Biosensors and Bioelectronics 67 (2015) 763–768

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Short communication

A paper strip based non-invasive glucose biosensor for salivary analysis

Anuradha Soni, Sandeep Kumar Jha *

Centre for Biomedical Engineering, Indian Institute of Technology Delhi, New Delhi 110016, India

ARTICLE INFO

Article history:
Received 6 June 2014
Received in revised form
7 September 2014
Accepted 16 September 2014
Available online 28 September 2014

Keywords:
Saliva glucose
Glucose biosensor
Non-invasive biosensor
Paper strip immobilization
Smartphone based biosensor

ABSTRACT

In our present study, we developed an optical biosensor for direct determination of salivary glucose by using immobilized glucose oxidase enzyme on filter paper strip (specific activity 1.4 U/strip) and then reacting it with synthetic glucose samples in presence of co-immobilized color pH indicator. The filter paper changed color based on concentration of glucose in reaction media and hence, by scanning this color change (using RGB profiling) through an office scanner and open source image processing software (GIMP) the concentration of glucose in the reaction medium could be deduced. Once the biosensor was standardized, the synthetic glucose sample was replaced with human saliva from donors. The individual's blood glucose level at the time of obtaining saliva was also measured using an Accuchek™ active glucometer (Roche Inc.). In this preliminary study, a correlation of nearly 0.64 was found between glucose levels in saliva and blood of healthy individuals and in diabetic patients it was nearly in the order of 0.95, thereby validating the importance of salivary analysis. The RGB profiling method obtained a detection range of 9–1350 mg/dL glucose at a response time of 45 s and LOD of 22.2 mg/dL.

1. Introduction

As per International Diabetes Federation, 371 million people worldwide have confirmed diabetes and the number is expected to rise to 366 million people by 2030 (WHO/IDF 2006. Danaei et al., 2011). In addition, an estimated more than 200 million people have undiagnosed diabetes. The blood glucose concentration is the major diagnostic requirement for diabetes patients with elevated HbA1c level and is essential for patient monitoring. Self-monitoring of blood glucose (SMBG), aided by advancement in biosensor technology in the past two decades has been established as a valuable tool for diabetes management. These methods are fast and reliable compared to time and reagent consuming spectrophotometric techniques. Such measurements are particularly important for clinical patients who have to monitor blood parameters on a daily basis and sometimes multiple times a day. The goal of SMBG is to help the patient maintain normal blood glucose concentrations in order to delay or prevent the progression of micro vascular (retinopathy, nephropathy and neuropathy) and macro vascular complications (stroke and coronary artery disease). In addition, it can also be useful in providing real time information for adjusting medications, dietary uptake, and physical activities in order to achieve optimum glycemic levels.

However, majority of glucose biosensors, including those are commercially available, either rely on invasive technique for blood extraction, or the monitoring is dependent on often unreliable and interference prone near infrared scanning of glucose using wearable sensors. Although the cost of such invasive glucometer test strips has drastically gone down, but still unaffordable for most people in developing and underdeveloped countries. Therefore, there is still the need to find low cost biosensor technology for diabetes monitoring. Alternate body fluids such as saliva and tears hold vital information about the disease (Rao et al., 2010) and should be targeted for monitoring. Amongst non-invasive estimation of glucose, salivary analysis is more convenient than painful extraction of blood from fingertip and through veins and can be collected by individuals with limited training, including the patient. Moreover, significant glucose level is present in saliva only in diabetic condition (Yamaguchi et al., 1998). The reason being that mammalian salivary glands are highly resistant to the passage of glucose from the blood into their secretion. Nearly all of the authors who have studied this problem agreed that, although the cells of the salivary glands are not entirely impermeable to glucose, they are very nearly so, since the amount of glucose which has at any time been detected in the saliva is so slight as to be insignificant. But that in the pathological condition of diabetes in individuals sugar appears in the saliva. Most of the previous studies have demonstrated the raised salivary glucose level in diabetes. However, there are very few reports on correlation of salivary glucose level (SGL) and blood glucose level (BGL) in diabetes (Hebb and Stavraky, 1936; Carda et al., 2006; Yamaguchi et al., 1998). In-fact the exact correlation between blood and salivary glucose is that diabetic patient contain glucose

* Corresponding author.
E-mail address: sandeepjha@cbme.iitd.ac.in (S.K. Jha).

http://dx.doi.org/10.1016/j.bios.2014.09.042
0956-5663/

in saliva whereas in non- diabetics condition patient does not contain significant glucose in saliva. A significant glucose concentration in saliva from the patients with insulin dependent diabetes mellitus (IDDM type1) was also reported by Twetman et al. (1992). The presence of glucose in the saliva of the diabetics probably reflects the high serum glucose concentrations (Hebb and Stavraky, 1936).

