



A Distance-Based Microfluidic Paper-Based Biosensor for Glucose Measurements in Tear Range

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Abstract

The prevalence of diabetes has increased over the past years. Therefore, developing minimally invasive, user-friendly, and cost-effective glucose biosensors is necessary especially in low-income and developing countries. Cellulose paper-based analytical devices have attracted the attention of many researchers due to affordability, not requiring trained personnel, and complex equipment. This paper describes a microfluidic paper-based analytical device (μ PAD) for detecting glucose concentration in tear range with the naked eye. The paper-based biosensor fabricated by laser CO₂; and glucose oxidase/horseradish peroxidase (GOx/HRP) enzyme solution coupled with tetramethylbenzidine (TMB) were utilized as reagents. A sample volume of 10 μ l was needed for the biosensor operation and the results were observable within 5 min. The color intensity-based and distance-based results were analyzed by ImageJ and Tracker to evaluate the device performance. Distance-based results showed a linear behavior in 0.1–1.2 mM with an $R^2=0.9962$ and limit of detection (LOD) of 0.1 mM. The results could be perceived by the naked eye without needing additional equipment or trained personnel in a relatively short time (3–5 min).

Keywords Microfluidic paper-based analytical device (μ PAD) · Glucose · Colorimetric detection · CO₂ laser · Paper · Tears

Introduction

Diabetes is a metabolic disorder that is the leading cause of mortality and health-related problems in developing countries [1–3]. According to the World Health Organization (WHO), approximately 422 million people have diabetes all around the world [4]. The self-monitoring of blood glucose, which can be obtained by glucose biosensors, is a technique to control this disease [5]. Other biofluids consisting of saliva, tears, sweat, interstitial fluid (ISF), and urine can be used to determine the blood glucose concentration [3, 6]. The main reason for utilizing these biofluids is to avoid finger pricks in the elderly, newborns, and

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hemophiliacs [7–9]. The important point about alternative biofluids is the correlation of glucose analyte in the biofluid and blood [10].

In comparison with other biofluids, the tear is more accessible and does not need any preparation, whereas the low glucose concentration in the tear and the low volume of tear are a number of the disadvantages of using the tear as biofluid in glucose sensors [9, 11–14]. The glucose concentration in the tear differs in various studies due to the sampling method [9, 15]. According to a study, a tear-based glucose biosensor should have a linear behavior in 0.1–3 mM [11].

Microfluidics has drawn the attention of many biosensor researchers over the past decade. Sensors with microfluidic technology can detect molecules in small sample volumes. Reduction of sample volume, response time, and improving sensitivity are some positive features of microfluidic diagnostic devices [16, 17]. The WHO has established ASSURED guidelines for such devices which stands for Affordable, Sensitive, Specific, User-friendly, Rapid and Robust, Equipment-free, and Deliverable to the end-users [18–20].

According to these guidelines, paper is an appropriate substrate for microfluidic devices due to its low cost, availability even in countries with limited resources, the capability to perform chemical reactions on the substrate and achieve acceptable sensitivity, not requiring preparation when performing assays, and fluid wicking through capillary action without the need to external pumps [7, 19, 21–24]. Microfluidic paper-based analytical devices (μ PADs) were first introduced by Whitesides in 2007 [25, 26]. μ PADs are widely used in colorimetric assays to detect glucose, uric acid, and other analytes in medicine. The easy immobilization of the reagents is another advantage of the porous structure of the paper, for example, the immobilization of glucose oxidase through a simple adsorption mechanism [22, 27–29].

Colorimetric assay is the most common detection method in the applications of μ PADs because of several advantages including employing affordable equipment for image acquisition (e.g., smartphone cameras, standard scanners, digital cameras, or portable microscopes). Color inhomogeneity is a challenge in colorimetric assays [15, 30].

For the glucose colorimetric detection, glucose oxidation occurs in the presence of glucose oxidase (GOx) enzyme; hence, hydrogen peroxide (H_2O_2) and gluconic acid are produced. The second reaction is based on the type of chromogenic reagent that can be pH-based (methyl red) or H_2O_2 -based. Potassium iodide (KI), 2,4,6-tribromo-3-hydroxybenzoic acid (TBHBA), 4-aminoantipyrine (4-AAP), 3,5-dichloro-2-hydroxy-benzenesulfonate (DHBS), 3-aminopropyltriethoxysilane (APTMS), 3,3'-diaminobenzidine (DAB), and 3,3',5,5'-tetramethylbenzidine (TMB) are a number of the well-known chromogenic reagents for H_2O_2 . When H_2O_2 -based reagent is used, HRP catalyzes the reaction of chromogenic reagent, and the H_2O_2 and a colored product are produced [21, 31–33].

In the colorimetric assay, the results can be analyzed by color analysis software or naked eye. ImageJ, GIMP, Quantity one software, and Adobe Photoshop are some well-known software for color analysis [26, 33–36]. In several studies, RGB and grayscale are mainly utilized to analyze color intensity [30, 33, 36]. In the naked-eye colorimetric methods, the results are analyzed without any need for complex equipment or software [6]. Time-based methods, ladder bar-based detection, and distance-based methods are some naked-eye detection assays in which the concentration of the analyte is related to the analysis time, the number of colored columns, and the length of the color change, respectively [30, 37–39]. In the distance-based methods, the analyte quantification is carried out by the length of the color change to decrease the user's individual error [38]. It can also adapt the dynamic range by changing the reagent concentration and geometry [39].

