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# Smartphone based non-invasive salivary glucose biosensor

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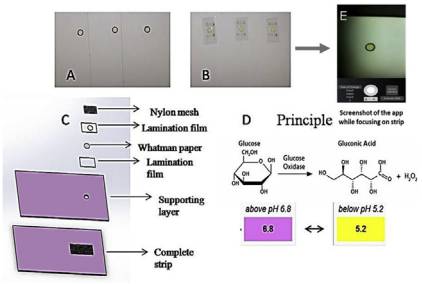
## HIGHLIGHTS

- A smartphone based non-invasive glucose biosensor was developed using saliva samples for diagnosis of diabetes.
- The biosensor was fabricated by immobilizing *Glucose oxidase* enzyme along with pH indicator on a paper strip.
- Color change of pH indicator upon reaction with glucose was estimated by using in-house developed android app.
- Clinical validation of biosensor was performed on healthy & diabetic subjects to correlate blood & salivary glucose levels.
- Use of smartphone enabled on-site determination of glucose levels without involvement of any specialized instrument.

## GRAPHICAL ABSTRACT

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## ABSTRACT

The present work deals with the development of a non-invasive optical glucose biosensor using saliva samples and a smartphone. The sensor was fabricated with a simple methodology by immobilization of *Glucose oxidase* enzyme along with a pH responsive dye on a filter paper based strip. The strip changes color upon reaction with glucose present in saliva and the color changes were detected using a smartphone camera through RGB profiling. This standalone biosensor showed good sensitivity and low interference while operating within 20 s response time. We used various means for improvements such as the use of slope method instead of differential response; use of a responsive pH indicator and made numerous tweaks in the smartphone app. Calibration with spiked saliva samples with slopes for (R + G + B) pixels revealed an exponentially increasing calibration curve with a linear detection range of 50–540 mg/dL, sensitivity of 0.0012 pixels $sec^{-1}$/mg $dL^{-1}$ and LOD of 24.6 mg/dL. The biosensor was clinically validated on both healthy and diabetic subjects divided into several categories based on sex, age, diabetic status etc. and correlation between blood and salivary glucose has been established for better standardization of the sensor. Correlation of 0.44 was obtained between blood and salivary glucose in healthy individuals whereas it was 0.64 and 0.94 in case of prediabetic and diabetic patients respectively. The developed biosensor has the potential to be used for mass diagnosis of diabetes especially in such areas where people remain prohibited from routine analysis due to high healthcare

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cost. Apart from that, a smartphone would be the only device the user needs for this measurement, along with a disposable low cost test strip.



## 1. Introduction

In today's developing world, more and more people are adopting sedentary lifestyle making them prone to several diseases and metabolic disorders such as *Diabetes mellitus* or diabetes. If left untreated for a long time, it can lead to several complications which can be categorized into microvascular (retinopathy, neuropathy, nephropathy etc.) and macrovascular complications (cerebrovascular disease, coronary heart disease, peripheral vascular disease, myocardial infarction etc.). According to International Diabetes Federation, about 415 million people in the world are found to be affected with diabetes including about 193 million people with undiagnosed diabetes and the number may rise to around 642 million by 2040 [1]. Therefore, monitoring of glucose levels on a daily basis is important, especially for individuals with confirmed diabetes, in order to avoid various complications associated with the disease.

The most common laboratory tests for the diagnosis of diabetes include Fasting Plasma Glucose test (FPG), Oral Glucose Tolerance test (OGTT), Random blood sugar test and Hemoglobin A1c test (HbA1c test). Although in recent years, Self-Monitoring of Blood Glucose (SMBG) has emerged as a better option. The main goal of SMBG is to monitor a person's blood glucose at different time intervals which can aid a doctor in adjusting medication/insulin dose and is also useful to a patient in evaluating his/her response to therapy [2]. The most common method of SMBG involves finger pricking to ooze out blood which may be inconvenient and painful to subjects. In recent years, saliva, tears, urine etc. have emerged as better alternatives to blood [3].

