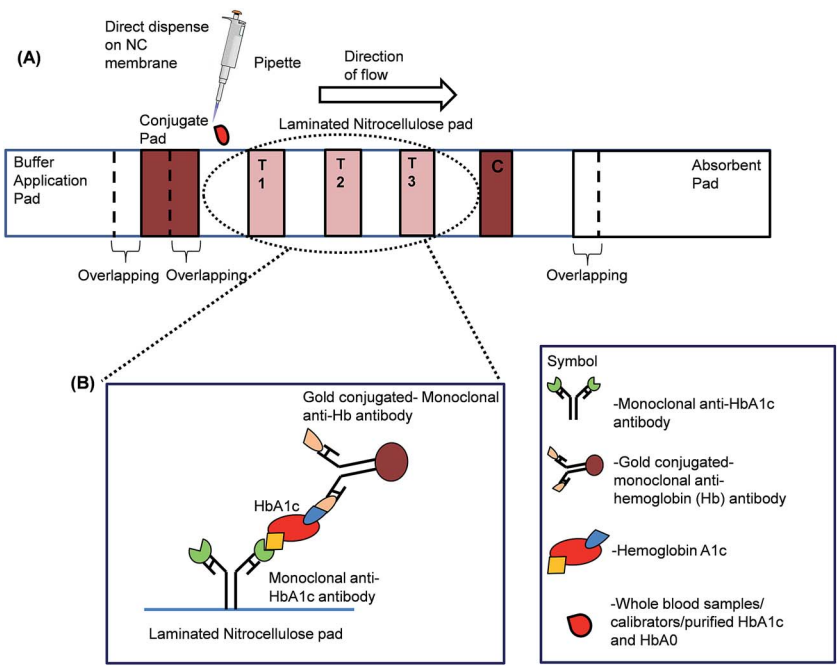

Fig. 1 Schematic of the fabrication of the HbA1c lateral flow immunosensor. (A) Top view of the HbA1c immunosensor. (B) The sandwich format formed on the nitrocellulose membrane in the fabrication of the hemoglobin A1c immunosensor.

with one control line consisting of polyclonal goat-anti-mouse antibody. Three concentrations of HbA1c were selected for testing. In the preliminary study on test lines optimization, the 4.6%, 6.0%, and 7.6% calibrators were diluted ten times (with the haemolysis reagent), and 5 μL of that dilution was directly dispensed onto half dipsticks (without the buffer application pad). Then, the half dipsticks were immersed into 10 μL of gold conjugates, followed by 100 μL of phosphate buffer to complete the test.

Due to the viscosity of the whole blood samples, the dilution factor used was very critical not only to lyse the cellular content of whole blood to release HbA1c, but also to improve the fluidity of whole blood on the test strip. The haemolysis reagent containing blood stabilizers was used to perform manual dilutions on whole blood and calibrators. Calibrators (lysed packed human red blood cells) with assigned values (4.6%, 6.0%, 7.6%, 9.1%, 10.7%, and 15.1%) of HbA1c were tested for different dilution factors to determine the quality of the calibration curve produced. Once the dilution factor was identified with calibrators, assessed by the quality of the calibration curves generated, the same dilution factor was used to determine the ideal dilution factor for whole blood testing on our immunosensor.