For the fabrication of a cellulose paper-based device, cost and resolution are the most important factors in process selection. There are several methods for the fabrication of μ PADs. These fabrication techniques are classified into two general classifications: physical and chemical. In physical manufacturing processes, a hydrophobic material is added to the paper and hydrophilic channels are developed, while in chemical methods, adding chemical materials change the hydrophilic property of the paper [40, 41].

In laser cutting, the cellulose paper is cut, and a two-dimensional device is fabricated. The high resolution, not requiring hydrophobic materials for channel fabrication, rapid fabrication, and mass production, is the interesting feature of laser CO₂ cutting. A disadvantage of this physical fabrication technique is the need for expensive equipment and careful selection of the power and laser rate to prevent paper burning [40, 41]. The paper-based device which is fabricated by the laser is not as rigid as μ PADs fabricated by other methods. Thus, a plastic film as backing or sealing is required for the packaging. The packaging has various advantages such as preventing μ PAD contamination, sample evaporation, and leakage. Pressure-sensitive tapes are mainly utilized as sealing films [30, 41].

This study aims to design and fabricate a colorimetric glucose μ PAD for tear analysis. The device is fabricated from cellulose filter paper by CO₂ laser, and pressure-sensitive adhesive tape is utilized for sealing. GOx, HRP, and TMB are reagents, and tear range concentrations of glucose solutions are used. The assay is recorded by a smartphone camera, while the color intensity-based and distance-based results are analyzed by ImageJ (in greyscale) and Tracker, respectively. Various factors such as the type of chromogenic reagent, immobilization, method of adding TMB and its concentration, and other factors are examined.

Materials and Methods

Materials and Chemical Agents

Glucose oxidase (GOx) (Merck, Germany) from *aspergillus niger*, horseradish peroxidase (HRP) (Merck, Germany), 3,3,5,5-tetramethylbenzidine (TMB) (BioBasic Inc, Canada), glucose (Merck, Germany), polyvinylpyrrolidone (PVP) (Merck, Germany), potassium iodide (KI), silica nanoparticle (SiNP, Fadak, Iran), methanol (Arman Sina, Iran), and phosphate-buffered saline (PBS, pH=7.6) (Merck, Germany) were purchased. All chemicals were used as received. Filter paper (80 g/m²) with 50% porosity and transparent pressure-sensitive adhesive tape (TPSA) were used as the substrate (hydrophilic channel) and sealing film, respectively.

Equipment and Software

A CO₂ laser engraving cutting machine (Perfect, China) operating at a wavelength of 10.64 μ m at 14 W was utilized for cutting cellulose filter paper. Images and videos were captured by a smartphone camera (iPhone 11, USA). CorelDRAW (Corel Corporation, 2018), Tracker (Open Source Physics Java framework, 5.1.3), and ImageJ (National Institutes of Health, 1.52a) were used for the channel design, distance-based measurement, and color intensity-based image processing, respectively. The images were captured in a specific room and under a particular light and finally subtracted by a defined value in ImageJ to eliminate the background effect.

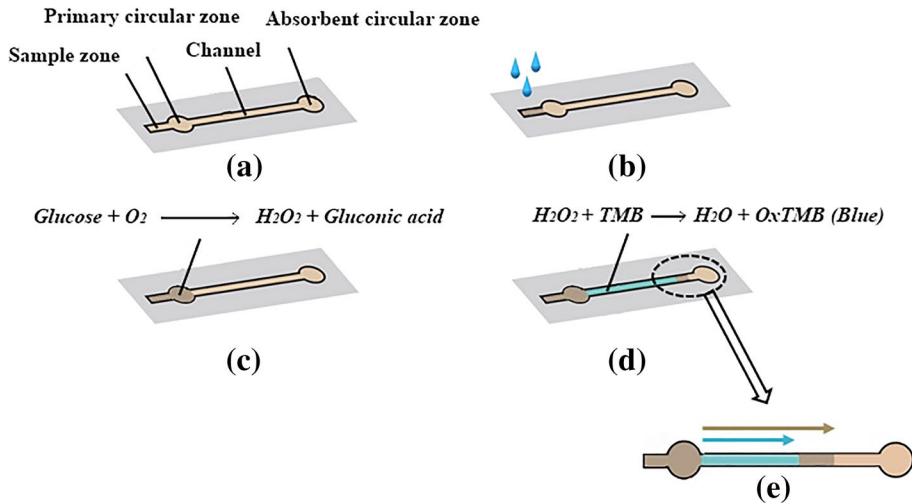


Fig. 1 The simplified schematic of the colorimetric reaction on the designed device in the presence of enzyme solution (GOx and HRP) and chromogenic reagent (TMB) (a) Schematic view of the designed device and different zones. (b) Addition of glucose sample to the device. (c) Glucose oxidation in the presence of enzymes and H_2O_2 production in primary circular zone. (d) H_2O_2 reaction with TMB and production of blue oxidized TMB in the channel. (e) The colored length and the wetted length vectors are displayed in blue and brown vectors, respectively

