Advances on the Development of Microfluidic Paper-based Analytical Device for Detection of Salivary Glucose

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Abstract— **The people living with *Diabetes Mellitus* (DM) has considerably increased over the last decades, becoming a major health problem for most countries. The frequent monitoring of blood samples is essential, but the available current technology is based on invasive sample collection, which can discourage the monitoring and lead to poor management of the disease. In this work we present the advances of the development of a Microfluidic Paper-based Analytical Devices (µPAD) for monitoring of DM through salivary samples. The low-cost device was created by transferring a hydrophobic material over a Whatman filter paper in a designed pattern with a stamping process. Obtained patterns were modified with chitosan in order to create a biocompatible environment for immobilization of glucose oxidase-peroxidase byenzimatic system. Two chomopheres were tested in order to achieve the high sensibility required for detection of glucose in salivary samples. The resulted µPADs were evaluated with stock glucose samples and real salivary samples showing a distinctive color change. The results suggest the developed devices can be optimized and applied as low-cost, easy to use and non-invasive test for DM monitoring.**

Keywords—**Diabetes, salivary glucose, µPADs, chitosan, enzyme**

I. Introduction

According to the World Health Organization, the number of people with *Diabetes Mellitus* (DM) increased from 108 million in 1980 to 422 million in 2014 [1,2]. In Mexico, given the high number of incidences, DM has been declared a national emergency with high priority for the public health system [3]. Frequent monitoring of glucose levels is essential for proper of DM management and to prevent complications such as limb amputation, blindness, kidney damage, and cardiovascular disease. Current methods for monitoring blood glucose are based on a puncture in the fingertip to collect a drop of blood on a test strip, which lead to an enzymatic reaction that can be measured to provide a glycemia result. However, the invasive nature of the sample collection can discourage patients from self-monitoring of glucose leading to a poor control of DM. Thus, non-invasive monitoring through fluids like urine, sweat, tears and saliva has been proposed to replace blood samples and monitor the state of the patient. Among these body fluids, saliva has attracted attention given the studied correlation between salivary glucose and blood glucose in diabetic individuals presented by several clinical studies [4]. Thus, salivary glucose detection can potentially bring the possibility of a monitoring method with a cost effective, easy, fast and non-invasive sample collection [4]. However, the low concentration of glucose found in saliva (0.03-0.08 mM/L) as compared with blood glucose (2-30mM/L) avoids the application of the current available self-monitoring technology for salivary analysis and only high sophisticated instrumental techniques such as UV-Vis-NIR, HPLC and GC respectively has been effectively applied to study salivary glucose in volunteers. However, these methods are time and reagent consuming, non-portable and require trained personal to be executed. So the development of novel, sensitive, portable, low-cost, easy to use analytical methods is desirable in order to perform non-invasive monitoring of DM through saliva.

Recently, the development of low-cost analytical devices based on paper substrates has been proposed [5]. The so called Microfluidic Paper-based Analytical Devices (µPADs) are paper strips modified with hydrophobic barriers which transport fluidic samples into a defined detection zone modified with chemical reagents. This produces a color change as result of analyte detection. The advantages of this technology are the cost-effective fabrication, disposability, low sample quantity, portability and easiness of interpretation through colorimetric indicators. These devices are promising analytical tools, especially for remote areas in developing countries. The first µPADs presented consist only on single hydrophilic surfaces (i.e. paper) isolated by hydrophobic barriers (e.g. paraffin) in order to create a detection zone; however, novel geometries for parallel detection as well as modification of the detection zone to improve the sensitivity of the devices have been proposed [6].

In this work, we present the advances on the development of a bienzymatic µPAD intended for non-invasive monitoring of DM through salivary glucose. Microfluidic patterns were created by a stamping process and detection zone was modified with chitosan in order to get a uniform spot on the detection zone and a biocompatible environment for the enzymatic reaction. Given the low concentrations of glucose reported for salivary glucose, two chromophores were tested to select the one with the best response. Once the colored spot was obtained, it is possible to perform the analysis with common image processing software or by naked eye.

II. Methodology

1) *Chemicals and materials*: Glucose oxidase (GOx) type X-S from *Aspergillus niger* (128.2 U/mg), Peroxidase type II from horseradish (HRP) (210 U/mg), sodium3,5-dichloro-2-hydroxy-benzenesulfonate (DHBS) ≥ 98%, 2,4,6-tribromo-3-hydroxybenzoic acid (TBHBA) 97%, chitosan powder, D-(+)-Glucose, 4-aminoantipyrine (4-AAP), paraffin and # 40 Whatman™ filter paper were purchased from Sigma-Aldrich. PBS buffer (100 mM, pH 7.0 at 20 °C) was prepared from potassium phosphate monobasic and sodium phosphate dibasic, both obtained from Sigma-Aldrich.