Various groups around the world have been working on non-invasive diagnostics in the field of glucose monitoring. Agrawal et al. tried to correlate blood glucose and saliva glucose concentration using known and commercial methods. They reported a correlation of +0.58 and + 0.40 among non-diabetic and diabetic subjects respectively [4]. Clark et al. developed iQuickit Saliva Analyzer, which is a standalone glucose biosensor for salivary glucose monitoring which can sync data to mobile devices.-Whitaker et al., Sanofi-Aventis reported iBGStar, a standalone electrochemical biosensor device which can be attached to a smartphone to draw power and send data to the mobile device [5]. Christopher Wilson and his team reported a tear based electrochemical glucose sensing medical device that uses a flexible wireless sensor system with an electrode resembling the shape of a hollow coil [6]. An electrochemical biosensor was fabricated by Claussen et al. to detect glucose in saliva and tears using complex photolithographic technique involving nanosheets of graphene, platinum nanoparticles and *Glucose oxidase* enzyme [7].Similarly, a device was developed by Liakat et al., which measured blood glucose levels by targeting the dermal interstitial fluid using mid-infrared quantum cascade laser spectroscopy [8]. Wang et al. described a saliva glucose monitoring system based on electrochemical sensing using carbon nanotubes functionalized with metal nanopartocles, polymer layers and *Glucose oxidase* enzyme [9]. However, major drawbacks of these sensors are involvement of complex procedures and costly reagents in sensor fabrication. Some of them involve electrochemical techniques for detection which are more prone to chemical interferences. Especially, the tear based sensors need to be worn on the eyelid and involve extraction of tear fluid which is inconvenient to most subjects. Most of these developed sensors are expensive and bulky in nature, hence can't be used for onsite diagnosis conveniently, except for the commercially developed iQuickit Saliva Analyzer, however, due to its closed source, authors could not verify its relevance to affordability vs. accuracy.

It was consistently felt across the research community that among these non-invasive body fluids, saliva was most significant in glucose determination because of its good correlation with blood glucose as reported by various groups [10–13]. Keeping this fact in mind, our group had developed in past a paper strip based glucose biosensor for non-invasive determination of salivary glucose by scanning color changes due to pH using an office scanner and RGB profiling using open source image processing software (GIMP) and a good correlation has been obtained between blood glucose level (BGL) and salivary glucose level (SGL) in case of diabetics [13]. However, this work was merely a proof of concept and needed to be developed further to make it a standalone solution for non-invasive glucose monitoring.

Recently, smartphones have emerged as portable, inexpensive and convenient platforms for point of care testing of various analytes. With the rapidly advancing technological era, smartphones have gained high importance among common people. According to ComScore, 67% of the mobile phones sold today are smartphones. It is estimated that by 2017, over one third of world's population will own a smartphone accounting to almost 2.6 billion smartphone users in the world. More than 40,000 mobile health applications are presently available and their number is increasing rapidly. The importance of smartphones in diagnostic testing lies on the fact that these are small, self-contained, standalone devices which are ubiquitous and most of them contain a high quality built in camera and possesses computational features; most of these are GPS enabled which integrate them to various public health projects. Thus smartphones act as robust, inexpensive, miniaturized systems which can help achieve the goal of “personalized medicine” where a layman can easily conduct the testing himself at home with limited training and also lead to *in situ* diagnosis in poor as well as remote areas devoid of conventional equipments and health facilities [14–17]. The major players in the field of smartphone based diagnostics include Agamatrix Inc., Alere Inc., CellScope Inc., Genomic Health Inc., Lifescan Inc., Oasis Scientific Inc., QIAGEN, Telecare etc. [16]. Such diagnostic methods include that for lactate [18,19], cortisol [20,21] etc. using sweat, saliva and blood. These are also used for the detection of pathogens [22,23] and biomarkers for diseases such as cancer [24,25] in non-invasive body fluids as well as serum. Both optical as well as electrochemical techniques have been employed for detection. An optical smartphone based biosensor for glucose has been developed by Chun et al. where they have fabricated the strips using wax printing and microfluidics and color changes have been detected using Image J software. Testing of the biosensor has been carried out in human serum [26]. Some smartphone based glucose monitors have also been commercialized such as the iBGStar by Sanofi Aventis, C8 Medisensors non-invasive glucose monitoring system etc. and many smartphone based applications for diagnosis of diabetes are also available such as Diabetes Buddy by Krodzone Technologies, Diabetes Pilot by

Digital Altitudes, Diabetes log by Distal Thoughts, Glucose Buddy by SkyHealth, Diamedic by Nicholas Martin, iDiabetes by iHealth Ventures, WaveSense Diabetes Manager by Agamatrix etc. [27]. However, major drawbacks of these reported sensors are involvement of complex micro fluidic and wax printing techniques for strip preparation and costly reagents for diagnosis such as the one reported by Chun et al.; use of interference prone techniques such as dynamic electrochemistry and Raman spectroscopy involving human skin where end results vary from user to user and even site to site and hence are inaccurate, unreliable as well as expensive in nature. Existing smartphone based apps also had limitations such as lack of personalized feedback, security issues and usability issues such as data entry and integration with patients and electronic health records [28].

Therefore, after weighing in the drawbacks of reported non-invasive sensors and intended usability, portability and accuracy of the device, we have conducted a major overhaul of our previous method [13] and developed a standalone smartphone based optical biosensor for the detection of glucose in saliva samples. Here, we have tried to make the biosensor more user friendly by incorporating a smartphone based platform, thus eliminating the need of any dedicated instrument for diagnosis and also increased the sensitivity by using a better pH indicator than methyl red used in our previous study. We developed our own android application, while incorporating response slope based detection instead of using differential method to further enhance the sensitivity and minimize interferences due to ambient light. Besides, we validated the developed sensor on larger number of patient's samples to confirm any clinically significant BGL to SGL correlation.