μPAD Design and Fabrication

Hydrophilic patterns were designed in CorelDRAW 2019. The device consisted of a sample zone ($2 \times 5 \text{ mm}^2$), a primary circular zone (diameter = 5 mm), a detection zone (channel, $2 \times 25 \text{ mm}^2$), and a circular absorption zone (diameter = 5 mm). The designed μPAD and different zones are demonstrated in Fig. 1a. The patterns were cut by a CO_2 laser engraving cutting machine ($\lambda = 10.64 \text{ μm}$, 14 W, 14 mm/s). Reagents were added to different zones and the device was packed with a common TPSA to prevent the sample from leakage and evaporation.

Enzymatic Reactions of Glucose Colorimetric Detection

The device was dipped in a PVP solution to improve the immobilization of the chemical agents. An enzyme solution containing 120 U/ml of GOx and 30 U/ml of HRP was added to the primary circular zone. The chromogenic solution containing TMB was dissolved in methanol and added to the channel called the detection zone. The role of the final circular zone was to absorb the extra fluid. The enzyme solution and chromogenic agent zone was investigated in various experiments and adding them to the primary circular zone and channel, respectively, was selected to achieve acceptable distance-based results.

Different concentrations of 10-μl glucose solution were added to the sample zone and wicked towards the primary circular zone by capillary action. Glucose was oxidized in the presence of GOx and the products (gluconic acid and hydrogen peroxide) entered the detection zone. H_2O_2 reacted with TMB in the presence of HRP and the blue oxidized TMB was

produced in the detection zone. Second, circular zone absorbed excessive water and by-products. The schematic of the reactions is illustrated in Fig. 1b, Fig. 1c, and Fig. 1d.

Procedure for Color Detection

The colorimetric assay video was captured by a smartphone camera and analyzed by ImageJ and Tracker to achieve intensity-based and distance-based results, respectively. For the intensity-based results, the video frame was imported into ImageJ and digitized in grayscale.

The cellulose paper is white, and the color intensity decreases after the chemical reaction, so more glucose concentration results in lower color intensity. The inverse grayscale was used to directly correlate color intensity and glucose concentration (inverse grayscale = 255—grayscale).

The video was imported in Tracker for the distance-based results. A vector determined the wetted length or colored length at different time intervals (from 0 to 240 s, 0 assumed the moment that the sample entered the channel). The initial and terminal points of the vector were the beginning of the channel and the end of the wetted or colored area (Fig. 3b). Finally, the magnitude of the vector over time diagram was plotted.

The wetted-length diagram showed the possibility of the glucose sample wicking feasibility along the channel, whereas the colored-length diagram indicated the presence of glucose and its reaction with other reagents. Therefore, when the wetted length is more than the colored length, it means the glucose had been finished during the reactions, and the sample without glucose was wicked towards the end of the channel as can be observed in Fig. 1e.

Evaluation of Different Factors

Various parameters were investigated to realize the performance of the μ PAD through color intensity-based and distance-based results. The type of the chromogenic reagent, type of the immobilization solution, method of adding the TMB reagent, TMB solution concentration, enzyme solution volume were investigated.

Type of Chromogenic Reagent

TMB (15 mM) and KI (1.2 M) were used as chromogenic reagents. The color intensity- and distance-based results were the evaluation factors. Each test was carried out four times for statistical clarity.

Type of the Immobilization

The disadvantage of not using an immobilizer is that the color change could flow along the channel, and consequently, the intensity-based and distance-based results could be inaccurate. Therefore, three types of immobilizations were utilized, and wetted length, colored length, and color intensity were compared with the immobilization-free experiment.

The immobilization agents were PVP solution (concentration 1%, dissolved in PBS) and SiNP (10–15 and 100 nm, dispersed in deionized water). Each test was carried out four times for statistical clarity.

Method of Adding TMB

The amount of chromogenic solution can affect the colored-length and intensity-based results. Four methods of adding TMB were defined:

1. Adding solution by a micropipette uniformly as much as the channel became wet; it is called uniform method (UM).
2. Adding the solution by a micropipette every 5 mm of the channel; it is called non-uniform method (NUM).
3. Adding solution twice by a micropipette uniformly with an interval of 5 min to allow the first series of TMB solution to be dried; it is called twice uniform method (2UM).
4. Dipping the secondary circular zone in the TMB solution and allowing the TMB to wick into the channel is called saturated method (SM) because most of the paper pores were filled and saturated with the chromogenic solution.

Each test was carried out four times for statistical clarity.

The Concentration of TMB

Different concentrations of TMB solutions (2.5, 3, 3.75, 5 mM) were used as chromogenic reagents to have an appropriate colored length for the tear glucose concentration. Each test was carried out four times for statistical clarity.

The Volume of Enzyme Solution

The enzyme solutions were added to the primary circular zone to investigate the effect of the enzyme volume on the color intensity-based results. For this purpose, 3 μl , 4 μl , and dipping the circular zone in enzyme solution (saturated method, SM) were examined to obtain adequate volume for the solution. Each test was carried out four times for statistical clarity.