Apart from saliva, tears, urine and sweat are the other two body fluids where glucose levels can be monitored, however, the composition of these fluids are non-uniform between person to person or for same person during different time of the day and external conditions. Also, extraction of tears is by far not entirely a non-invasive technique as it requires procedures which are cumbersome and at times inconvenient to the patient. While saliva is considered more suitable option for non-invasive measurements due to ease in extraction compared to tears and sweat, and considering that measurement of alcohol, lactate and glucose have been measured successfully in saliva (Guilbault et al., 1995). Therefore, if fresh saliva is drawn for analysis each time, it can prove more significant for non-invasive monitoring of body metabolites.

Amongst the transducers that are used for developing a biosensor, optical transducers are more suitable for analysis of saliva due to its transparency. Optical transducers are also much more reliable, convenient and sensitive compared with electrochemical transducers (Kumar et al., 2006), as the latter are more prone to interferences from redox-active substances (Jha et al., 2009) present in the saliva.

Various attempts have been made by researchers in developing glucose monitoring systems for salivary measurements. However, their approach was either in the direction of conventional measurement through colorimetric assays such as by Lambert and Borchert (2001); detection through NIR/Raman based spectroscopic techniques (Lipson et al., 2009), or Clarke type electrode based amperometric measurement (Yamaguchi et al., 1998) etc. Amongst the non-invasive detection method, salivary measurement is fast becoming a popular research area (Amer et al., 2001; Panchbhai, 2012; Yamaguchi et al., 1998).

Therefore, taking clues from these developments, we developed an optical non-invasive glucose biosensor for salivary analysis. In this study, filter paper strips were used to co-immobilize glucose oxidase and a pH sensitive dye which would change color due to gluconic acid formation during enzymatic reaction. This color change could be estimated through an office scanner and an open source software (GIMP) after pixelation of the image into RGB (red–green–blue) profile and then correlating it with glucose concentration to deduce biosensor calibration curve. The RGB color model is an additive model in which primary colors—red (R), green (G) and blue (B) are added in various proportions to reproduce a broad range of colors. Therefore, such a simple approach of using RGB profile of paper strip for glucose monitoring is not only novel, but shall be cost-effective and convenient to patients who would like to monitor their body glucose level in a non-invasive way, but also can be used with any smartphone that would eliminate the need for procuring a dedicated instrument for glucose analysis. Moreover, this approach may be useful in rapid screening of large populations for diabetes.

2. Materials and methods

2.1. Material

The office scanner (model Scanjet 2300c) was from HP India Ltd., commercial glucometer (Accuchek Active) was procured from Roche India Ltd. Filter paper (Whatman no. 1), Polyvinyl alcohol (PVA) (Cat. no. 363138-500G), Methyl red (Cat. no. 250198-100G), Dithiothreitol (DTT) (sigma Cat. no. Do632-1G), Glucose oxidase (GOD) (Cat. no. G7141-50ku), dextrose and phosphate buffer were purchased from Sigma Aldrich India.

2.2. Methods

2.2.1. Immobilization and characterization of GOD

At first a stock solution of glucose oxidase was prepared by dissolving 10 mg of glucose oxidase in 5 ml of 1 mM phosphate buffer maintaining the pH at 7, so that a 50 μl of solution contains 100 enzyme units. The working solution of glucose oxidase was a 10 times dilution of this stock solution. The glucose oxidase (GOD) was immobilized on paper strip via the adsorption method. In the working solution of glucose oxidase 2 mg of methyl red dye along with 1.5 mg of reducing agent DTT were added. The solution was then mixed thoroughly. Whatman's filter paper was cut into 3 cm × 0.5 cm in size to prepare paper strip for immobilization. After that 50 μl enzyme solution was poured over the paper strip and was dried. The dried strip was then dipped in 1% PVA and was then again dried and stored at 4 °C until use.