## 2. Materials and methods

### 2.1. Materials

Smartphone (model Samsung Galaxy SIII) was of Samsung India Limited make; commercial glucometer (Accuchek active™) was from Roche India Limited; Laminator (Model ECO 12) was from Excelam™.Filter paper (Whatman number 1), Polyvinyl alcohol (Cat. No.563900), Bromocresol purple solution (Cat. No.860891), *Glucose oxidase* (G7141-250 KU), dextrose (Cat. No.G8270), sodium phosphate dibasic (Cat. No.V800397), sodium phosphate monobasic (Cat. No.V800376), dithiothreitol (Cat. No.43819) were purchased from Sigma Aldrich India.Card stock sheets, lamination film, double sided tape, nylon mesh, ear buds (Johnson and Johnson) were purchased from local market.

### 2.2. Methods

#### 2.2.1. Preparation of test strips and immobilization of glucose oxidase

The test strips were fabricated using a card stock having dimensions of 8.9 cm × 5 cm and thickness of about 0.35–0.5 μm which act as supporting layer. A hole of 4.5 mm was created on the supporting layer at a distance of 1.5 cm from the top which was then laminated using a thin lamination film to constrict the sample within the detection zone. Another layer of lamination film with a similar hole (4.5 mm) was used to constrict the Whatman filter paper (circle with 5 mm diameter) between the two films. The filter paper acts as detection zone. The detection zone was then covered with a nylon mesh (1.2 cm × 0.8 cm) held on both sides with the help of a double sided tape. The remaining part of the mesh excluding the detection zone may be covered with the help of a white adhesive tape. The front and back view of the strip as well as layered structure along with reaction mechanism are depicted in Fig. 1A-D.

For immobilization on 100 such strips, 500 μl of solution was prepared by mixing 5 mg of *Glucose oxidase*, 200 μl of dye (bromocresol purple), 200 μl of phosphate buffer (1 mM, pH 7) and 100 μl of 0.25% PVA. To increase the stability of the strips, 1 mg of dithiothreitol may also be added and the solution is then mixed thoroughly. A 5 μl of the above solution was then immobilized on the detection zone through the mesh with the help of a micropipette such that the enzyme loading is 10U per strip and then dried and stored desiccated at 4 °C until use. The enzyme activity of the strips was estimated using DNS (dinitrosalicylic acid) based spectroscopic method and protein content in the strip was determined using Lowry's method of protein estimation.

#### 2.2.2. Development of smartphone application for RGB profiling

The RGB profile of the strips with respect to glucose concentration was determined using our own smartphone based android application (app) developed in-house. The application was designed to estimate glucose levels with respect to changes in RGB pixel intensity using slope method. Slope method (change in sensor response within a pre-specified time interval) was chosen as compared to differential method (change in sensor response between two time values) of obtaining response in order to minimize ambient light interferences which may result in false readings. Another advantage of slope based method was that no baseline correction was needed while calibrating, as the slope of sensor response curve remains almost constant over the time interval in which the reaction takes place, especially in the linear range of the sensor. While in differential method, the response keeps changing with respect to time and a 90% of the saturated sensor response is to be taken for calibration purposes.

The app was designed on an Android platform incorporating the camera as well as the flashlight of the smartphone. For estimation of glucose levels through the app, sample was added on the strip and then the strip was placed in close proximity to the smartphone camera. To minimize variations due to distance of detection zone on the strip to the smartphone camera, especially when the strip was to be placed against a surface or held in hand, we used a dark cardboard box with a depth of 3 cm and a hole proportional to smartphone camera and flash areas taken together. The strip was placed in the box after application of sample and the sequence of detection was initiated in the app thereafter by setting the time interval within which the slope was to be taken. The application automatically recognized the detection zone. In the background, app took two snapshots of detection zone at pre-specified time intervals and then calculated the average slopes for red, green and blue pixel changes at 20 random points within the detection zone. Finally glucose concentration in the sample was deduced and the result is displayed on the smartphone screen. Screenshots of the app while detecting glucose (Fig. 1E) and flowchart of these steps are depicted in Supplement Fig. S1 & S2.

#### 2.2.3. Biosensor measurements

Calibration of the biosensor was carried out using spiked saliva samples. For calibration purpose, saliva sample from a healthy individual was spiked with 5% volume of glucose solutions (in phosphate buffer saline pH 7) of varying concentrations (0–750 mg/dL) to minimize variations in viscosity and other salivary parameters. Glucose concentration already present in saliva of the healthy donor was determined using DNS based spectroscopic method and the values obtained were added respectively to each of the spiked values in order to obtain actual glucose concentration in the spiked sample. For biosensor measurements, 5 μl of spiked saliva sample (0–750 mg/dL) was applied on the strip through the mesh and the color changes were scanned on other side of the strip using our developed smartphone application for RGB profiling.