Distance-Based Biosensor Evaluation

According to previous experiments, the final test was carried out by adding the 3 μl of the enzyme solution to the primary circular zone, and the channel of the device was saturated with TMB solution, as the chromogenic factor, with the concentration of 3.75 mM. Various concentrations of glucose samples in tear range (0.1, 0.2, 0.4, 0.8, and 1.2 mM) were added to the sample zone and the colored length was measured. Each test was carried out four times for statistical clarity.

Results and Discussion

Evaluation of Different Factors

To achieve an appropriate performance for the μPAD glucose biosensor, various factors were evaluated and color intensity-based and distance-based results were obtained considering the type of the test.

Type of Chromogenic Reagent

TMB and KI were used as the chromogenic solution and glucose solutions with a concentration of 2.5, 5, 10, and 20 mM were used as samples (only length-based results of 10-mM glucose sample are displayed to reduce the article volume). The velocity of the glucose solution (slope of the plot) in the wetted-length plot (Fig. 2a) was more when TMB was used as a chromogenic solution and it reached the end of the channel earlier. Moreover, the color change occurred immediately after adding the glucose sample in the presence of TMB, while for KI it occurred 3 min after the sample addition; therefore, the colored bar for KI was showed up at 120 s (Fig. 2b); furthermore, the colored length for

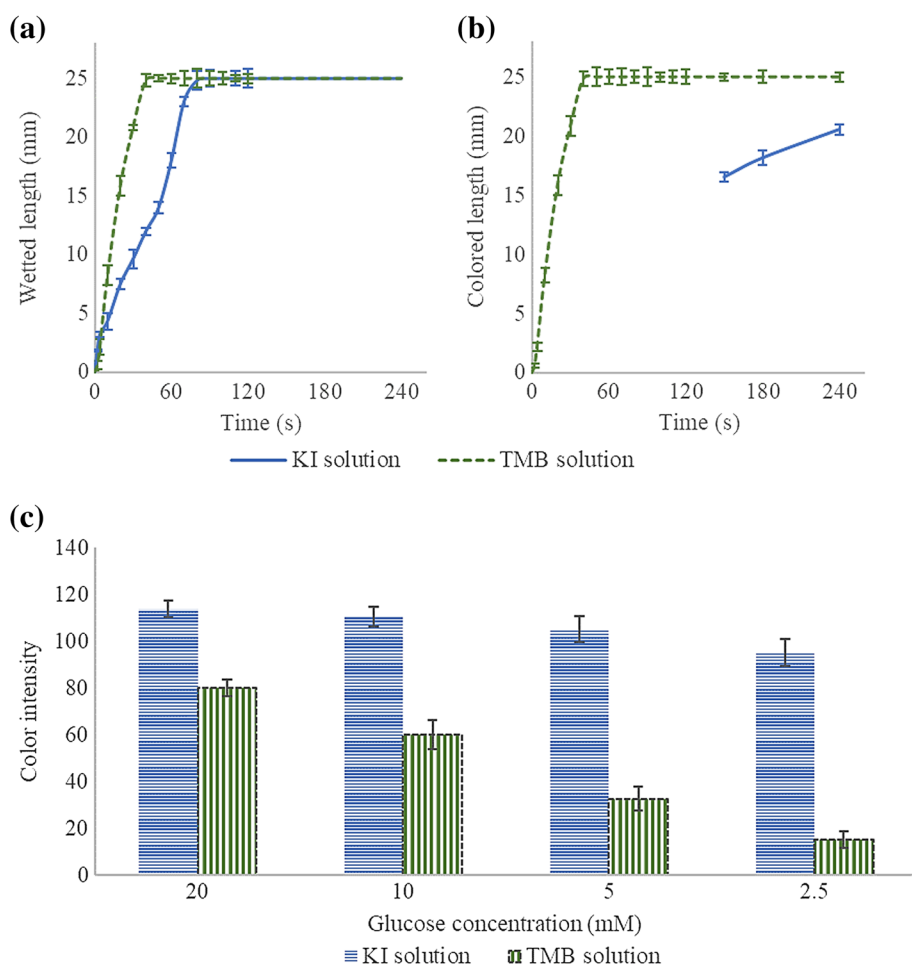


Fig. 2 (a) Wetted-length diagram for 10 mM of glucose solution, the solution moved faster in the presence of TMB compared with KI solution. (b) Colored-length diagram for 10 mM of glucose solution, earlier color change in TMB solution compared with KI solution. (c) Color intensity diagram for various glucose concentrations, more discrepancy in color intensity of various concentrations when TMB solution is used as a chromogenic reagent

the TMB solution was significantly longer. As indicated in the color intensity diagram (Fig. 2c), the color intensity was more when KI was used as a chromogenic solution but the color intensity discrepancy was low. According to results, although KI solution resulted in higher color intensity (Fig. 2c), TMB solution made the color change appear earlier (Fig. 2b); therefore, TMB solution is used for further analysis.