2) *Fabrication of µPADs*: a stamping process was used to create the µPADs similar to the one described by de Tarso-García *et al* [7]. A punch of aluminum was designed on Solid Works® and manufactured at IDF Industrial® (Chihuahua, Chihuahua, Mexico) to create the microfluidic patterns with a flower-like shape. Circles of 5cm of diameter were cut from Whatman filter paper and dipped into liquid paraffin for 2 seconds to create waxed paper. To stamp the microfluidic pattern, the aluminum punch was heated at 113°C and pressed against the waxed paper attached with non-coated Whatman filter paper. An aluminum cylinder of 5 kg was used to add pressure for 5 s. The result is a flower-like geometry stamped over the non-coated Whatman paper, with hydrophobic barriers created by the transfer of paraffin from the waxed paper to the non-coated paper. All the process was performed over a flat surface covered with Teflon paper. To modify the detection zone, solutions of chitosan at 0.5%, 0.75% and 1% (m/v) in acetic acid 2% (v/v) were prepared. 3µL of chitosan solutions were spotted on each detection zone and allowed to dry at room temperature while a mixture of GOx (120 U/mL) and HRP (30U/mL) in PBS buffer for glucose assay was prepared. 1.0µL of the enzymatic mixture was spotted on each detection zone and allowed to dry at 4°C. Two different chromogen agents were employed for glucose detection. A first chromogenic solution was prepared from 4mM 4-AAP and 8mM of DHBS as described by Gabriel *et al* [6]. The second solution was based on the procedure described by Cha *et al* [8] with 1.3mL of PBS buffer, 2.0mL of TBHBA at 5mg/mL and 0.5mL of 0.1M 4-AAP. After enzyme immobilization, 1.0µL of each chromogen solution was spotted on each detection zone, under dark conditions since these solutions are light sensitive.

3) *Glucose detection*: to verify the ability of the developed µPADs for detection, stock solutions of glucose in PBS buffer (pH 7.0) from 0.07mg/dL to 18mg/dL were prepared. A volume of 90µL of glucose solution were spotted on the central zone and distributed to the detection zones by capillarity. Then, the produced color change was recorded with a smartphone camera in order to get analytical information through image processing with the RGB (Red-Green-Blue) model for color description. By relating the RGB components with glucose concentration, calibration curves for each modified zone (chromophere+chitosan) was obtained. Finally, a group of 4 volunteers were recruited to monitor their value of fasting salivary glucose concentration. All subjects gave their informed consent for inclusion before the samples were collected and arrived at fasting condition with normal oral hygiene. Unstimulated saliva was collected on 1.5 mL microtubes and deposed over the central channel of µPAD to monitor salivary glucose with the described procedure. Since GOx and HRP enzymes usually work in pH ranges from 5 to 7.4, thus since pH of salivary samples usually ranges from 6.5 to 7 no pretreatment of samples was required.

III. Results and Discussion

A. Fabrication of µPADs

Obtained µPADs with optimized conditions of 113°C heating and 5 s of pressure are shown in Figure 1A. An augmented view of the channels is presented in Figure 1B. The typical fibrillar morphology of cellulose paper in the hydrophilic zone can be appreciated on the right side, while the hydrophobic zone appeared coated by paraffin on the left side.

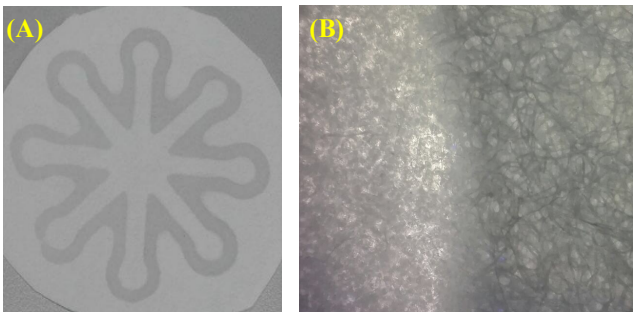

Figure 1. (A) Flower-like shape obtained with 5 seconds of stamping and 113ºC. (B) A 40X augmented image for hydrophobic and hydrophilic zone of fabricated µPAD