3. Results and discussion

3.1 Design and principle of the lateral flow immunosensor

The lateral flow immunosensor was designed to meet the demand for sustainable diabetes care in a resource-limited setting. The HbA1c biosensor was made of paper and, with large-scale production, the cost of the test strip can be within the range of $0.10 to $3.00 (depending on the antibody production cost),[11] which makes it relatively inexpensive. Our HbA1c lateral flow immunosensor consists of a sample pad, conjugate pad, nitrocellulose (NC) membrane, and absorbent pad. On the NC membrane, three test zones contain the capturing antibody for semi-quantitative, visually interpretable HbA1c detection, while a control zone with the secondary antibody (goat anti-mouse antibody) serves as an internal control for each test strip (Fig. 1). In our lateral flow HbA1c immunosensor, we chose to perform the detection in the sandwich format (Fig. 1), which is known to be a more selective and sensitive way to detect molecules with more than one epitope.[12] Haemoglobin consists of four subunits, and HbA1c is formed *via* glycation of the terminal valine residue in β subunit(s).[13] Because HbA1c constitutes only a small fraction (approximately 5%) of the total haemoglobin[14] (for a detailed description on the biological aspect of the haemoglobin pool, please refer to Ang *et al.*[15]), to amplify the test signal, we chose the anti-haemoglobin antibody as the detecting antibody. To maintain the specificity and selectivity of our assay, the anti-haemoglobin A1c (HbA1c) antibody was immobilized on the NC membrane as the capturing antibody (Fig. 1b). As the sample ran through the assay, the capturing antibody binds the glycated epitopes exposing the non-glycated sites or non-specific glycation sites (non-terminal valine residues in β subunits or other residues in any haemoglobin subunits), which were then recognized by the gold nanoparticle (40 nm)-conjugated detecting antibody, thus generating a signal at the test zone(s).

3.2 Haemoglobin A1c immunosensor evaluation

3.2.1 Selectivity and interference test. To evaluate the selectivity of the assay, diluted purified non-glycated haemoglobin (HbA0), glycated species of HbA0 (which consists of glycation sites other than β-chain N-terminal valine) and haemoglobin A1c (HbA1c) were tested. Because the gold conjugated-anti-haemoglobin antibody binds specifically to haemoglobin, the selectivity test is thus necessary to make sure that the signals generated on the NC membrane are purely from glycated haemoglobin (HbA1c). Non-glycated haemoglobin and other glycated species can bind to the gold conjugated-anti-haemoglobin antibody, hence posing the chance to generate false positive results on the strip. Other than that, because glycation can occur at other amino acids, the selectivity of the anti-HbA1c antibody towards the glycation site at β-chain N-terminal valine is important to ensure that the signal is specifically from HbA1c in samples. To ensure the selectivity in detection of HbA1c, purified HbA1c (human haemoglobin glycated at β subunit N-terminal valine), glycated species of HbA0 (glycation sites other than the β-chain N-terminal valine) and non-glycated haemoglobin (HbA0) were tested on the haemoglobin A1c immunosensor that was developed. As shown in Fig. 2A, strip 2 with glycated species of HbA0 and HbA0 (strip 3) showed no signal on the immunosensor, indicating that the developed immunosensor was very selective to HbA1c. Hence, the test lines that showed up in the immunosensor were representative of the presence of HbA1c (Fig. 2A).

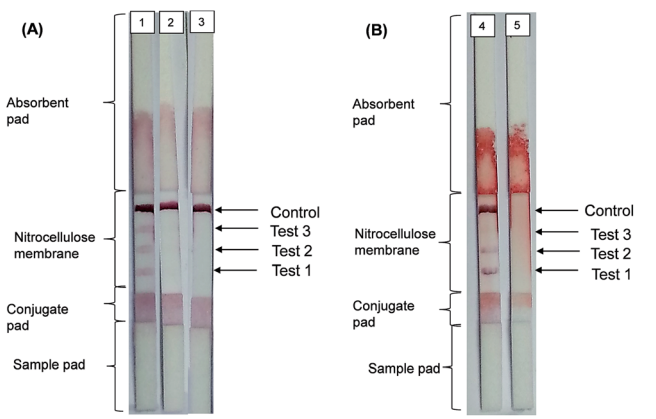

Fig. 2 (A) Selectivity test. Purified HbA1c (strip 1), purified glycated species of HbA0 (strip 2) and purified HbA0 (strip 3) were tested on the developed hemoglobin A1c immunosensor. No cross-reactivity was observed from the glycated species of HbA0 (strip 2) and non-glycated hemoglobin (HbA0) (strip 3), indicating that the immunosensor developed was very specific to HbA1c. (B) Interference test. Strip 4 is the developed hemoglobin A1c immunosensor while strip 5 was the control strip with anti-hemoglobin antibody without conjugation to gold nanoparticles, Fig. 2B shows that the signal generated from the assay was only from the bound gold conjugated-anti-hemoglobin antibody, the whole blood did not contribute to the redness observed either on the test lines, or the control line.