Type of the Immobilizer

Three types of immobilizers (PVP solution and SiNP in different sizes of 10–15 nm and 100 nm) were utilized for 2.5, 5, 10, and 20 mM of glucose (only results of 10 mM are depicted). As indicated in Fig. 3a, the immobilizer affected the sample velocity and color intensity. The glucose solution could reach the end of the channel faster in immobilizer-free assay. Moreover, increasing nanoparticle size resulted in the deceleration of glucose solution wicking along the channel when SiNP was utilized as an immobilizer. The color

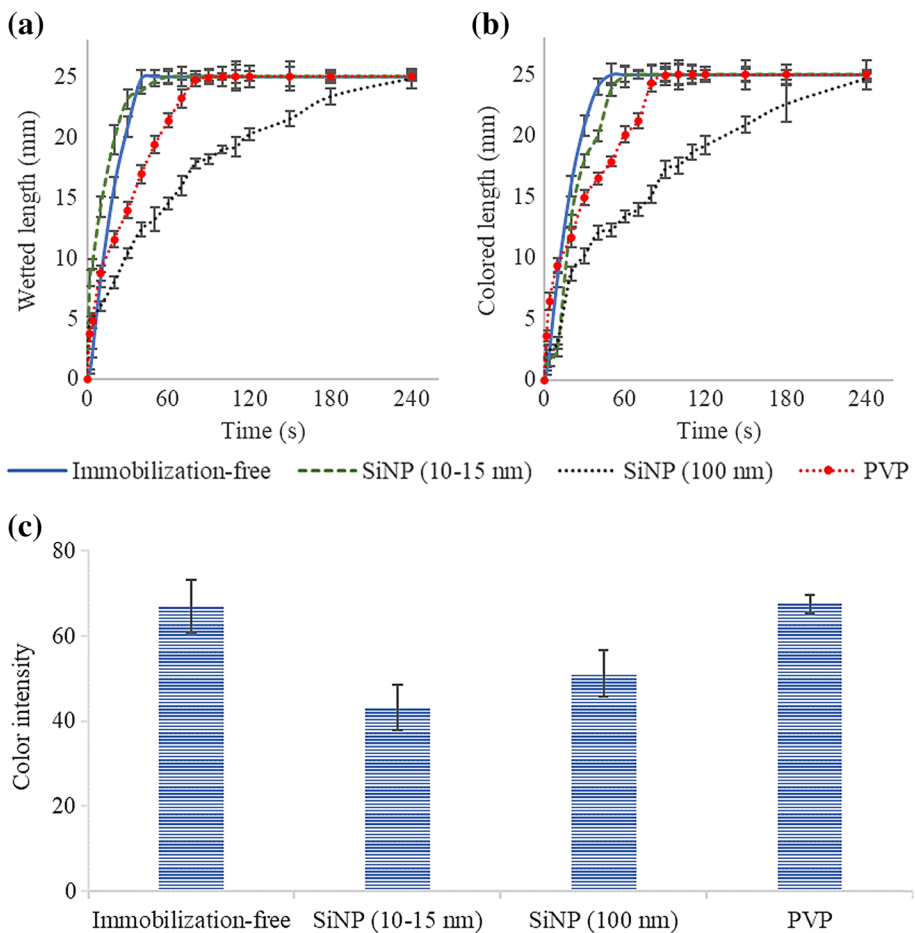


Fig. 3 (a) Wetted-length diagram for 10 mM of glucose when the effect of immobilization was examined. (b) Colored-length diagram (c) The greyscale color intensity when various immobilization solutions were used for 10 mM of glucose

change occurred faster when the immobilization agent was not used (Fig. 3b). According to the color intensity diagram (Fig. 3c), using a PVP solution for immobilization enhanced the color intensity compared to other assays. Besides, when the immobilizer was not utilized, the color change could flow along the channel and affect the distance-based results. Therefore, the PVP solution is used for future investigations.

Method of Adding TMB

Four methods of adding TMB by the concentration of 15 mM (UM, NUM, 2UM, and SM) were investigated for 2.5, 5, 10, and 20 mM of glucose (only results of 10 mM are depicted). As illustrated in Fig. 4a, the velocity of the sample decreased with increasing TMB owing to the reduction of pores that help fluid wicking; therefore, faster wicking of glucose sample was observed when lower TMB solution was used. Additionally, colored length was significantly longer in SM in comparison with UM due to the consumption and running out of the whole glucose in a shorter channel length. In UM assay, the final colored length equaled the channel length which shows that all the glucose did not participate in the reaction, and consequently, the amount of TMB was not adequate for such amount of glucose (Fig. 4b).

When a colored-length diagram for various glucose concentrations in the SM is plotted, a gentle behavior can be observed between the colored length and glucose concentration (Fig. 4c). According to Fig. 4c, the colored length increased in the presence of more glucose concentration and did not change after 150 s. The colored length in a specified time (150 s) over the glucose concentration is illustrated in Fig. 4d, and more colored length was observed in the presence of more glucose concentration. Since the tear glucose range is less than 1.2 mM and also the colored length for this concentration is under 5 mm, the concentration of TMB solution should be calibrated and different TMB concentrations should be examined. The effect of TMB concentration is investigated in future tests. The color intensity for different assays was approximately the same except when TMB was added nonuniformly (NUM) because of the lower TMB solution volume and finally the lower products and color change (Fig. 4e).