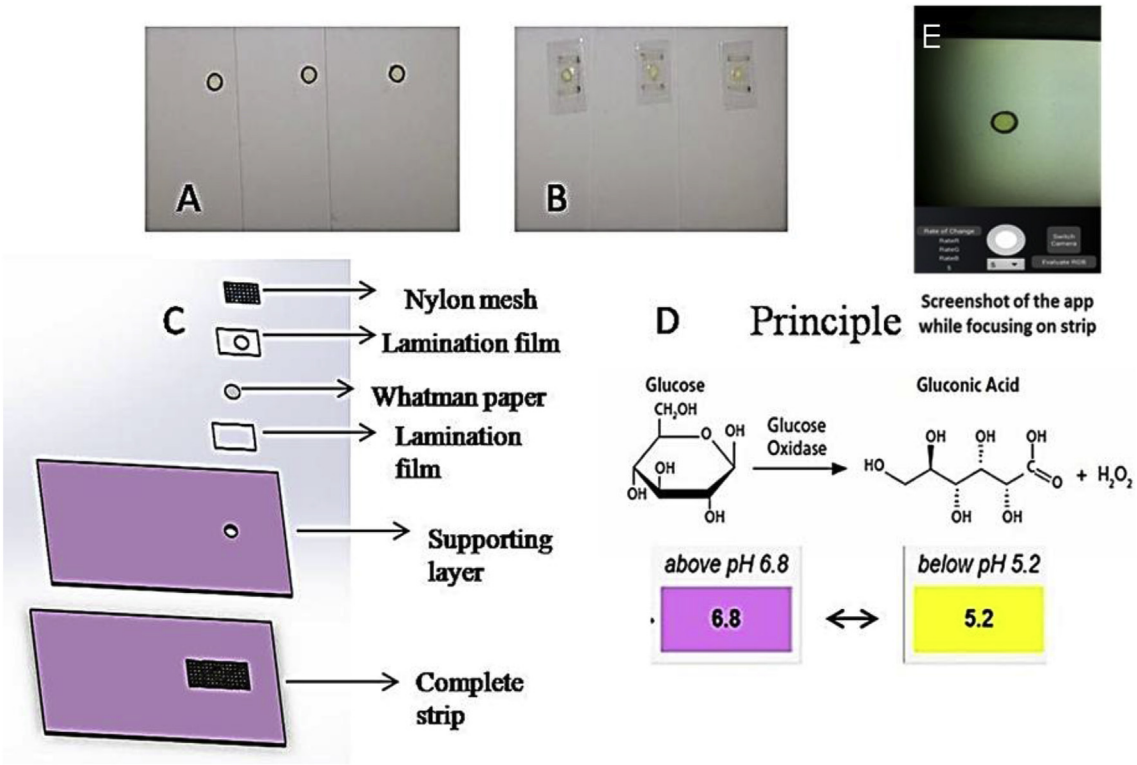


**Fig. 1.** (A & B) Front and back view of the biosensor strips, (C) Layered structure of the strip (D) Principle involved in color change of biosensor strip due to *Glucose oxidase* reaction, (E) Screenshot of developed android app focusing on detection zone of test strip.

Usually 10 s is the minimum time required for sample handling (sample application and absorption on the strip), thereafter response slope was obtained for 10 s (after initial 10 s of sample handling), thus giving a response time of 20 s total. Change in slope for red (R), green (G) and blue (B) pixels were obtained for different glucose concentrations and calibration curves were then plotted using Originlab 7.5 software. As R, G and B together form an additive model of color change, different combinations of these colors were used (total pixels changes for R + G, G + B, R + B and R + G + B) and calibration curves were obtained against glucose concentration v/s change in slope within 10 s for all these combinations separately to increase the sensitivity. Calibration was carried out five times and data plotted with standard deviation as error bars.

### 2.2.4. Shelf-life studies and effect of camera sensor, ambient light and chemical interference on biosensor response

Interference studies were carried out using ascorbic acid and lactic acid which are commonly found interferents present in human saliva. Interference from lactose, a common disaccharide found in milk products, suspected to interfere with the sensor response when it breaks down into glucose and galactose due to the presence of salivary lactase was also checked. For these studies, 5 μl of sample was added onto the strip with or without spiking with glucose. These samples contained various concentrations of interferents (0–5 mM) and 125 mg/dL glucose was used for spiking.

