A distance-based microfluidic paper-based biosensor for glucose measurements in tear range

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Research Article

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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 for the detection of glucose in tear with the naked eye. The paper-based biosensor fabricated by laser CO_2, and GOx/HRP enzymatic solution coupled with TMB was utilized as reagents. A sample volume of 10 µl was needed for the biosensor operation and the results were observable within 5 minutes. To evaluate the device performance, color intensity-based and distance-based results were analyzed by ImageJ and Tracker. Distance-based results showed a linear behavior in the range of 0.1–0.6 mM with an $R^2 = 0.967$ and LOD of 0.2 mM. The results could be perceived by the naked eye without any need to further equipment or trained personnel in a relatively short time (3–5 minutes). Moreover, glucose concentration could be obtained non-invasively by tears collected by this µPAD.

1. Introduction

Diabetes is a metabolic disorder that is the major reason for mortality and health-related problems in developing countries (Martinkova and Pohanka 2015; Turner and Fragkou 2008). According to the World Health Organization (WHO), about 422 million people have diabetes all over the world (2020). The self-monitoring of blood glucose, which can be obtained by glucose biosensors, is one way to control this disease (Yoo and Lee 2010). Microfluidics has attracted the attention of many biosensor researchers over the last decade. Sensors that have microfluidic technology can detect molecules in small sample volumes. Reduction of the sample volume, response time, and improving sensitivity are some of the positive features of microfluidic diagnostic devices (Demirci et al. 2012; Selmi et al. 2017). WHO established ASSURED guidelines for such devices which stand for Affordable, Sensitive, Specific, User-friendly, Rapid and Robust, Equipment-free, and Deliverable to the end-users (Walji and Science 2015).

According to such guidelines, paper is an appropriate substrate for microfluidic devices due to the low cost, availability even in low-resources countries, the capability to perform chemical reactions on the substrate and achieving acceptable sensitivity, not requiring preparation when performing assays, and fluid wicking through capillary action without the need to external pumps (Chandra Sekar et al. 2014; Liu et al. 2016; Songok and Toivakka 2016; Walji and Science 2015).

Microfluidic paper-based analytical devices (µPADs) were firstly introduced by *Whitesides* in 2007 (Chen et al. 2012). µPADs are widely used in colorimetric assays for the detection of glucose, uric acid, and other analytes in medicine. Moreover, these devices are advantageous in the field of environmental and food monitoring (Fan et al. 2018). Paper has a porous structure and the fluid can be wicked passively through pores by the means of capillarity. Easy immobilization of reagents is another advantage of the

paper porous structure, e.g., immobilization of glucose oxidase through a simple adsorption mechanism (Chandra Sekar et al. 2014; Kuek Lawrence et al. 2014; Zhou et al. 2014).

Colorimetric assay is the most common detection method in the applications of μPADs because of several advantages such as employing affordable and inexpensive equipment for image acquisition (e.g., smartphone camera, common scanners, digital cameras, or portable microscopes). Color inhomogeneity is a challenge in colorimetric assays (Gabriel et al. 2017).

For the colorimetric detection of glucose, the oxidation of glucose occurs in the presence of glucose oxidase (GOx) enzyme, and hence hydrogen peroxide (H_2O_2) and gluconic acid are produced (Gabriel et al. 2016; Liu et al. 2016; Morbioli et al. 2017). The second reaction is based on the type of chromogenic reagent that can be pH-based (methyl red) (Soni and Jha 2015) or H_2O_2-based (Liu et al. 2016). 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 some of the famous chromogenic reagent for H_2O_2 (Liu et al. 2016). 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 (Gabriel et al. 2016; Liu et al. 2016; Morbioli et al. 2017).

Costa et al. used HRP, GOx, KI, 4-AAP, and DHBS as reagents. (Costa et al. 2014). In another study, a tree-like μPAD in the presence of GOx, HRP, 4-AAP, and TBHBA as reagents with the limit of detection (LOD) of 0.3 mM in serum was developed (Zhu et al. 2014). *Gabriel et al.* established a glucose μPAD and obtained LOD of 0.1 mM. GOx, HRP, and TMB were used as reagents and chitosan as an immobilizer (Gabriel et al. 2017).

In the colorimetric assay, the results can be analyzed by color analysis software or naked-eye. ImageJ (Fatoni et al. 2016), GIMP (Soni and Jha 2015), Quantity one software (Zhu et al. 2014), Adobe Photoshop (Chen et al. 2012), Adobe Illustrator, GenePix, Digital Color Meter, and Corel Photo-Paint are some well-known software for color analysis (Nery and Kubota 2013). To analyze the color intensity, RGB (Soni and Jha 2015) and grayscale (Jin et al. 2017) are mostly utilized in various studies.