The Concentration of TMB

TMB solutions with concentrations of 2.5, 3, 3.75, and 5 mM were evaluated. The glucose concentration was in the tear glucose range (0.1–1.2 mM). As mentioned in previous experiments (Method of Adding TMB), results could be achieved in a distance-based manner, so the colored-length in 150 s (2.5 min) is measured, and wetted-length and colored-length results are neglected. As demonstrated in Fig. 5a, the colored length increased by reducing the TMB concentration. This is actually due to the earlier running out of the glucose in more TMB concentration through the reaction and consequently very low colored length; therefore, the concentration of 5 mM TMB was not appropriate for the tear range glucose. The colored length for 2.5, 3, and 3.75 mM was very similar. The color intensity was increased by increasing the TMB concentration (Fig. 5b). The concentration of 3.75 mM of TMB solution is selected for further tests because of more colored length and higher color intensity.

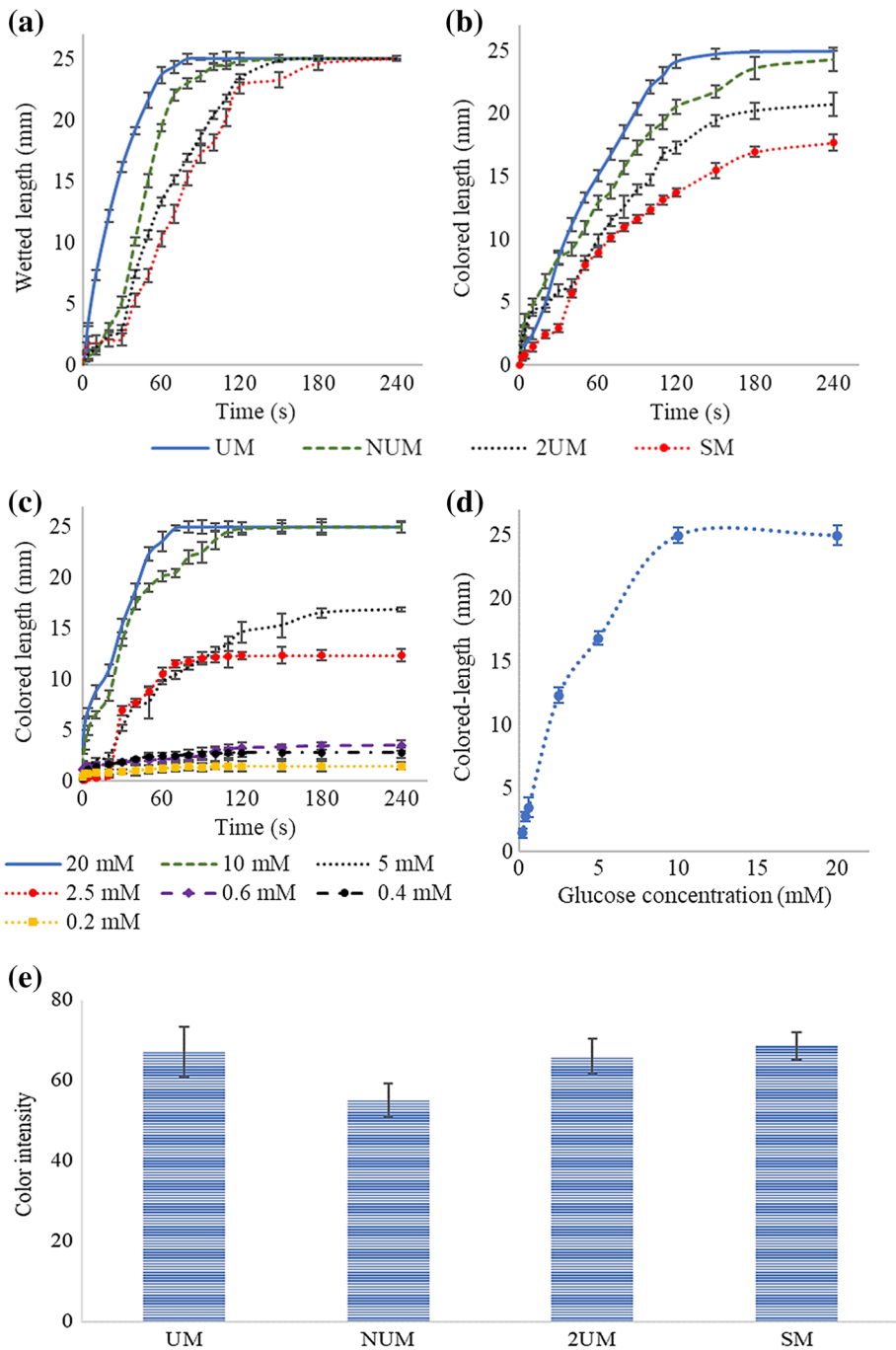


Fig. 4 (a) Wetted-length diagram in the method of adding TMB solution experiments for 10 mM of glucose. (b) Colored-length diagram, decrease colored length in the presence of more TMB as earlier running out of the glucose. (c) Colored-length over time diagram for various glucose concentrations. (d) Colored length diagram for various glucose concentrations in 150 s. (e) Color intensity diagram for different methods of adding TMB solution for 10 mM of glucose