Because the whole blood samples are red in colour, there was a possibility that the signals generated on the test lines on the developed immunosensor were contributed partly by the redness of the whole blood sample. While the measurement of the reflectance on test lines can be performed using the ESQuant lateral flow reader, which is designed to be specific for detecting gold conjugates, the visual interpretation by household end-users can be cumbersome without the reader if blood interferes with the colour generated on the test lines. From our study, we noticed that the background colour of whole blood did not contribute to the colour generated on the test line (refer to Fig. 2B); thus, the colour generated on the test line was solely dependent on the bound gold conjugates. In short, it was confirmed that our developed immunosensor is very specific to HbA1c and only bound gold conjugates that formed "sandwiches" with HbA1c and the capturing antibody (monoclonal anti-HbA1c antibody) will result in the visibility of the test lines.

3.3 Optimization of experimental parameters

3.3.1 Optimization of gold conjugates. We improved the detection sensitivity and specificity by optimizing the generation of stable gold conjugates. To determine the ideal conditions to synthesize stable gold conjugates, a flocculation assay was performed as described in Section 2.3.1. In the flocculation assay, electrolytes such as sodium chloride were used to mask the charges on the gold nanoparticles, causing the disruption in attraction and repulsive forces, resulting in gold nanoparticles collapsing and aggregating to show visual changes in colour.[10] On the other hand, the presence of the proteins inhibits the aggregation of gold nanoparticles by absorbing to the surface of the gold nanoparticles.[10] Hence, the smaller the difference in absorbance before and after treatment with 10% NaCl (termed as absorbance decrease), the better is the conjugation of proteins onto the surface of the gold nanoparticles and therefore the higher the stability of the gold conjugates. By performing the flocculation assay, we found out that at pH 8.0, with a concentration of 30 μg mL^{-1} of the anti-haemoglobin antibody, the gold conjugates formed had the least absorbance difference (Fig. 3). Therefore, this indicated that gold conjugates were very stable under the specified conditions, which were utilized later to prepare gold conjugates on a large scale for deposition onto conjugate pads to assemble a full haemoglobin A1c immunosensor.

3.3.2 Optimization of blocking reagents. To eliminate the high background, different types of blocking reagents were tested in the assay to select the optimal blocking buffer for the test strips. An ideal blocking buffer should be able to reduce the background noise while providing concentration-dependent detection of the analyte, *i.e.*, HbA1c. In our case, 1% of the western blocking reagent (WBR), which contains casein, was shown to efficiently block the unwanted non-specific signal. Little to no background was observed on the negative control strips (strips tested with phosphate buffer in place of purified HbA1c) blocked with 1% WBR. On the other hand, the unblocked strips or blocked strips with other blocking reagents (polyvinylpyrrolidone (PVP) and polyvinyl alcohol (PVA), bovine serum albumin (BSA), and a mixed blocking reagent that