Studies were also conducted on 100 mg/dL synthetic glucose under different light conditions to know the best mode of obtaining response; modes used were ambient light (flashlight on and off), box (flashlight on) and attached mode (strip attached onto camera lens directly with flashlight on and off). The dark box used in the study had dimensions 14.9 cm × 8 cm × 5.3 cm and the length and width of cut on the box were 5.1 cm × 2.1 cm through which camera was focused on the detection zone of the strip. The distance of cutout from edge of the box was 1.3 cm and the distance of camera from strip was 5 cm. Calibration was also carried out with different dyes and different smartphone models were used and a comparison between the responses obtained in all these cases was also carried out. For repeatability, reproducibility and other studies, saliva sample was obtained from few healthy subjects and testing was done using our biosensor on consecutive days. The shelf life study on the strips was also carried out for a period of 90 days. All the results were plotted using Origin lab software and correlation obtained by *t*-test using Microsoft Excel software.

### 2.2.5. Saliva sample collection and measurement technique

For estimation of glucose in real sample, saliva sample was applied on the strip using a clean ear-bud and color changes were scanned on the other side of the strip using our developed application. Sample application using an ear bud had more uniformity as compared to a pipette; hence response was also better in case of using buds. Usually about 5.28 μl of saliva sample (SD = 0.86, n = 6) is transferred on the strip using an ear-bud through gentle touch. Fresh saliva was obtained every time after rinsing of mouth with drinking water and the sample was applied directly on the strip without involving any centrifugation or filtration step. For estimating color changes in the strip through the app, the strip was placed inside a dark box after adding saliva sample; time for obtaining slope (10 s) was already fixed before adding saliva. Then the smartphone was placed over the box while the camera and flash light areas could oversee the strip through the hole. The app then automatically sensed the detection zone and calculated change in response slopes and displayed unknown glucose concentration according to the calibration curve equation fed into the app (Supplement Fig. S2).

*2.2.6. Clinical validation of the biosensor on real samples*

Validation of the biosensor was carried out on both healthy and diabetic subjects. Sampling was carried out on 91 subjects between age group 20–80 years at Outpatient Department of Indian Institute of Technology Delhi hospital, New Delhi following institute's ethical guidelines; written consent was obtained from all the subjects, no money was paid to them neither were their identities revealed. Saliva samples were collected from all the subjects using a clean ear bud and glucose concentration determined using our developed app. Fresh saliva was obtained every time after rinsing of mouth with drinking water as per the process mentioned earlier. Individual's blood glucose level at the time of obtaining saliva was also determined using Accuchek active blood glucometer. The SGL obtained through our biosensor was then correlated with BGL obtained using Accuchek active glucometer using statistical analysis and *t*-test using Microsoft Excel software. In few samples, salivary glucose obtained using our biosensor was also correlated with salivary glucose obtained using DNS based spectroscopic method apart from blood glucose measurement and a comparison between all three methods was carried out.

## 3. Results and discussion

### 3.1. Strip preparation and characterization of enzyme

The immobilized strips were stored at 4 °C until use to maintain the enzyme activity. Enzyme activity of the strips was determined by dinitrosalicylic acid based spectroscopic method [29] whereas protein content was estimated by modified Lowry's method [30]. Enzyme activity and protein estimation was carried out on the strips in triplicates. The enzyme activity as reported by DNS based spectroscopic method was found to be 3.9 U/strip whereas the protein content per strip as estimated by Lowry's method was reported to be 234 μg. Shelf life study for the biosensor was carried out for a period of 90 days, the sensor was found to retain atleast 80% of its original activity during this period, which was expected because glucose oxidase is known to be a stable enzyme (Supplement Fig. S3). Study of batch variation was carried out among the strips immobilized at four different time intervals, results indicate around 15% error rate among the batches (Supplement Fig. S4), especially as the fabrication of strip was done manually. Automation of strip fabrication, and automated sample application is expected to bring down such batch wise variations further.

### 3.2. Biosensor measurements

Calibration of the biosensor was carried out using a pipette as well as using an ear bud. Studies indicate that approximately 5 μl of saliva sample gets transferred to the strip using an ear bud if applied gently (Supplement Fig. S5), therefore to maintain similar conditions, 5 μl sample volume was fixed during calibration using a pipette. Calibration curve obtained using an ear bud gave approximately 30% better response as compared to a pipette due to uniformity in sample application on the strip using an ear bud (Supplement Fig. S6). This was since the best auto-pipettes these days have error probability of 10–15% in low volume applications.

Testing was also carried out using two different pH indicators on the test strip-bromocresol purple and bromothymol blue having similar pH ranges. The strip changed color due to the decrease in pH upon the formation of gluconic acid as a result of reaction of Glucose oxidase enzyme with glucose present in saliva sample. The larger the concentration of glucose present in the sample, more amount of gluconic acid is formed and hence color change was more profound (Fig. 1D). During comparison of dyes, it was observed that bromocresol purple produced around 20% better response than bromothymol blue as well as found to saturate at higher concentrations when compared to the latter (Supplement Fig. S7). Therefore, final calibration was carried out inside a dark box using bromocresol purple as pH indicator using ear bud.