Dominguez et al. established a low-cost glucometer for the salivary samples with LOD of approximately 0.009 mM using the RGB scale. *Fatoni et al.* recorded the color change by a scanner and analyzed the colorimetric results by ImageJ (RGB scale) (Jiang et al. 2012). In another study, researchers used a scanner for image acquisition and GIMP (RGB scale) for color intensity analysis (Soni and Jha 2015). *Chen et al.* established a μPAD for the detection of glucose and uric acid. Images were recorded by the Gel Documentation system and camera and the results were analyzed by Quantity One (greyscale) and Photoshop, respectively (Chen et al. 2012).

In the naked-eye colorimetric methods, the results are analyzed without any need to complex equipment or software (Radhakumary and Sreenivasan 2011). Time-based methods, ladder bar-based detection, and

distance-based methods are some of the 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 (Cate et al. 2013; Lewis et al. 2012; Tian et al. 2016).

In the distance-based methods, the analyte quantification is carried out by the length of the color change. This can decrease the individual error and dependence of results to the user (Cate et al. 2013). It can also adapt the dynamic range by changing the reagent concentration and geometry (Tian et al. 2016). *Cate et al.* have developed a simple distance-based glucose μPAD. Results could be read after 15-20 min with the LOD of 0.6 mM (Cate et al. 2013).

To obtain the blood glucose concentration, other biofluids such as saliva, tear, sweat, interstitial fluid (ISF), and urine can be used (Radhakumary and Sreenivasan 2011). The main reason for utilizing these biofluids is to avoid finger prick in the elderly, newborns, and hemophiliacs (Jung et al. 2017). The important point about alternative biofluids is the correlation of the glucose analyte in the biofluid and blood (Dominguez et al. 2017).

In comparison with other biofluids, the tear is more accessible and does not require further preparation. Whereas, low glucose concentration in tear and low volume of tear, are some of the disadvantages of using the tear as biofluid in glucose sensors (Bandodkar and Wang 2014; La Belle et al. 2016; Lane et al. 2006; Zhang et al. 2011). The glucose concentration in the tear in various studies is different due to the method of sampling (Gabriel et al. 2017). According to a study, a tear-based glucose biosensor should have a linear behavior in 0.1-3 mM (Lane et al. 2006).

The glucose concentration in the tear in various studies is different due to the method of sampling. In a study, the glucose concentration was found 0.1-0.6 mM (Gabriel et al. 2017), while in another study the fasting tear glucose was 0.1-0.3 mM in healthy people and 1.0-1.2 mM in diabetic patients (Kang et al. 2017). According to a study, a tear-based glucose biosensor should have a linear behavior in 0.1-3 mM (Lane et al. 2006). *Gabriel et al.* and *Kang et al.* developed μPAD tear glucose biosensor and obtained LOD equal to 0.05 mM and 0.1 mM, respectively (Gabriel et al. 2017; Kang et al. 2017).

For the fabrication of a cellulose paper-based device, cost and resolution are the most important factors for the selection of the method. The cost involves the cost of equipment and materials, and the appropriate resolution should be lower than 0.2 mm. There are various methods for the fabrication of μPADs. These fabrication techniques are classified into two general classifications: physical and chemical (He et al. 2015).

In physical fabrication techniques, a hydrophobic material is added to the paper and hydrophilic channels are developed. In chemical methods, the hydrophilic property of the paper is changed by adding chemical materials (He et al. 2015; Yetisen et al. 2013).

In laser cutting, the cellulose paper is cut and a two-dimensional device is fabricated. 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. On the other hand, the high resolution, not requiring hydrophobic materials for channel fabrication, rapid fabrication, and mass production are some of the interesting features of laser CO_2 cutting (He et al. 2015; Yetisen et al. 2013).

The paper-based device which is fabricated by the means of the laser is not as rigid as μPADs fabricated by other methods. Thus, a plastic film as backing or sealing is required for the packaging. Packaging has various advantages such as preventing μPAD contamination, sample evaporation, and leakage. Pressure-sensitive tapes are mostly utilized as sealing films (Yetisen et al. 2013).

This study aims to design and fabricate a colorimetric glucose μPAD for tear analysis. The device is fabricated from cellulose filter paper by CO_2 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 investigated.

2. Methods

2.1. 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.

2.2. Equipment and software

A CO_2 laser engraving cutting machine (Perfect, China) operating at a wavelength of 10.64 μm at 14 W was utilized for cutting cellulose filter paper. Images and videos were captured by a smartphone camera (iPhone 11, USA).

CorelDRAW (2018), Tracker (5.1.3), and ImageJ (1.52a) were used for the channel design, distance-based measurement, and intensity-based image processing, respectively.