The protein content in the paper strip was estimated as per Lowry's method (Lowry et al., 1951). In order to ascertain extent of enzyme immobilization, enzyme assays were conducted with immobilized paper strip using synthetic glucose solution and then residual glucose concentration was estimated using the Dinitrosalicylic acid reagent (DNS) based spectrophotometric method. The strips were also checked for enzyme's stability for a period of 60 days. The same DNS method was also used for determination of salivary glucose concentration in samples from donors. In that case, 1 ml of fresh saliva sample was used as test solution. While enzymatic methods are often used for determination of salivary glucose concentration, modified DNS method (Mohun and Cook, 1962) has a detection range of 7.6 mg/dL onwards, which is similar to our requirement and sufficient to determine SGL in absence of other reducing sugars in sample.

2.2.2. Biosensor measurement

The GOD-immobilized filter paper strips were used for biosensor measurements in synthetic glucose samples. For this, 50 μl of synthetic glucose sample (0.5–500 mM) prepared in 1 mM PBS was dropped onto the filter paper strip. The color change on the strip was scanned with respect to time using an office scanner and the image was pixelated using GIMP (Version 2.8) software into RGB profile. The optimum response time (time to reach 90% of saturated sensor response) was first calculated (time studied were 30, 45, 55, 60 s, and 5 min) and then the calibration curve of biosensor was obtained with respect to R/G or B color intensity on paper strip.

For the estimation of glucose in saliva, we used the paper strip with immobilized glucose oxidase and 50 μl of saliva samples collected from healthy persons and diabetic patients (type- I or II) was applied onto it before scanning it using the office scanner as before. The blank for synthetic sample was only buffer and blank for test samples (saliva) was the baseline response in sample. This was followed for the reason that the baseline shifted when we did not immobilize GOD on the membrane.

For validating the biosensor on real samples, 36 volunteers of age-group 20–45 years were enrolled at Outpatient Department of Indian Institute of Technology Delhi hospital, New Delhi, following institute's ethical procedures, wherein the individuals signed consent form and no money was paid to them, neither their identity was revealed. Concurrent blood glucose analysis was performed for the volunteers using Accuchek Active glucometer (Roche Inc. India) and the data was compared with corresponding saliva glucose value obtained using our biosensor. The method was

also validated with the DNS reagent method in case of few volunteer's samples, wherein 1 ml of fresh saliva had to be drawn as test sample for colorimetric assay. As it was unavoidable to have interferences from food and other substances in saliva that could alter the pH of a person's saliva, therefore, individuals were asked to draw fresh saliva each time after rinsing the mouth with drinking water. The individuals could draw saliva with a cleaned finger themselves before applying it onto the filter paper strip. No further treatment on saliva samples (such as centrifugation etc.) were needed.

The enzymatic assays were performed in triplicates and biosensor calibration was performed five times unless and otherwise mentioned and data was plotted along with standard deviation as error bars. The baseline for biosensor was the initial RGB values (0th second plus human error of 3–5 s until scan) after application of sample. For finding correlation between salivary and blood glucose levels, concurrent measurements (blood as well as saliva) for a sample was performed only once. The data analysis were performed and graphs were plotted using origin lab 7.5 software. For establishing interferences from lactic acid (produced due to bacterial fermentation in mouth) and ascorbic acid (common interferent for glucose measurement), different concentrations of these compounds were applied on strips with or without glucose in presence of buffer. The biosensor based method was then compared with DNS reagent method for SGL analysis and also with commercial blood glucose meter (Accuchek Active glucometer, Roche Inc.) to establish correlation with BGL analysis. While one drop of either saliva or blood was required for biosensor and commercial glucometer, 1 ml of saliva had to be used for DNS reagent based estimation. The reproducibility of sensor was obtained using concurrent analysis of same sample in triplicate without time lag. The statistical correlation analysis and student's t-test were performed using Microsoft Excel software.