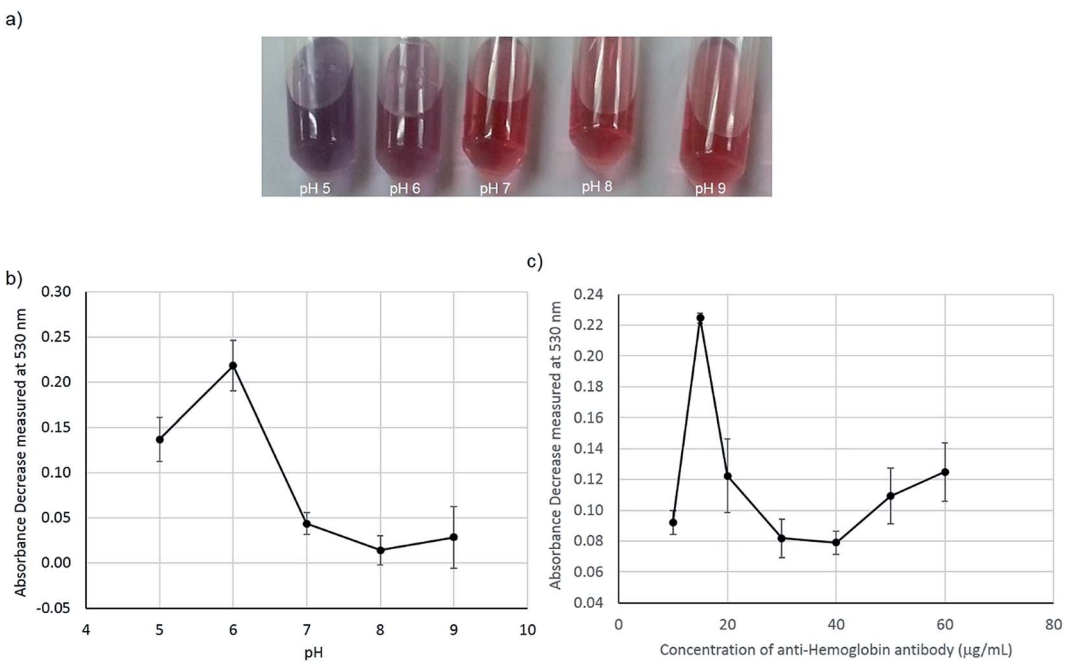

Fig. 3 (A) The variation of the colors across a range of pH, after the treatment of 10% NaCl. The gold conjugates formed at pH 5 and pH 6 were unstable, indicated by the purplish blue color. (B) Absorbance decrease *versus* pH (measured at 530 nm). The optimum pH to solvate and stabilize gold conjugates was identified as 8.0. (C) Absorbance decrease *versus* detecting antibody concentrations (measured at 530 nm). 30.0 μg mL^{-1} of the anti-hemoglobin antibody was determined to be the least concentration of antibody needed to form stable gold conjugates at pH 8.0. Error bars display the standard deviation of triplicates for each data point.

consisted of 1% BSA, 0.02% polyvinylpyrrolidone (PVP), 0.05% casein, and 0.002% Tween-20 in 1× Tris-buffered saline (pH 7.4)) yielded a high signal in negative control strips, indicating that the signal observed from the positive test strips (strips tested with purified HbA1c) did not necessarily originate from the binding of HbA1c (Fig. 4). In most lateral flow studies, the remaining protein-binding sites on the NC membrane can be blocked efficiently by BSA (bovine serum albumin);[16–18] however, our test strips showed significant interference from the background noise (non-specific binding) when the strips were blocked with BSA. In other words, BSA and other blocking reagents did not sufficiently block the protein-binding sites on the strips. On the other hand, 1% WBR-blocked strips which had little to no signal on negative control strips, indicated that 1% WBR is the best blocking reagent for our test strips, similar to the observation by Ang *et al.*[19] Therefore, we can then be confident that the signal generated from the immunosensor blocked with 1% WBR was specifically originating from HbA1c "sandwiched" between the gold conjugated-anti-haemoglobin antibody and anti-HbA1c antibody.

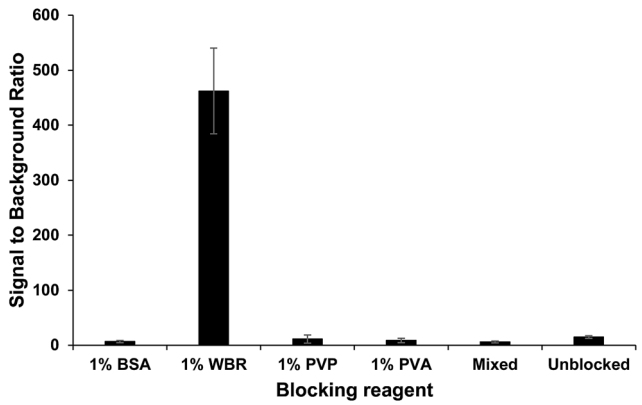

Fig. 4 Signal to background ratio *vs.* blocking reagents. All the blocking reagents were prepared in dilution using 0.01 M phosphate buffer, pH 7.4. Mixed blocking buffer was prepared using 1% BSA, 0.02% polyvinylpyrrolidone (PVP), 0.05% casein, 0.002% Tween-20 in 1× Tris-buffered saline (pH 7.4). All the other blocking buffers, except 1% WBR (western blocking reagent), resulted in a visually detectable signal on strips. 1% WBR was thus selected as the best blocking buffer for our fabricated HbA1c immunosensor. Error bars represent standard deviation triplicates associated with each data point.