2.3. μPAD design and fabrication

Hydrophilic patterns were designed in CorelDRAW 2019. The device consisted of a sample zone, primary zone, detection zone, and absorbent zone, Fig. 1. The patterns were cut by a CO_2 laser engraving cutting

machine (λ=10.64 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.

2.4. Enzymatic reactions of glucose colorimetric detection

The device was dipped in a PVP solution to improve the immobilization of the chemical agents. An enzymatic 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 absorbing the extra fluid. The zone of the enzymatic solution and chromogenic agents was investigated in various experiments and adding them to the primary circular zone and channel, respectively, was selected to achieve acceptable distance-based results.

Three μl of glucose solution in different concentrations was 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. The excessive products (containing water) were absorbed by the second circular zone to prevent changing the color intensity.

2.5. 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 frame of the video was imported into ImageJ and digitized to grayscale.

The cellulose paper is white and the color intensity decreases after the chemical reaction, so more concentration of glucose results in lower color intensity. The inverse grayscale was used to have a positive correlation between color intensity and glucose concentration (inverse grayscale = 255 - grayscale).

The video was imported in Tracker for the distance-based results. The wetted length or colored length was determined by a vector at different time intervals (from 0 to 360 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 shows the possibility of the glucose sample wicking feasibility along the channel, whereas the colored-length diagram indicates 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.3.

2.6. Evaluation of different factors

Various parameters were investigated to realize the performance of the μPAD 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, concentration of the TMB solution, and volume of the enzymatic solution were investigated.

2.6.1. 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.

6.6.2. Type of the immobilization solution

The disadvantage of not using an immobilization solution 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 immobilization solutions were utilized and the color intensity and wetted-length and colored-length results were compared with the immobilization-free experiment.

The immobilization agents were PVP solution (concentration 1%, dissolved in PBS), SiNp (10-15 and 100 nm, dispersed in deionized water).

2.6.3. Method of adding TMB solution

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

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

2.6.4. The concentration of TMB solution

Different concentrations of TMB solutions (2.5, 3, 3.75, 5mM) were used as chromogenic reagents to have an appropriate colored length for the tear glucose concentration.

2.6.5. The volume of enzymatic solution

The enzymatic 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 enzymatic solution (saturated-type) were examined to obtain adequate volume for the solution.

2.6.6. Distance-based biosensor evaluation

According to previous experiments, the final test was carried out by adding the enzymatic solution to the primary circular zone, and TMB solution as the chromogenic factor to the channel of the device. Various concentrations of glucose samples in tear range glucose (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 at least 3 times for statistical clarity.

3. Results And Discussion

3.1. 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.

3.1.1. Type of chromogenic reagent

TMB and KI were used as the chromogenic solution and glucose solutions with a concentration of 2.5, 5, and 20 mM were used as samples (to reduce the article volume, only length-based results of 2.5 mM glucose sample are displayed). The channel color change in the presence of glucose solution can be observed in Fig. 4a.

The velocity of the glucose solution (slope of the plot) in the wetted-length over time plot (Fig. 4b), is more when TMB is used as a chromogenic solution and it reaches the end of the channel earlier. Moreover, the color change occurred immediately after adding the glucose sample in the presence of TMB solution (Fig. 4c).

As indicated in the color intensity over glucose concentration diagram (Fig. 4d), the color intensity is more when KI is used as a chromogenic solution but the color intensity discrepancy is low and even the color intensity for 5 and 20mM glucose sample is equal. As explained in length and intensity-based results, the TMB solution is used for further analysis.

3.1.2. Type of the immobilization solution

Three types of immobilization solutions (PVP solution and SiNp in different sizes of 10-15 nm and 100 nm were utilized. As indicated in Fig. 5a, using an immobilization solution can affect the sample velocity and color intensity. The glucose solution could reach the end of the channel faster in an immobilization-free assay. Moreover, increasing nanoparticle size leads to the deceleration of glucose solution wicking

along the channel when SiNp is utilized as immobilizer. The color change occurs faster when the immobilization agent is not used (Fig. 5b).

According to the color intensity diagram (Fig. 5c), using a PVP solution for immobilization enhances the color intensity in comparison with 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.

3.1.3. Method of adding TMB solution

Four methods of adding TMB solution by the concentration of 15 mM (uniform, nonuniform, twice uniform, and saturated) were investigated for 2.5, 5, and 20 mM of glucose (only results of 2.5 mM are depicted). As illustrated in Fig. 6a, the velocity of the sample decreases with increasing TMB owing to the reduction of pores that help fluid wicking. Additionally, this decrease can be observed for the colored-length diagrams in the assays with more TMB solution due to the consumption of the whole glucose in a shorter channel length. In the uniform method, the final colored length equals 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. 6b).