3. Results and discussion

3.1. Preparation and stabilization of biosensor strips

First of all the enzyme was immobilized on the filter paper strip and was preserved at 4 °C. A schematics showing immobilization and biosensor measurement method is shown in Fig. 1. The specific enzyme activity of immobilized enzyme on paper strips was obtained with the help of protein estimation and enzyme assay. The protein content per strip was found to be 540 μg, while 7.7 U of enzyme activity was estimated per strip. This translated a specific activity of immobilized glucose oxidase as 1.4 U/strip. This value remained nearly constant for up to 60 days when the strip was kept desiccated at 4 °C, suggesting that the enzyme was stable under refrigerated condition, especially when protein stabilizer DTT was used during immobilization and preservation stages. Also, a coating of PVA over the strip helped in increasing adsorption stability, as this extra hydrophilic coating prevented desorption of co-immobilized GOD-pH dye.

3.2. Biosensor measurements

For calibration of biosensor, one drop (50 μl) of synthetic glucose samples (9–1350 mg/dL) prepared in phosphate buffer was added on top of one strip and the liquid was allowed to spread on the whole strip by capillary action. Once the reaction was complete, the paper was scanned using an office scanner and the image was stored in computer. Then the stored image was cropped to its edges and entire area was analyzed for RGB histograms to obtain the pixel density for each primary color (Fig. 1B). Though, due to manual preparation method, enzyme and samples may not always get dispersed on the strips homogenously, yet by analyzing the entire strip area for color change, an average RGB histogram could be obtained using GIMP software, thereby greatly reducing the variations in measurements. A plot between glucose concentration and R/G/B pixel count was the basis of calibration curve for this biosensor (Fig. 2A and B). Once the biosensor was standardized, the synthetic glucose sample was replaced with human saliva from donors.

Before the standardization, optimum response time of the biosensor was calculated on the basis of RGB peak pixel values obtained for 30 s to 5 min intervals. Although calibration could be performed within the linear detection limits for all the time intervals, yet, as the procedure required manual scanning of paper strip, the minimum time delay (human delay) between application of sample until analyzing the pixel intensity was found to be 3–5 s. Hence, the biosensor baseline time was fixed at this level. Beyond this level, biosensor response was noted down at different time intervals and biosensor response time of 45 s was calculated on the basis of obtaining 90% of saturated response from highest