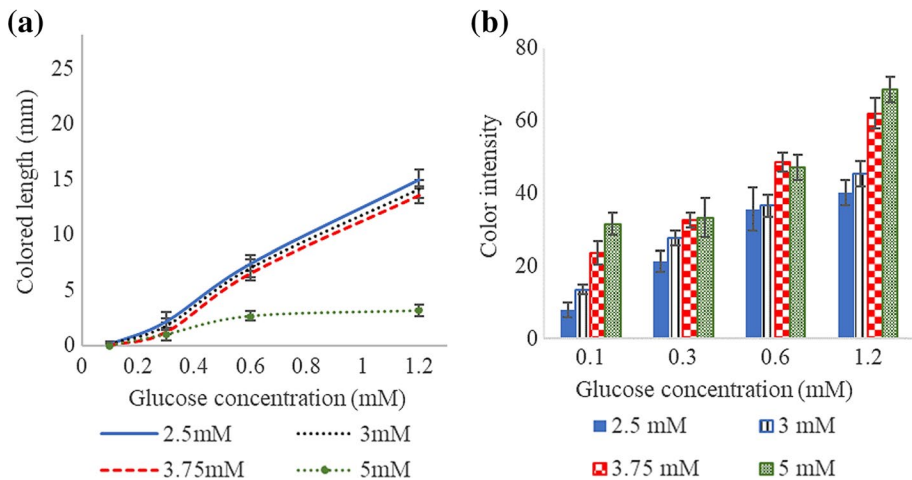


Fig. 5 (a) Colored-length diagram in 150 s when different TMB concentrations were used, more colored length in lower TMB concentration. (b) Color intensity diagram, more grayscale intensity for the 3.75 mM of TMB

The Volume of the Enzyme Solution

The enzyme solution volumes of 3 μl , 4 μl , and SM were examined. According to the colored-length over glucose concentration diagram, using a lower amount of enzyme solution offered more colored length (Fig. 6a). The color intensity was decreased by increasing the enzyme solution amount specially in higher concentrations of glucose (Fig. 6b). Therefore, 3 μl of the enzyme solution was selected.

Distance-Based Glucose Detection Assay

The glucose solution with a concentration of 0.1–1.2 mM was utilized. The colored length showed a linear relationship to glucose concentration. The linear equation obtained colored length = $13.4 \times (\text{glucose concentration}) - 0.7653$ with an $R^2 = 0.9962$ (Fig. 7).

Conclusion

Diabetes is a common disorder all over the world. Self-monitoring of blood glucose is one of the ways of controlling diabetes and is carried out by glucose biosensors [1, 17]. Nowadays, μPADS are widely used in the field of medicine, environment, and food monitoring because of various features such as paper substrate material abundance, fluid flow through capillary action passively, and easy enzyme immobilization [18, 22, 33, 42]. In the present study, a minimally invasive glucose biosensor was fabricated to determine the tear range glucose sample through a distance-based colorimetric assay. The glucose biosensor was fabricated on paper by a CO_2 laser. The enzyme solution contained GOx 120 U/ml and HRP 30 U/ml. TMB with a concentration of 3.75 mM was selected as a chromogenic solution. The reagents were added to the specified zones after dipping the μPAD in

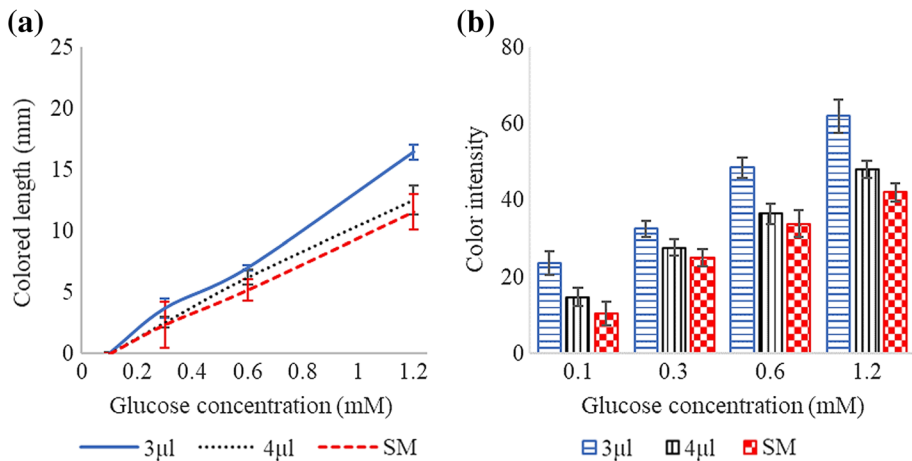


Fig. 6 (a) Colored-length diagram in 150 s when different volumes of enzyme solution were used, increasing colored length by decreasing enzyme solution volume. (b) More color intensity by lowering amount of enzyme solution in various glucose concentrations