When a colored-length diagram for various glucose concentrations in the saturated method is plotted, a gentle behavior can be observed between the colored length and glucose concentration. According to Fig. 6c, the colored length does not change after the 150s. The colored length in a specified time (150 s) over the glucose concentration diagram is illustrated in Fig. 6d. Since the tear glucose 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. The effect of TMB concentration is investigated in future tests. The color intensity for different assays is approximately the same except when TMB is added nonuniformly (Fig. 6e). The non-uniform method has lower color intensity because of the lower TMB solution volume and finally the lower products and color change.

3.1.4. The concentration of TMB solution

TMB solutions with a concentration 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 solution) results can be achieved in a distance-based manner, so the colored-length in a specified time (150s) is measured, and wetted-length and colored-length results are neglected.

As demonstrated in Fig. 7a, the colored length increases by the reduction of the TMB concentration. This is actually due to the earlier running out of the glucose in more TMB concentration through the reaction. The colored length for 2.5, 3, and 3.75 mM are very similar and 3.75 mM of TMB solution is selected for further tests owing to the higher color intensity in lower glucose concentrations (Fig. 7b).

3.1.5. The volume of the enzymatic solution

The enzymatic solution volumes of 3 µl, 4 µl, and saturated-method were examined. According to the colored-length over glucose concentration diagram, using 3 µl of enzymatic solution offers linear behavior (Fig. 8a). A significant color intensity change is not observed in different volumes of enzymatic solution (Fig. 8b).

3.1.6. Distance-based glucose detection assay

The glucose solution with a concentration of 0.1-1.2 mM was utilized. The colored length shows a linear relationship to glucose concentration. The linear equation obtained colored length = 14.399 × (glucose concentration) -1.736 with an R^2=0.967.

4. 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. In the present study, a minimally invasive glucose biosensor was fabricated to determine the tear glucose through a distance-based colorimetric assay. The microfluidic paper-based device was fabricated by a CO_2 laser. The µPAD consists of a sample zone, primary circular zone (addition of enzymatic solution), channel (addition of chromogenic solution), and secondary circular zone (absorbent zone).

The enzymatic 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 an immobilizer (1% PVP). According to the assay, the wetted-length, colored-length, and inverted grayscale color intensity were measured.

10 µl of various concentrations of glucose solution was added to the sample zone. For the distance-based assay, the colored length was measured at a specific time (150s). 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 an LOD of 0.2 mM.

Gabriel et al. fabricated a wax-printed µPAD for tear glucose detection. TMB and chitosan were utilized as chromogenic and immobilization solutions, respectively. The sample volume was 5 µl 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 µl [38].

James et al. fabricated a stamp patterning µPAD 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 0.05-1.25 mM. 2 µl glucose sample was used and TBHBA and 4-AAP were the chromogenic reagents [43].

In another study, the distance-based results were obtained with 20 µl of glucose solution. The dynamic range was 0.6-15 mM and LOD of 0.6 mM. DAB was a chromogenic solution [31]

Declarations

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Conflict of interests

Authors have no conflict of interests to declare.

Authors' contributions

Samira Allameh was 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.

Availability of data and material

All useful data obtained during the study are available.

Ethics approval (Not applicable)

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

Consent to participate (Not applicable)

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

Consent for publication (Not applicable)