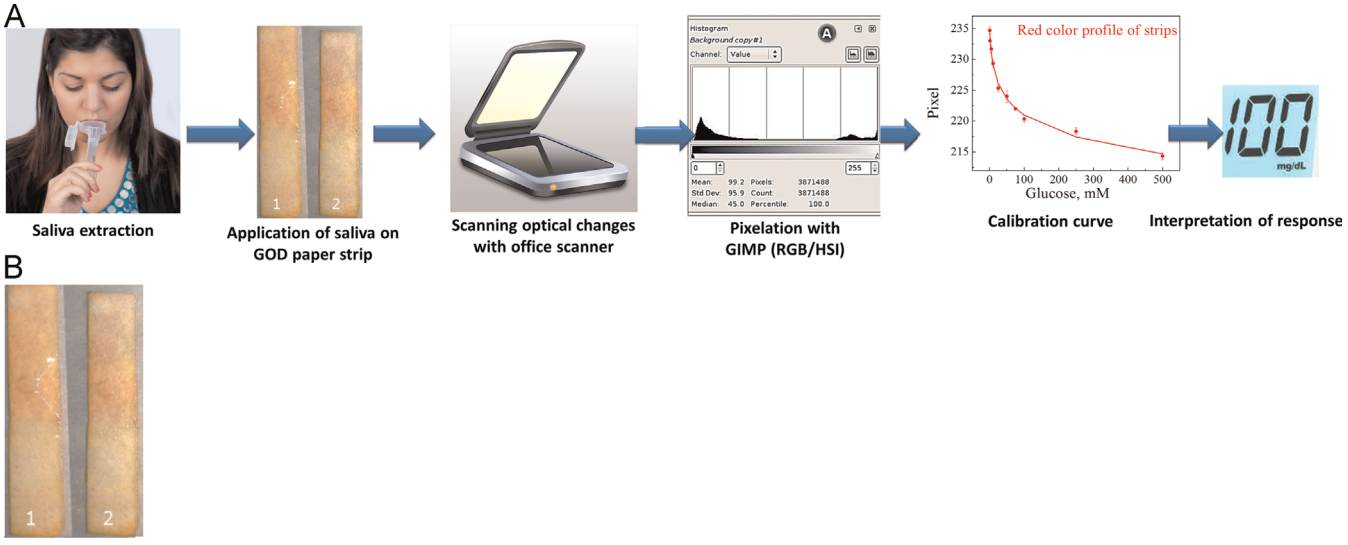

Fig. 1. (A) Schematics of salivary glucose detection using paper strip based biosensor. (B) Color of filter paper strips used in the study at the time of application of sample (1) and after 45 s (2). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

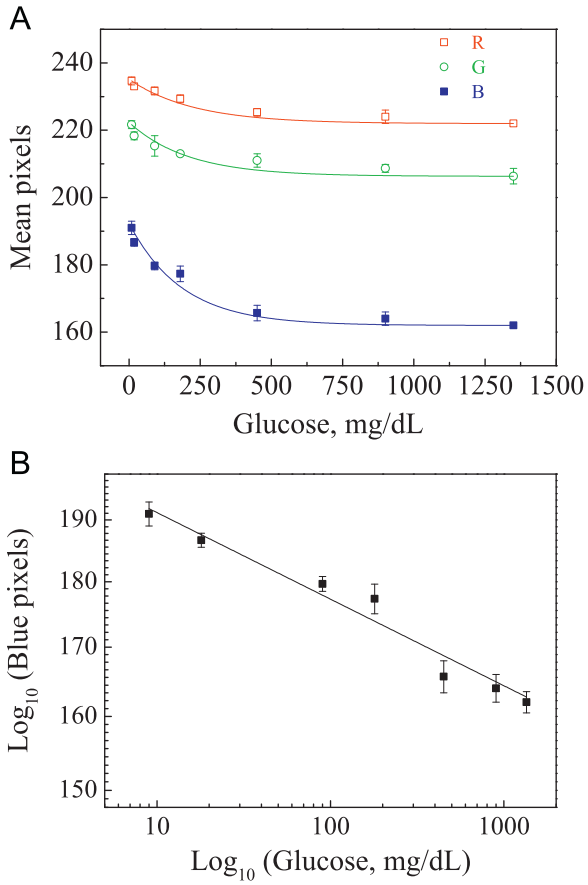

Fig. 2. (A) Calibration curve of paper strip based biosensor in terms of concentration (mg/dL) vs. pixel intensity of red (-□-)/green(-○-)/blue(-■-) color components in RGB profile. (B) Linearized double log (Log_{10}) calibration curve for blue pixels. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

measured concentration. Amongst R, G and B color pixels, blue color profile showed highest sensitivity of 0.0033 pixel/mg dL^{-1} glucose in the clinically significant concentration range of 36–540 mg/dL. Although in the wide concentration range of 9–1350 mg/dL, the calibration curve pertaining to blue pixels resembled exponential curve (Fig. 2A), it could be linearized by using logarithmic (Log_{10}) scale on both axes (Fig. 2B). The regression coefficient of this linear calibration curve was 0.96 (Eq. 1).

$$\mathrm{Log}_{10}(\mathrm{Blue\ \ pixels}) = 2.3144\text{–}0.0328\ \left[\mathrm{Log}_{10}(\mathrm{glucose,\ \ mg/dL})\right] \quad (1)$$

The limit of detection (LOD) of a sensor is usually calculated as the concentration at which sensor signal to noise ratio is just above 3. Therefore, while measuring the pixel density changes in samples devoid of glucose (buffer only), sensor noise was found to be 0.2 pixel (standard deviation 0.5 pixel, $n=15$) which was equivalent to 7.4 mg/dL glucose as per Eq. (1). Hence the LOD of biosensor was calculated as 22.2 mg/dL of glucose ($S/N=3$). The SGL measurements by other groups had better LOD values, such as Yamaguchi et al. (1998) reported a LOD of 0.1 mg/dL with a range of 0.1–10 mg/dL glucose using electrochemical transducer. Under normal physiological condition, the fasting plasma glucose concentration is in the range 36–540 mg/dL (Heller, 1999; Makaram et al., 2014), whereas usual salivary glucose levels have been previously reported as 0.15–3.78 mg/dL (Yamaguchi et al., 1998; Makaram et al., 2014). Though, due to high LOD of 22.2 mg/dL, such a low SGL cannot be detected with our method, the primary aim of this study was to develop a biosensor for mass screening of

Table 1
Statistical correlation (*t*-test) between SGL (biosensor method) and BGL (commercial glucometer method).