3.3.3 Optimization of test lines. To achieve visual interpretation of HbA1c levels based on the number and the intensity of the test lines shown, the test zone on the NC membrane was expanded to contain three lines of the immobilized capturing (HbA1c-specific) antibody for the sandwich reaction. As the first test line was saturated, the remaining HbA1c moved to the next line, where it is again sandwiched between the capturing and detecting antibodies; thus, the concentration of HbA1c in a sample can be assessed by the number and colour intensity of the lines in the test zone(s). Optimization of test lines involved testing on different formats to determine which format would allow for a more user-friendly interpretation, without the requirement of a lateral flow reader for measurement. Fig. 5A–C show different test line formats across different concentrations of HbA1c. While the interpretation of intensity can be very subjective, we found that the number of lines better aided the visual interpretation. It is important to note that one- (Fig. 5A) and two-line formats (Fig. 5B) showed a gradient of intensity across an increasing concentration of HbA1c; however, it was subjective to tell which strip has the darker or more intense test line(s) with either the one- or two-line format. On the other hand, the three-line format allowed a clear interpretation of the strip tested with higher levels of HbA1c. The least concentrated HbA1c (4.6%) had not only a less intense first test line, but the 2nd and 3rd test lines were almost invisible, leaving a good intensity gap for the next higher level (6.0%) of HbA1c tested for the ease of distinguishing (Fig. 5C). To distinguish between the 6.0% and 7.6% HbA1c in the three-line format, the third line was the determining line. Because all three lines showed up, we relied on the intensity of the 3rd line to judge which strip had a higher level of HbA1c tested. The third line clearly showed up with a higher intensity in the strip with a higher level of HbA1c. In short, we decided to use the three-line format for better visual interpretation in the household setting.

3.3.4 Generation of calibration curves. To measure HbA1c levels in blood samples, the relationship between the signal intensity and HbA1c levels was investigated using commercial calibrators. To generate a calibration curve for quantitative analysis, six points of HbA1c concentrations that covered 4.6% to 15.1% HbA1c were used. Optimization at different dilutions was performed to determine the best calibration curve to perform the quantitative analysis. The calibrators were purchased (lysed human erythrocytes) with designated HbA1c

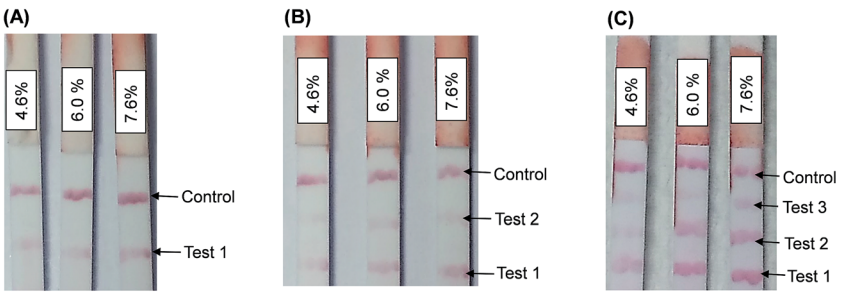

Fig. 5 (A) Strips with the 1-test line format tested with increasing concentration of HbA1c. (B) Strips with the 2-test line format tested with increasing concentration of HbA1c. (C) Strips with the 3-test line format tested with increasing concentration of HbA1c.

values (4.6%, 6.0%, 7.6%, 9.1%, 10.7%, and 15.1%). Optimization of the calibration curves was performed based on different dilution factors using the haemolysis reagent (water with stabilizers). Another study by Chen *et al.* also reported on using water to perform dilution on the whole blood sample prior to testing.[8] The calibration curves at different dilutions demonstrated distinct patterns, indicating that dilution factors can directly affect the immunosensor performance in terms of sensitivity in our assay.