Calibration curves were obtained for change in slope for R, G and B pixels with respect to glucose concentration. Among R, G and B, exponentially increasing curves were obtained for R and G with respect to glucose whereas in case of B, the response does not vary significantly with increase in glucose concentration (Fig. 2A). To increase the sensitivity, calibration curves were also plotted for various combinations of slope (R + G), (G + B), (R + B) and (R + G + B) with respect to glucose concentration (Fig. 2B). Among these combinations, (R + G + B) was found to provide maximum response, therefore clinical validation and other studies on the biosensor were carried out by plotting Slope (R + G + B) against glucose concentration.

An exponentially increasing calibration curve has been obtained by plotting Slope (R + G + B) against glucose concentration (Fig. 2C) which can be represented with the help of equation (1) where x denotes unknown glucose concentration and y stands for Slope of response curve (R + G + B). The response time has been fixed as 20 s where 10 s is the time required for sample application and absorption of sample on the strip and then slope was taken for the next 10 s.

$$y = 1.45\ (x)^{0.54} / (48.03)^{0.54} + (x)^{0.54} \quad (1)$$

The calibration curve can be linearized by taking double log ($\log_{10}$) on both the axes (Fig. 2D) which can be represented by equation (2).

$$\text{Log}_{10}\ (R + G + B)\ \text{Slope} = 0.53\ \log_{10}\ (\text{Glucose}) - 1.54 \quad (2)$$

This could be simplified for concentration range 90–540 mg/dL by plotting a linear curve as equation (3)

$$y = 0.29349 + 0.00104\ x \quad (3)$$

Limit of detection of a biosensor is usually calculated as the concentration at which sensor signal to noise ratio is just above 3, but practically it can also be calculated by measuring the change in Slope (R + G + B) for samples devoid of glucose. For this purpose, healthy individual's saliva sample was allowed to react with *Glucose oxidase* for some time so that whole glucose present in the sample gets consumed. The sensor noise level for this sample was found to be −0.196 pixels/sec (n = 5) which was equivalent to 24.6 mg/dL. The sensitivity for the biosensor in terms of (R + G + B) Slope between the clinically relevant range of 50–540 mg/dL was found to be 0.0012 pixels $\text{sec}^{-1}$/mg $\text{dL}^{-1}$.

### 3.3. Effect of camera sensor, ambient light and chemical interference on biosensor response

Various factors such as location of smartphone camera, ambient light as well as chemical interferences such as presence of ascorbic and lactic acids and lactose in mouth can affect biosensor response. To estimate the effect of all these parameters on sensor response, studies have been carried out. To estimate the effect of chemicals on sensor response, interference studies were carried out using various concentrations of ascorbic and lactic acid and lactose (0–5 mM). In samples devoid of glucose, an increasing trend in Slope (R + G + B) has been reported with increase in lactic acid or ascorbic acid concentration due to decrease in pH. However, in samples containing glucose along with interferents (ascorbic or lactic acid), saliva was found to act as a buffer with concentrations