Condition	Correlation coefficient	*t*-Test *p* values	Inference for SGL to BGL correlation
Healthy individuals	0.64	0.028	Significant difference in levels
Diabetic patients	0.95	0.46	Near identical levels
Fasting condition	0.78	0.008	Significant difference in levels
Post breakfast	0.96	0.49	Near identical levels

diabetes in individuals, in whose cases BGL and SGL are quite high (Table 1, Fig. 3) and within the detection range of our biosensor.

With regards to possible interferences, it was very likely that food and other interfering substances present in saliva could alter the pH of person's saliva, and hence influence the sensor reading. Therefore, we conducted interference studies on synthetic glucose samples (250 mg/dL) in presence of either ascorbic acid or lactic acid in different concentrations (0–250 mM) and found significant interference from these compounds on sensor response (Supplement, Fig. S1 and S2). While the sensor response with samples devoid of glucose showed an increasing trend (δ pixel density), indicating that with lowering of pH levels due to these weak acids, the blue pixel intensity reduces exponentially. The test strips had an enzyme loading of 7.7 units of GOD, capable of producing 115.5 mM gluconic acid in the microenvironment consisting of 50 μL of sample. On top of that, addition of another acidic specie in the medium should have increased pixel counts further. But in presence of glucose along with interferents, pixel density rather decreased with high concentrations of interferents, thus indicating possible enzyme inhibition. Therefore, it was advised that the individuals should not eat or drink anything, especially the interferents like ascorbic acid (such as chewing Vitamin C tablet or drinking Vitamin C fortified drinks) 30 min prior to measurement and draw fresh saliva each time after rinsing the mouth with drinking water to remove accumulated lactic acid and residual food particles. However, as an advantage of present methodology, no pre-treatment of saliva samples (such as centrifugation etc.) was necessary prior to these measurements.

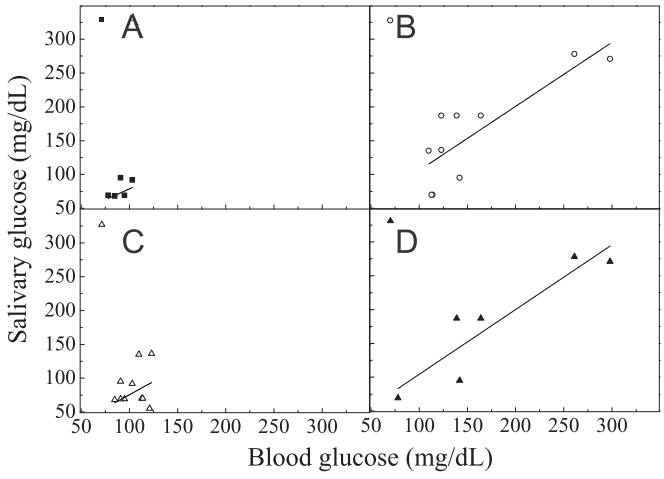

Fig. 3. Correlation between salivary (SGL) and blood glucose levels (BGL) in clinical patients and healthy individuals: the correlations for (A) non-diabetic (-■-) (0.64), (B) Diabetic (-○-) (0.95), (C) before breakfast (fasting glucose) (-Δ-) (0.78) and (D) post breakfast (PP) (-▲-) (0.96) samples were calculated.

Table 2
Statistical correlation (*t*-test) between three methods for glucose detection.