Fig. 6A–C show the overall detection range of the calibration curves for different dilution factors, while the insets of Fig. 6A–C depict the linear dynamic range useful for future quantitative HbA1c level measurements. Note that for undiluted calibrators, the calibration curve generated had a low coefficient of determination, R^2, of 0.61 (inset of Fig. 6A), indicating that the linear relationship between the HbA1c levels and the signal on the assay was weak. Also, the standard deviations were consistently large throughout the HbA1c levels tested (Fig. 6A). Since the accuracy in quantification of the HbA1c levels by using a lateral flow assay can often be affected by the sample composition (matrix effect),[20] we presumed that the high viscosity of the undiluted calibrators had contributed to the high degree of variability for each test across the HbA1c levels (Fig. 6A). Hence, we concluded that the assay could be saturated with undiluted calibrators and therefore optimization on the dilution factors was necessary to create a calibration curve. By diluting the calibrators at 1 : 2 (inset of Fig. 6B) and 1 : 5 (inset of Fig. 6C) dilution factors, we found that the resultant calibration curves yielded a reasonably good fit within the range of 4.0% (20 mmol mol^{-1}) to 12.0% (108 mmol mol^{-1}) HbA1c. In comparison, with both 1 : 2 and 1 : 5 diluted calibration curves in the same range for linear detection (4.0% to 12.0%), the 1 : 2 diluted calibration curve assumed a higher R^2 of 0.95, while the 1 : 5 diluted curve showed a R^2 of 0.78. However, with the consideration of a better assay time (1 : 2 diluted calibrators took 45 min to eliminate the background for analysis, 1 : 5 diluted calibrators required as low as 20 min to be ready for measurement) for better sensitivity observed across the HbA1c levels (assessed by the higher slope of the linear graph and the visual interpretation shown in the inset of Fig. 6D), we decided that the 1 : 5 diluted calibration curve was the best calibration curve to perform future quantitative analysis.

The results showed that the calibration curve at 1 : 5 dilution assumed a reasonably good fit with the highest sensitivity to establish a linear relationship between the signal and HbA1c levels within the range of 4% (20 mmol mol^{-1}) to 12%