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Figures

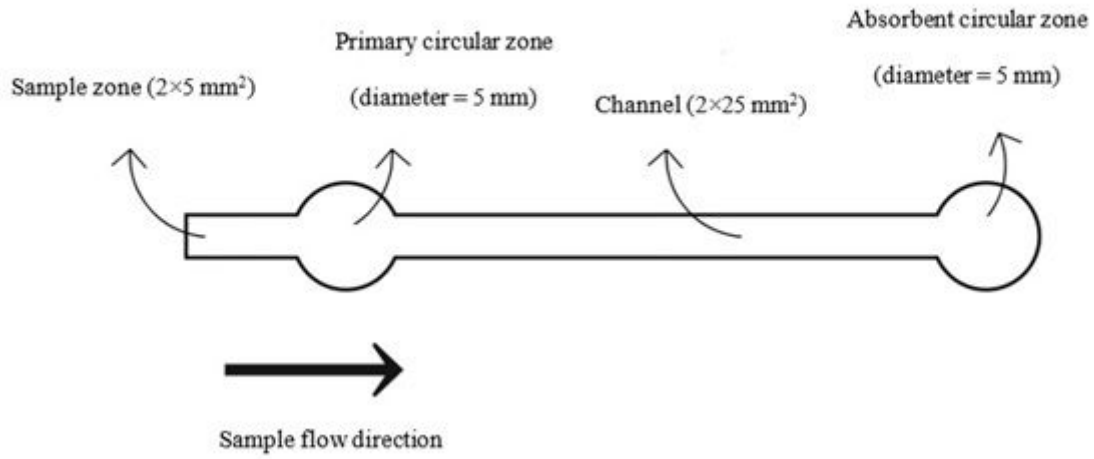

Figure 1

The schematic view of the designed device and different zones

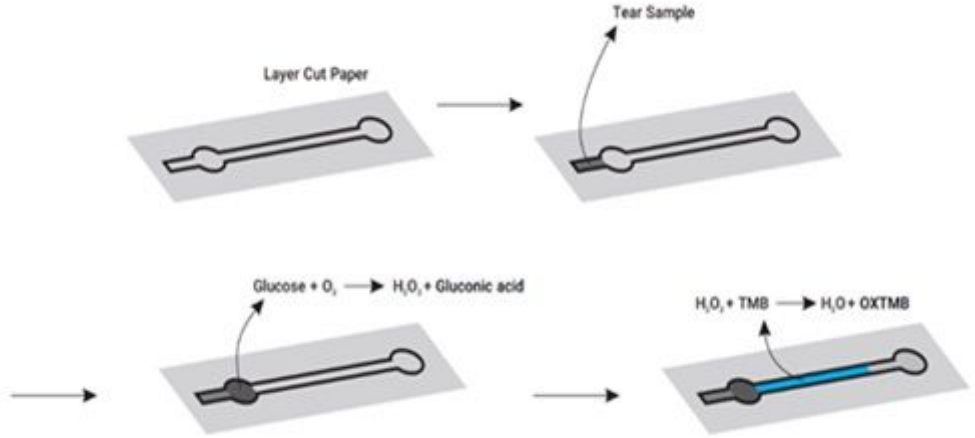

Figure 2

The simplified schematic of colorimetric reaction in the presence of enzymatic solution (GOx and HRP) and chromogenic reagent (TMB)

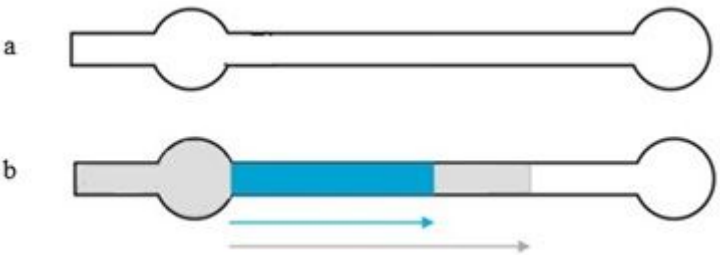

Figure 3

a) The distance-based glucose biosensor at different time intervals and vectors at time=0 s b) The colored length and the wetted length are displayed in blue and grey vectors, respectively