Methods	Biosensor method (SGL)	Commercial glucometer (BGL)
Commercial glucometer (BGL)	$p=0.16$ (insignificant difference)	
DNS reagent method (SGL)	$p=0.48$ (insignificant difference)	$p=0.11$ (insignificant difference)

3.3. Measurements with clinical samples

For the estimation of glucose in saliva, 50 μl of saliva samples collected from healthy as well as type-I/II diabetes patients (36 volunteers) were applied onto the filter paper strip and the color change was observed after 45 s using the office scanner. The SGL results were also obtained for individuals under fasting condition or after having breakfast. The results were compared with an commercial Accuchek blood glucose analyzer (which was routinely being used in hospital) for BGL values and the data was statistically compared using *t*-test. A correlation of 0.64 was found between glucose levels in saliva (SGL) and blood (BGL) of healthy individuals (*t*-test, $p=0.028$, $n=6$, indicating significant difference) and for diabetic patients it was nearly in the order of 0.95 (*t*-test $p=0.46$, $n=10$, indicating insignificant difference) (Fig. 3). While a skewed correlation between SGL and BGL in case of non-diabetic individuals was indicated in literature by various groups before, where they reported anywhere between 1:100 and 1:10 ratio between SGL to BGL (Darwazel et al. 1991; Panchbhai, 2012). A significant glucose concentration in saliva from the patients with insulin dependent diabetes mellitus (IDDM type1) was also reported (Amer et al., 2001; Carda et al., 2006). However, a statistically significant match between SGL and BGL levels of glucose in diabetic patients have been confirmed for the first time in the present study. In continuation of these correlation study, the responses for fasting and post meal SGL, the correlation between SGL and BGL were 0.78 under fasting condition (*t*-test, $p=0.008$, $n=12$, showing significant difference) and 0.96 for post breakfast samples (*t*-test, $p=0.49$, $n=6$, indicating insignificant differences). These correlations have been summarized in Table 1.

Though saliva is considered as ultra-filtrate of blood and glucose concentration in it should be proportional to blood glucose, yet, there are two possible explanations of such unexpected correlation. First, the mammalian salivary glands are highly resistant to the passage of glucose from the blood into their secretion. All the authors who studied this correlation agreed that, although the cells of the salivary glands are not entirely impermeable to glucose, they nearly behave so, since the amount of glucose which has at any time been detected in the saliva is nearly insignificant. But during pathological condition of diabetes in humans, sugar appears in the saliva in significant proportion (Rathery and Binet, 1920) possibly due to damage to the epithelial lining of salivary gland. And second, that there is natural decrease of the salivary stream in diabetics caused by the increase of diuresis or polyuria, that make the extracellular liquid decrease notoriously, and as a consequence, the production of saliva. This in turn concentrates glucose in the saliva and other excretory body fluids in diabetic individuals. On the other hand, occurrence of significant post meal SGL for healthy individuals indicates that further studies should be undertaken to find the reasons behind this phenomena.

The method for estimation of glucose in saliva was also validated by using the samples from same volunteer (to maintain homogeneity in samples) using DNS reagent (SGL), biosensor (SGL) and commercial blood glucose (BGL) (Accuchek Active, Roche Inc.) methods (Supplement Fig. S3). While one of the samples was obtained 2 h post meal on day one, others were pre breakfast samples obtained on day one and two. This comparison was shown to illustrate day to day and pre/post meal variation of SGL/BGL in same individual. The statistical t-test analysis between the different methods showed an insignificant difference between the three methods (Table 2), thereby validating the efficacy of present method vis-à-vis existing protocols for SGL measurement. The sensor response was also reproducible for more than 95% (Supplement, Fig. S4) when measured concurrently on same sample in triplicates without any time gap.