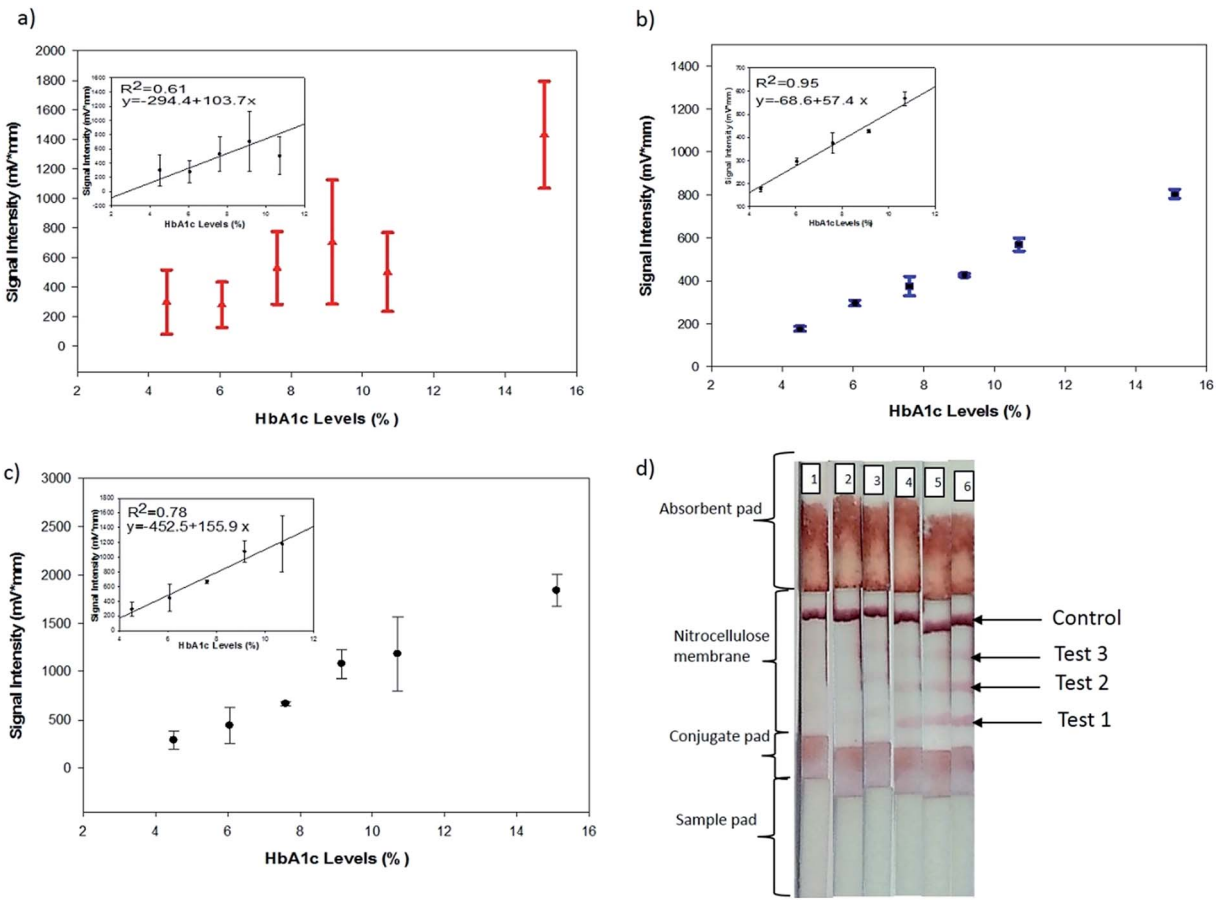

Fig. 6 (A) Signal intensity of test lines measured *versus* concentrations of HbA1c, without dilution. (B) Signal intensity of test lines measured *versus* concentration of HbA1c, at 1 : 2 dilutions. (C) Signal intensity of test lines measured *versus* concentration of HbA1c, at 1 : 5 dilutions. Error bar represents the standard deviation for triplicates of each data point. (D) Visual representation of strips tested with the calibrator diluted at 1 : 5 dilution factors. 1 to 6 indicated the increasing HbA1c levels (1 : 4.6%; 2 : 6.0%; 3 : 7.6%; 4 : 9.1%; 5 : 10.7%, and 6 : 15.1%).

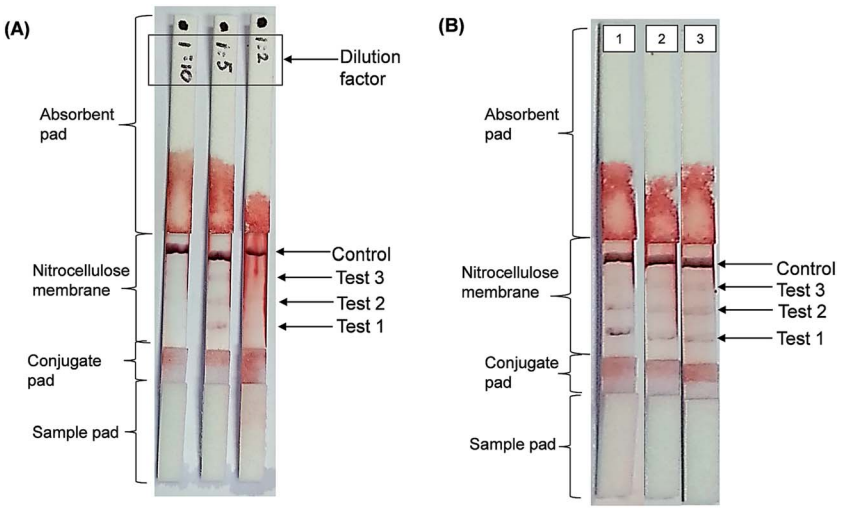

Fig. 7 (A) Dilution factors 1 to 2, 1 to 5, and 1 to 10 were tested to determine the ideal dilution factor for whole blood. Note: picture shown after 20 min of washing. (B) The hemoglobin A1c immunosensor tested with whole blood at 1 to 5 dilution factor, with three different concentrations (strip 1: 5% HbA1c; strip 2: 6.5% HbA1c; strip 3: 9.9% HbA1c).