Usually a main drawback of µPADs is the irregular color spot formation on the detection zone, which can prevent an efficient quantification of analytes. Thus, detection zone of fabricated µPADs were modified with 0.5%, 0.75% and 1% (m/v) of chitosan in order to provide a biocompatible environment for enzyme immobilization and to improve the color spot formed on the detection zone. Fig. 2A shows the FT-IR specters for Whatman paper prior and after modification with chitosan as well as after enzyme immobilization. Most of the bands presented in Fig. 2A belong to the cellulose of the filter paper: the wide band between 3600 and 3200 cm^{-1} was attributed to

O-H groups; 2915, 2850 and 1312 cm^{-1} bands were related with C-H_2 group; 1278 cm^{-1} band was attributed to C-H group; 1055 and 1028 cm^{-1} bands were related with C-O group and finally 1159 cm^{-1} band was attributed to C-O-C group [9]. For chitosan, the two characteristic bands were located at 2873 cm^{-1} for O-H as well as C-H groups [10]. No significant bands were observed after enzyme immobilization, suggesting a superficial interaction rather than chemical bonding interaction of enzyme with µPAD detection zone. Figure 2B, 2C and 2D shows the modification of color spot in detection zone for chitosan addition. While color formation was achieved for each modified zone, only 1% chitosan achieved a regular defined color formation in detection zone, which might be attribute the hydrophobic nature of chitosan.

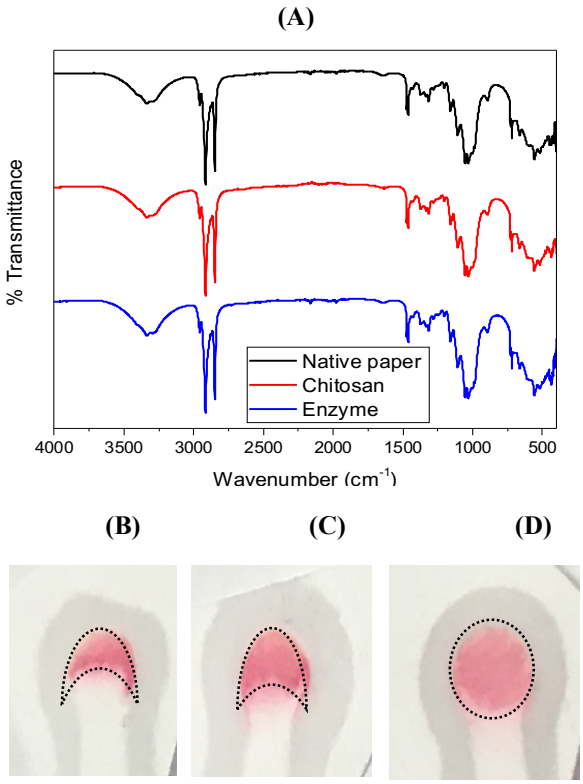

Figure 2. Modified detection zone with chitosan. FTIR specters for native paper, modified detection zone with chitosan and immobilized enzyme on detection zone. Closer view of detection zone spot with TBHBA chromophere for (B) 0.5% (C) 0.75% and (D)1% of chitosan.

C. Glucose Detection

The GOx-POD system is well established for biochemical analysis in liquid samples by enhancing color formation after interaction with a chromogen agent. In this work, the GOx-POD system was immobilized on paper based modified detection zone leading to a pink/purple color formation in the spot zone. The intensity was monitored with a smartphone camera to stablish a relationship with glucose content by the extraction of RGB components for each concentration of glucose.

Figure 3A, 3B,3C and 3D shows the calibration curves obtained for the combinations of chitosan and chromopheres in the modified zone.

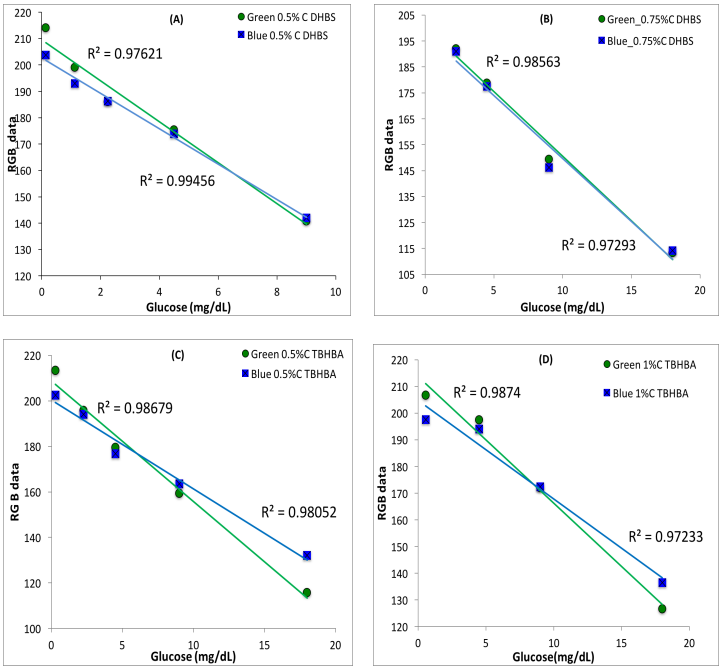

Figure 3. Calibration curves obtained for chitosan modified zones and TBHBA and DHBS chromopheres.