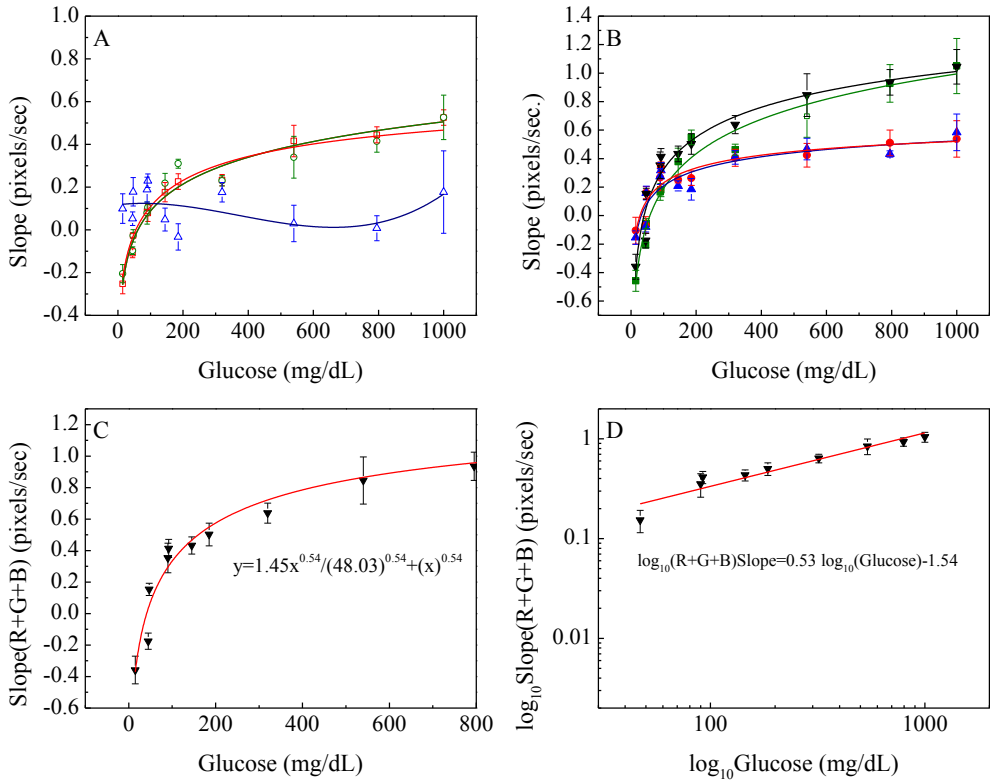


**Fig. 2.** (A) Calibration curve plotted against glucose concentration v/s change in slope for R (-□-), G (-○-) and B pixels(-Δ-) (B) Calibration curve plotted with respect to glucose concentration v/s change in slope for (R + G) (-■-), (G + B) (-●-), (R + B) (-▲-) as well as (R + G + B) (-▼-) pixels (C) Final Calibration curve plotted against glucose concentration with respect to slope (R + G + B) pixels (D) Linearized Calibration curve formed by taking double log ($\log_{10}$) on both the axes.

ranging from 0 to 1 mM (clinically relevant range of lactic or ascorbic acid usually present in mouth) [31,32] and thus very slight increase in pixel intensity occurs up to this range; pixel intensity was found to increase significantly due to decrease in pH at higher concentrations of ascorbic or lactic acid beyond the clinically relevant range (Fig. 3A–D). This was due to the fact that ascorbic acid and lactic acid are all weak acids with similar pKa values and the response factors of them are the same in lower concentration ranges. The higher concentrations however, were out of clinically relevant range. Thus interferents such as ascorbic and lactic acids were found to have negligible effect on the sensor response in clinically relevant range, though, as a precaution, the users were asked to rinse their mouth prior to saliva collection in standard operating procedure (SOP) so that to get rid of accumulated interferents. The developed sensor was also found to be selective for glucose with no interference due to the presence of disaccharides such as lactose (0–5 mM) (Fig. 3E). The pH of saliva may range from 4.5 to 7.5 under different pathophysiological conditions. If such saliva of diverse initial pH is to be applied on the test strip, it might cause interference in reading. However, there was a clear advantage of using slope based calibration of biosensor. If there is ever any increase in slope of response curve, it is due to enzymatic reaction, as response due to pH range of saliva alone would have been saturated within a few sec.

Studies were also carried out in different modes to estimate the effect of ambient light on sensor response. Although slope method reduces outside light interferences to some extent, still ambient light is a major source of error in smartphone based optical sensing and therefore best conditions need to be chosen in order to get accurate results. Results from our preliminary studies carried out on 100 mg/dL synthetic glucose in different modes indicate that attached mode (strip attached to smartphone camera) produced maximum sensor response whereas in ambient light (flashlight on) and inside a dark box (flashlight on) there is slight variation in pixel intensity (Supplement Fig. S8). Major drawback in attached mode was that the strip was out of focus with respect to camera, as the strip was physically stuck on it. The flashlight can diffuse in wet white filter paper and thus illuminated the paper more intensely than the ambient light could. This could be a reason for less variation in sensor response in this mode. Moreover, in ambient light still there were chances of error, especially if there was abrupt change in light intensity, such as if the user moves from well-lit area of room to darker corner during measurement. Besides, it may not be very hygienic to place saliva samples over camera lens especially if it is to be used in primary healthcare centers. Therefore, to avoid inconsistencies in camera focusing and to make the sensor more relevant to commercialization all the studies were carried out inside a light tight box with a hole to allow camera to focus on strip placed within it. With this, we suggest the requirement of a box type external magnetic latched attachment of sort to the camera to place the test strip before focusing the camera into it.

Effect of various smartphone models on sensor response was also tested. It was mainly carried out for the reason that most of the smartphone brands have different camera modules and from different manufacturers and specifications, which can bring deviations in sensor measurements. Three smartphone models were used for the purpose i.e. Samsung Galaxy SIII, HTC Desire 526G Plus and Gionee P5 mini. Study was carried out inside a dark box as well as under ambient light conditions so as to gather information about how camera position and flashlight intensity can affect sensor response. It was observed that inside a dark box there was only slight change in pixel intensity with respect to smartphone models