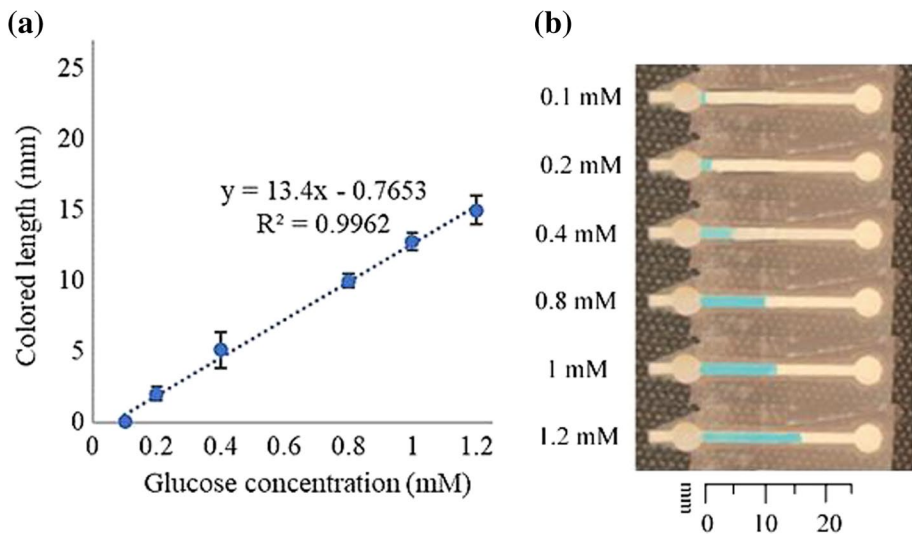


Fig. 7 (a) The linear behavior of the device for different glucose concentrations in tear range. (b) Different colored length in various glucose concentrations

an immobilizer (1% PVP). According to the assay, the wetted length, colored length, and inverted grayscale color intensity were measured. Various concentrations of 10-μl glucose solution were added to the sample zone. For the distance-based assay, the colored length was measured at a specific time (150 s). Since the color change of the TMB solution is unstable, the naked-eye results could be observed within 5 min. The dynamic range of the device was 0.1–1.2 mM with a limit of detection (LOD) of 0.1 mM.

Table 1 Summary of various studies and comparison with the present study

Sample	Sample volume (μl)	Reagent	Dynamic range (mM)	LOD (mM)	Assay duration (min)	Analyze method	Fabrication method	Novelty	Reference
Tear	5	GOx, HRP, TMB, Chitosan	0.1–1	0.5	15	Color intensity (Photo-Paint software)	Wax printing	• Glucose measurement in tear range	[15]
Tear	6	GOx, HRP, 4-AAp	0.1–1.4	0.1	-	Color intensity	Wax printing	• Using Schirmer test strips as the substrate	[43]
Saliva	2	GOx, HRP, TBHBA, 4-AAp	0.05–1.5	0.05	-	Color intensity (GIMP software)	Stamp patterning		[44]
Human serum	20	GOx, HRP, DAB, AgNP	0.6–15	0.6	< 10	Distance based	Wax printing	• Glucose measurement without need to equipment	[38]
Glucose solution in tear range	10	GOx, HRP, TMB, PVP	0.1–1.2	0.1	2.5	Distance based	CO ₂ cutting laser	• Low concentrations of glucose measurement without need to equipment • Possibility of sampling by the device • Easy fabrication technique without requirements to hydrophobes	Present study

Gabriel et al. fabricated a wax-printed μ PAD for tear glucose detection. TMB and chitosan were utilized as chromogenic and immobilization solutions, respectively. The sample volume was 5 μ l and color intensity-based results were achieved by Corel Photo-Paint software. The LOD was 0.5 mM with a dynamic range of 0.1–1 mM [15]. In another study, the Schirmer test strips were wax printed for channel preparation for tear glucose analysis. 4-AAP was used as the chromogenic reagent and LOD of 0.1 mM with the dynamic range of 0.1–1.4 mM was achieved. The results were analyzed by the RGB color codes and the sample volume was 6 μ l [43]. *Jiménez et al.* fabricated a stamp patterning μ PAD for the detection of glucose in saliva. The color intensity-based results were obtained by GIMP software with the LOD of 0.05 mM and a dynamic range of 0.05–1.5 mM. Two-microliter glucose sample was used and TBHBA and 4-AAP were the chromogenic reagents [44]. In another study, the distance-based results were obtained with 20 μ l of glucose solution. The dynamic range was 0.6–15 mM and LOD of 0.6 mM. DAB was a chromogenic solution [38]. The summary of recent studies is in Table 1.

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Author Contribution Samira Allameh was an MSc. student in biomedical engineering and she did this study as a master thesis under the supervision of Dr. Mohsen Rabbani. Samira wrote the manuscript and Mohsen modified and prepared it for publication.

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Availability of Data and Material All useful data obtained during the study are available.

Declarations

Ethics Approval This research does not contain any studies with human participants or animals performed by any of the authors.

Consent to Participate This research does not contain any studies with human participants performed by any of the authors.

Consent for Publication Not applicable.

Conflict of Interest The authors declare no competing interests.

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