(108 mmol mol^{-1}) of HbA1c. The linear concentration range between 4% and 12% HbA1c contained the critical treatment goal values of 6.5–7% HbA1c and therefore meets the demands of efficient diabetes control. However, there was a limitation for a semi-quantitative analysis *via* visual interpretation based on the number of test lines and its intensity beyond 9.1% HbA1c. As all the three test lines appeared and the degree of the intensity was quite similar after 9.1% HbA1c (Fig. 6D), it could be difficult for the end user to distinguish a higher level of HbA1c beyond this point. Nonetheless, by using the lateral flow reader to perform a quantitative measurement, the signal intensity of the test lines was found to be increasing in a HbA1c-dependent way beyond 9.1% of the HbA1c level.

3.4 Lateral flow immunosensor for real sample analysis (HbA1c in whole blood)

While the calibration curve is very useful to assess HbA1c levels in whole blood, it is also very important for the blood to readily flow through the strips without extensive and prolonged pre-treatment. Our study focused on measuring HbA1c levels in whole blood, which can often be too viscous to readily flow through the lateral flow test strips. To resolve this problem, manual dilution is required. In our experiment, the dilution of whole blood samples obtained from the University of Malaya Medical Center (UMMC) was performed with a haemolysis reagent (distilled water with blood stabilizers). The diluted whole blood (10 μL) was dispensed directly onto the NC membrane, which was washed with phosphate buffer containing Tween-20. At a 1 : 2 dilution, whole blood remains viscous to flow through the assay *via* capillary reaction. On the other hand, whole blood at a 1 : 10 dilution flowed readily across the assay matrix; however, the test lines generated were too weak for visual interpretation. Our results indicated that whole blood samples yield a HbA1c level-dependent signal at a dilution factor of 1 : 5 (Fig. 7A). A 1 : 5 dilution was the ideal dilution factor for whole blood to flow readily across the assay matrix with a low background while generating a visually interpretable signal within a reasonable assay time of approximately 25 min.

Once the ideal dilution factor was determined to be at 1 : 5, we performed a preliminary test on the whole blood samples that have different HbA1c values (5.5%, 6.5%, and 9.9% HbA1c). As shown in Fig. 7B, strip 1 tested with 5.5% HbA1c had only two test lines, a clear indication that it had the lowest HbA1c value tested. To distinguish which had higher HbA1c value tested, strip 3 (tested with 9.9% HbA1c) was shown to have higher intensity across all three test lines compared to strip 2 (tested with 6.5% HbA1c), thus reflecting that the HbA1c levels tested were highest among all of the strips. Hence, for diabetic patients with inadequately controlled blood glucose, *i.e.*, for those with HbAlc levels >6.5% (ref. 21) (or >7% in the United States[22]), the immunosensor will show intense dark red colour in all of the test zones, suggesting that immediate medical attention is needed. With distinctive patterns observed on the strip tested with groups of blood samples with different HbA1c levels, our immunosensor showed promising performance as a useful point-of-care device that caters to the need of user-friendly and inexpensive technology for better diabetic care in resource-limited settings.

4. Conclusions

In this work, we have manufactured a lateral flow HbA1c immunosensor that is simple, economical, and portable. The haemoglobin A1c lateral flow immunosensor has been optimized so that the gold conjugates were stabilized at pH 8.0, 30 μg mL^{-1}, and the ideal blocking reagent for the immunosensor was found to be 1% western blocking reagent (containing casein). In addition, the immunosensor was found to generate a specific signal from HbA1c, where blood colour did not interfere with the colour shown on the test lines and the control line. To

quantify the whole blood samples, the calibration curve at 1 : 5 dilution was found to be the ideal curve for the quantitative analysis.

With simple dilution from finger-prick blood, our prototype can be used with a standard image set (a similar concept to the pH paper colour scale) for certain ranges of HbA1c levels (normal range <6.0% HbA1c, under control range 6.5% to 7.0% HbA1c, elevated and needing medical attention range >7.0% HbA1c) where the patient can compare the colour intensity and interpret the result visually. With the construction of the calibration curve, future quantitative analysis can be conducted alongside with a lateral flow reader. We anticipate that our design developed here can be integrated with inexpensive detection technology like smartphone-based imaging[23] to enable point-of-care rapid evaluation for numerical results on HbA1c levels, thereby better facilitating on-site diabetes care in resource-limited settings.

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