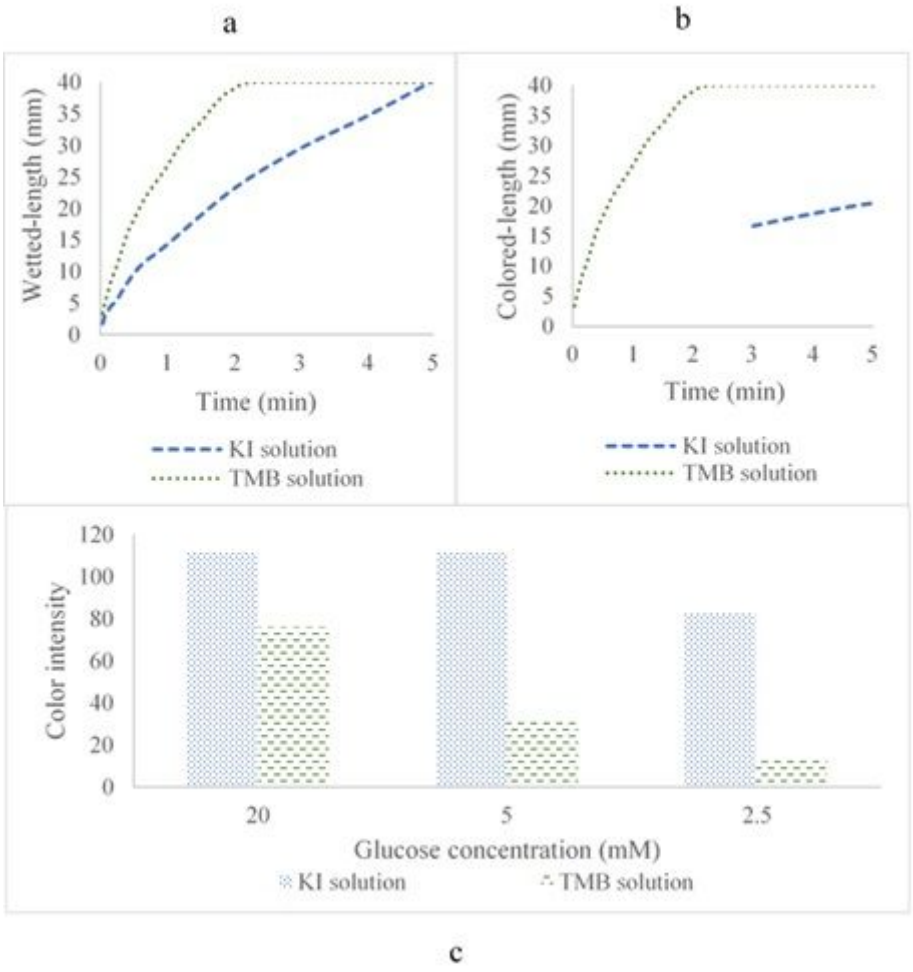

Figure 4

a) Wetted-length vs time diagram for 2.5 mM of glucose solution, the solution moves faster in the presence of TMB compared with KI solution b) Colored-length vs time diagram for 2.5 mM of glucose solution, earlier color change in TMB solution compared with KI solution c) The greyscale color intensity of different reagents diagram for various glucose concentrations, more discrepancy in color intensity of various concentrations when TMB solution is used as a chromogenic reagent

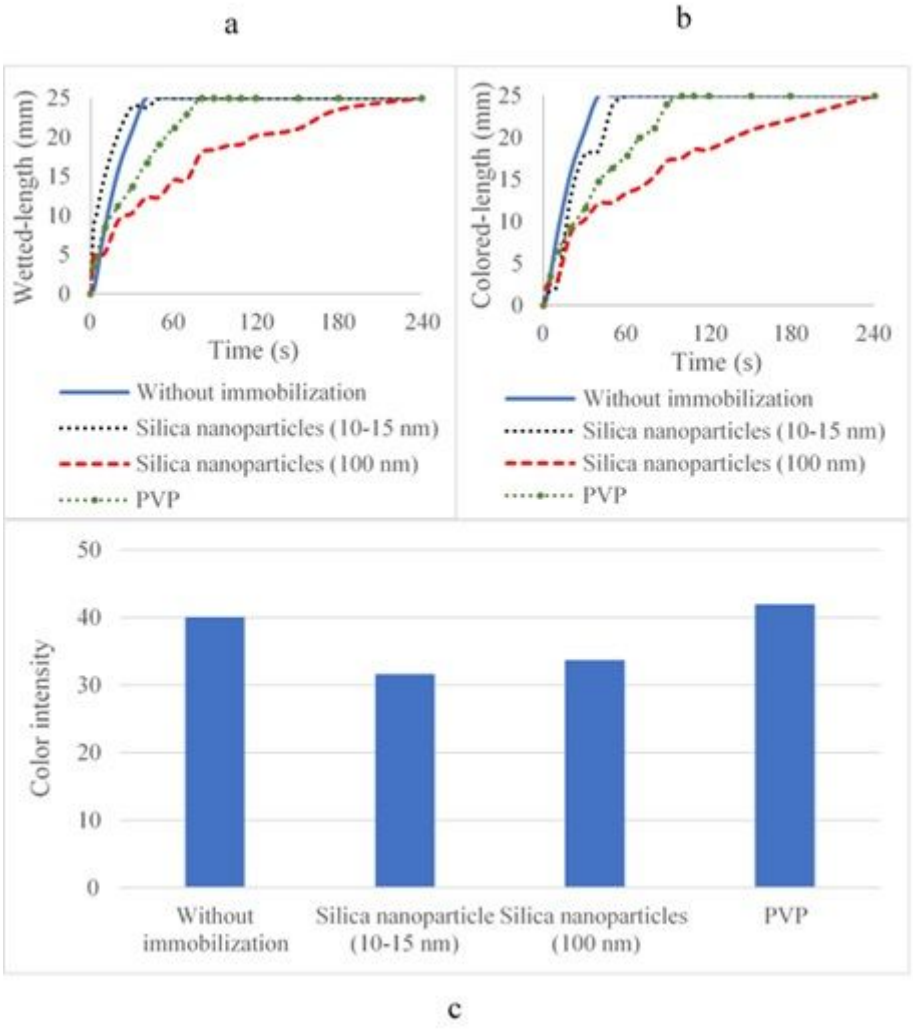

Figure 5

a) The wetted-length over time diagram for 2.5 mM of glucose when the effect of immobilization solution is examined, faster wicking and reaching the end of the device channel when immobilization solution is not used b) The colored-length over time diagram, faster color change in immobilizer-free assay c) The greyscale color intensity when various immobilization solutions are used for 2.5 mM of glucose, the higher grayscale intensity in the detection zone when PVP solution is used as an immobilizer