RGB color model has been extensively applied for color description in colorimetric quantitative applications. The model split the information in three channels namely Red (R), Green (G) and Blue (B) which can exhibit higher sensitivity for a determined color. In this work the red channel shows poor sensitivity for color description and was discarded. On the other hand, the green and blue channel exhibited good linearity for description of glucose concentrations within 0.14 mg/dL to 18 mg/dL. These concentrations cover the reported concentration range reported for salivary glucose and also all the tested combinations of chitosan+chromophere achieved a lower limit of detection (LOD) than current optical glucometers.

TABLE I
ANALYTICAL DATA FOR MODIFICATION OF DETECTION ZONE

DETECTION ZONE MODIFICATION	LOD	STD (n=4)	R^2
0.5% chitosan+DHBS	0.14 mg/dL	±7.25 RGB units	0.9762
0.75% chitosan+DHBS	2.25 mg/dL	±8.56 RGB units	0.9856
1 % chitosan+DHBS	0.28 mg/dL	±14.79 RGB units	0.8221
0.5% chitosan+TBHBA	0.28 mg/dL	±9.39 RGB units	0.9867
0.75% chitosan+TBHBA	0.56 mg/dL	±7.51 RGB units	0.8812
1% chitosan+TBHBA	0.56 mg/dL	±5.62 RGB units	0.9874

As stated from Table I, 0.5% chitosan+DHBS chromophere achieved the lowest LOD but 1% chitosan+ TBHBA chromophere showed the best reproducibility. Lower reproducibility was found for 1%chitosan+DHBS and 0.75% chitosan+TBHBA, which might be influenced by light conditions and camera angles during image capture. Thus, a special holder for μPAD image analysis must be considered to guarantee similar conditions for image analysis. With the calibration curve obtained salivary analysis for 4 volunteers was conducted with the fabricated μPADs. TBHBA chromophere and 1% chitosan was selected for analysis, given the low LOD, the higher reproducibility and the wide linear zone for quantification (0.56-18 mg/dL) which was not achieved with DHBS. The results for this screening test are showed in Table II.

TABLE II
DETECTION OF GLUCOSE IN SALIVARY SAMPLES

DETECTION ZONE MODIFICATION	Glycemia [mg/dL]	Salivary glucose [mg/dL]
SUBJECT 1	101	1.76
SUBJECT 2	160	4.38
SUBJECT 3	165	5.67
SUBJECT 4	95	1.18

Glycemia level for subject 1 and 4 can be classified as non-diabetic, while subject 2 and 3 can be classified as diabetic according with WHO criteria. As previously report, the results showed higher salivary glucose content for diabetic subject than for normal subject, even no strong correlation can be claimed from these data. However, detection of salivary samples can be further improved in terms of accuracy, sensitivity and reproducibility in order to apply the μPAD approach to higher number of volunteers. Thus the presented method is promising for a simple, low-cost and sensitive alternative for monitoring of salivary glucose.

V. CONCLUSION

In this work, μPADs with defined hydrophobic channels were obtained by a stamping process. The optimal conditions of time and temperature for fabricating repeatable patterns were obtained. The detection zone was modified with chitosan and tested with two different chromogen reagents prior of image processing analysis through RGB model. Even both chromopheres with each proposed chitosan percentage for modification achieved LOD suitable for salivary glucose detection, combination of 1% chitosan+TBHA was selected for real sample analysis because of the better reproducibility and higher linear range. Volunteer samples exhibited higher salivary glucose content for diabetic subjects than healthy subjects. This suggest a potential application of μPADs for analytical quantification of salivary glucose.

ACKNOWLEDGMENTS

The authors would like to thank to M.C. Luis de la Torre Sáenz for his assistance with the infrared measurements. They would also like to thank to Dr. Armando Zaragoza and M.C. Claudia Hernandez for the facilities provided within the polymer synthesis laboratory. The funding of this work was supported by the National Council of Science and Technology (CONACyT) in Mexico through the grant 215729.

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