4. Conclusion

In the present study we developed a simple paper strip based biosensor for salivary glucose analysis. The GOD-pH indicator dye co-immobilized paper strips were scanned using office scanner to deduce their RGB profile, which was correlated to glucose concentrations. The response time of the biosensor was 45 s. Clinical measurements through the biosensor revealed a SGL to BGL correlation of 0.64 for non-diabetic and 0.95 for diabetic individuals or 0.78 under fasting condition and 0.96 for post breakfast samples. The statistical t-test analysis showed that SGL in post breakfast samples from healthy individuals and SGL in diabetic patients were significantly related to their BGL. The finding indicates that whether the individuals are diabetic or non-diabetic, an elevated blood glucose level also increased secretion of glucose into saliva and hence, it can be a basis of designing a biosensor for rapid saliva glucose analysis. This would be particularly helpful in mass screening of diabetes in countries, where healthcare costs prohibit people from routine glucose monitoring and due to that reason, often they remain undiagnosed until diabetic condition progresses into irreversible stage. Since we simplified the diagnosis approach with the use of GOD based pH sensitive filter paper strip, it can lead to development of a cost effective biosensor which can be used along with office scanner and even cell phones having a camera. Amongst the drawbacks of this sensor, it had a high LOD of 22.2 mg/dL glucose, but due to good SGL to BGL correlation of 0.95 in diabetic patients, glucose levels that will be encountered by our method shall fall within the clinically significant range of 36–540 mg/dL BGL. It was significant as the primary aim of our study was to establish a rapid mass diabetes diagnosis and not just to detect low levels of salivary glucose concentrations. Another drawback of our biosensor was that it was influenced with common interferents such as lactic and ascorbic acid, that would prohibit individuals from consuming food 30 min prior to measurement with a condition that fresh saliva should be drawn for analysis by rinsing the mouth. Further, we shall continue this study by including more donors of different age, sex, geographical locations to ascertain exact pattern and correlation between blood and salivary glucose in healthy and diabetic individuals to develop better standardized sensor.

Acknowledgements

The work was supported by Indian Institute of Technology Delhi, through its intramural research funding scheme.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.09.042.

References

Amer, S., Yousuf, M., Siddiqui, P.Q.R., Alam, J., 2001. Pak. J. Pharma. Sci. 14 (1), 33–37.

Carda, C., Mosquera-Lloreda, N., Salom, L., Ferraris, M.E.G.d., Peydró, A., 2006. Med. Oral Patol. Oral Cir. Bucal 11, E309–E314.

Danaei, G., Finucane, M.M., Lu, Y., Singh, G.M., Cowan, M.J., Paciorek, C.J., Lin, J.K., Farzadfar, F., Khang, Y.-H., Stevens, G.A., Rao, M., Ali, M.K., Riley, L.M., Robinson, C.A., Ezzati, M., 2011. Lancet 378, 31–40.

Darwazel, A.M., MacFalane, T.W., McCuish, A., Lamey, P.J., 1991. J. Oral Pathol. Med. 20 (6), 280–283.

Guilbault, G.G., Palleschi, G., Lubrano, G., 1995. Biosen. Bioelectron. 10 (3–4), 379–392.

Hebb, C.O., Stavraky, G.W., 1936. Exp. Physiol. 26 (2), 141–153.

Heller, A. Annu, 1999. Rev. Biomed. Eng. 1, 153–175.

Jha, S.K., Kanungo, M., Nath, A., D'Souza, S.F., 2009. Biosens. Bioelectron. 24 (8), 2637–2642.

Kumar, J., Jha, S.K., D'Souza, S.F., 2006. Biosens. Bioelectron. 21 (11), 2100–2105.

Lambert, J.L., Borchert, M.S., 2001. In: USPTO (Ed.), US 6181957, US.

Lipson, J., Bernhardt, J., Block, U., Freeman, W.R., Hofmeister, R., Hristakeva, M., Lenosky, T., McNamara, R., Petrasek, D., Veltkamp, D., 2009. J. Diabetes Sci. Technol. 3 (2), 233.

Makaram, P., Owens, D., Aceros, J., 2014. Diagnostics 4, 27–46.

Mohun, A.F., Cook, I.J.Y., 1962. J. Clin. Pathol. 15, 169.

Panchbhai, A.S., 2012. J. Oral Maxillofac. Res. 3 (3), e3 (1–7).

Rao, P.V., Srinivasa, R.N., Roberts, C.T., 2010. Med. Update 10, 110–126.

Rathery, Binet, 1920. La Presse Med. 27, 263.

Twetman, S., Nederfords, T., Stahl, B., Aronson, S., 1992. Pediatric Dent. 3, 184–188.

Yamaguchi, M., Mitsumori, M., Kano, Y., 1998. IEEE Eng. Med. Biol., 59–63.