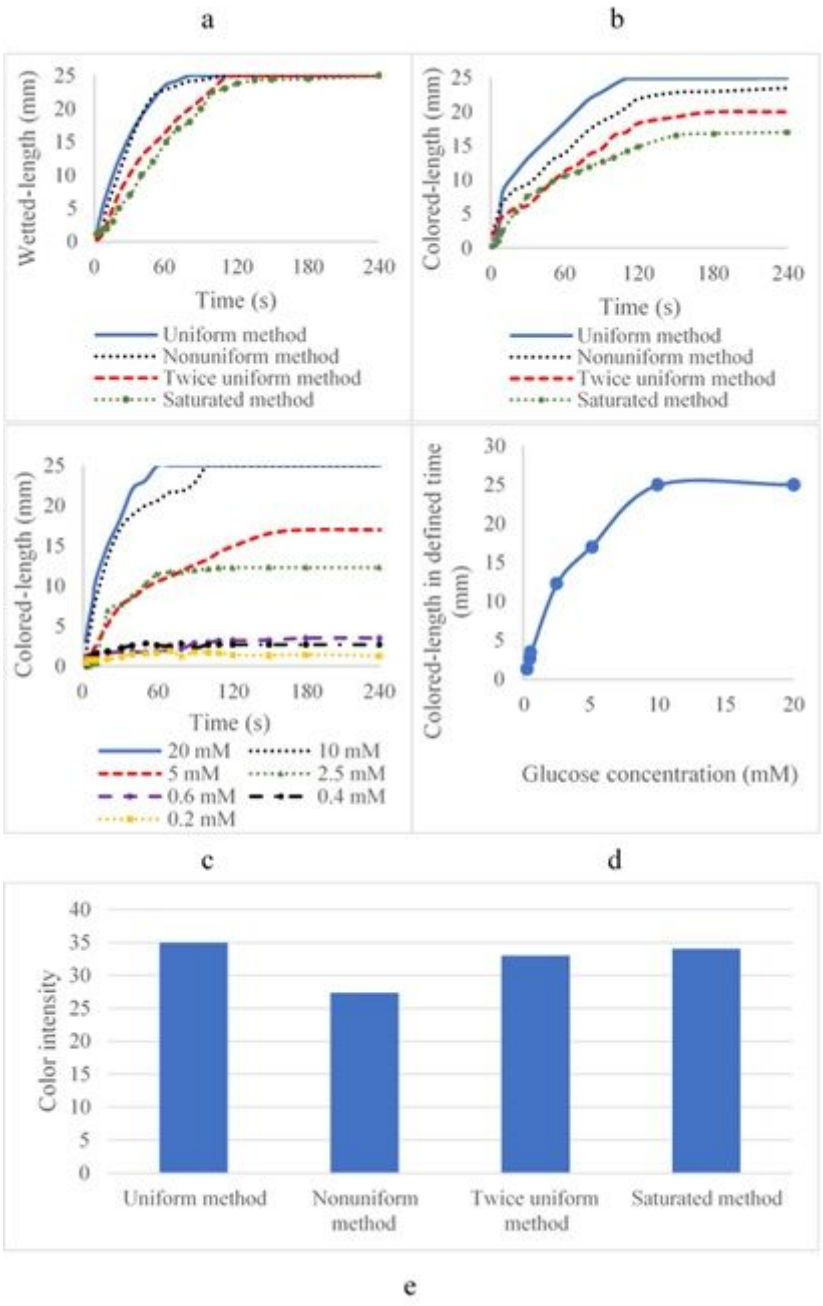

Figure 6

a) The wetted-length vs time diagram in the method of adding TMB solution experiments for 2.5 mM of glucose, faster wicking of glucose sample when lower TMB solution is used. b) The colored-length over time diagram, colored length decrease in the presence of more TMB solution as earlier running out of the glucose. c) The colored-length over time diagram for various glucose concentrations, colored length increase in the presence of more glucose concentration d) The colored-length vs glucose concentration diagram at a specific time (150s), more colored length is observed in the presence of more glucose concentration e) The color intensity diagram for different methods of adding TMB solution for 2.5 mM of glucose, lower color intensity correlates with lower TMB solution volume

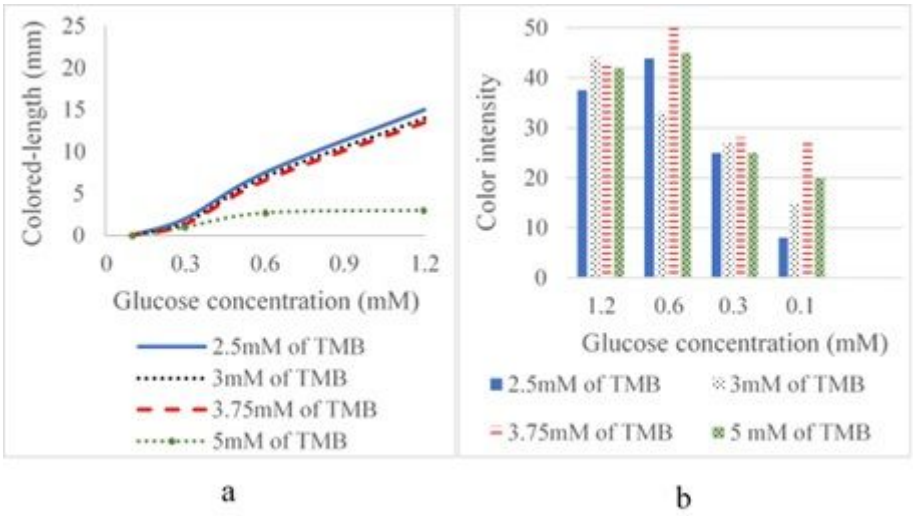

Figure 7

a) The colored-length vs glucose concentration diagram when different TMB concentration is used, more colored length in lower TMB concentration b) The greyscale color intensity over glucose concentration diagram, more grayscale intensity for the 3.75 mM of TMB

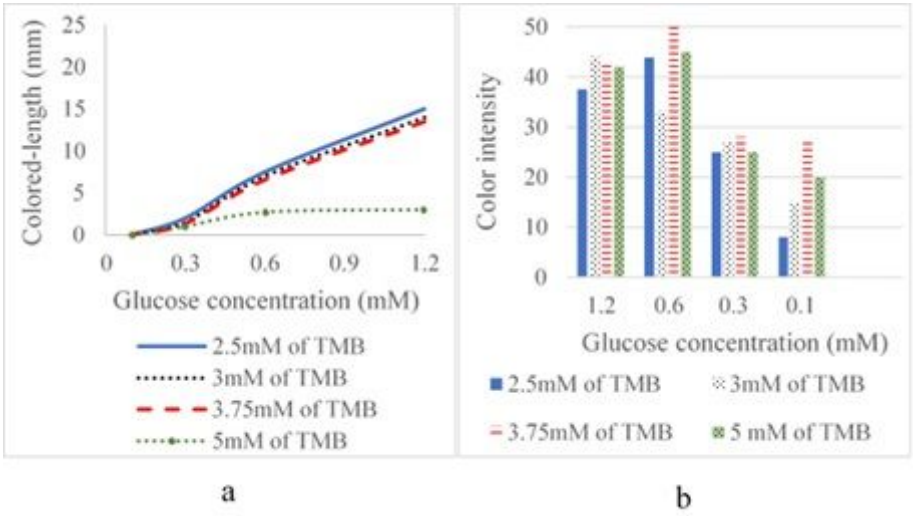

Figure 8

a) The colored-length over glucose concentration diagram when different volumes of enzymatic solution are used, selecting 3µl of enzymatic solution for further examinations due to linear behavior between colored length and glucose concentration b) Same color intensity when different amounts of enzymatic solution are used for various glucose concentrations

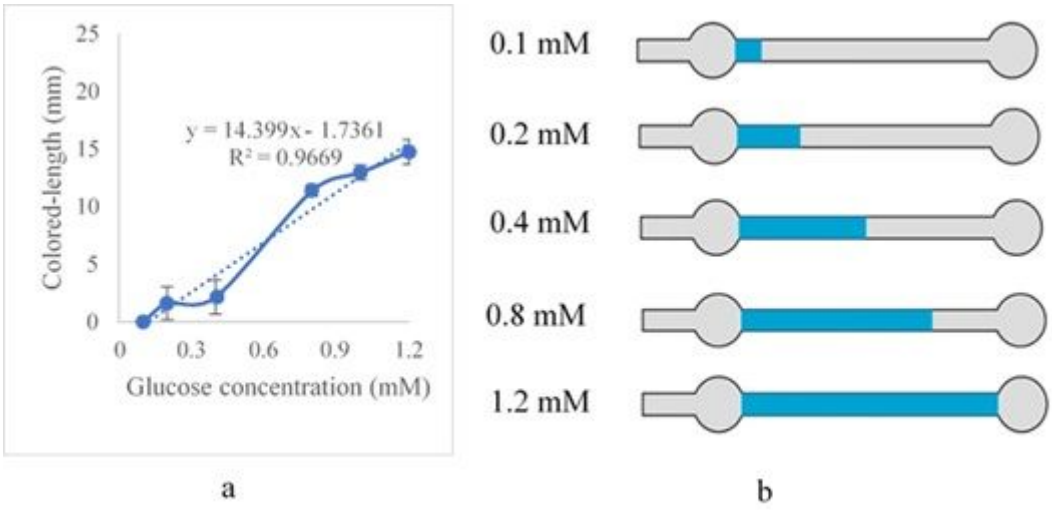

Figure 9

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