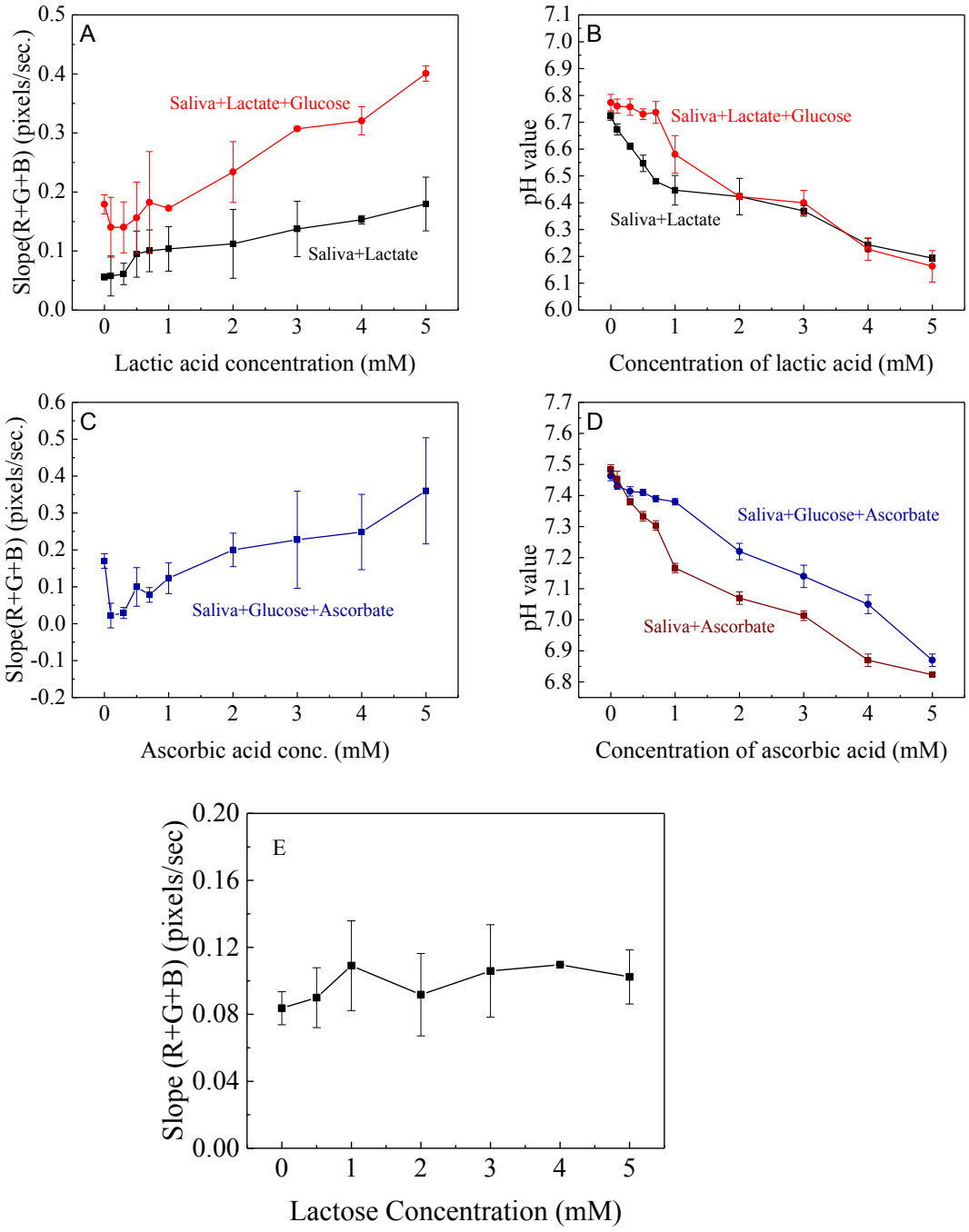


**Fig. 3.** Interference studies using acids such as lactic and ascorbic acid and disaccharides such as lactose. (A) Effect of lactic acid concentration on sensor response. In the clinically relevant range of lactic acid found in mouth (0–1 mM), saliva was found to act as a buffer to some extent while with higher concentrations of lactic acid, sensor response increases with decrease in pH. (B) Study of buffering action of saliva upon addition of lactic acid. (C) Effect of ascorbic acid concentration on sensor response. Similar behavior has been noted as in the case of lactic acid with increasing concentration. (D) Study of buffering capacity of saliva upon addition of ascorbic acid. (E) Effect of lactose concentration on sensor response.

while in ambient light conditions difference in camera position and intensity of flash leads to variation in response and it was reported that Gionee P5 mini which contained side camera gave more response as compared to Samsung Galaxy SIII and HTC Desire 526G Plus, both these models had camera positioned at the centre (Supplement Fig. S9). Thus, it was concluded that in order to use the biosensor in ambient light condition, internal compensation mechanism have to be incorporated to the developed app for each smartphone model - in the line that open source smartphone ROM developers such as Cyanogenmod and LineageOS developers do. Else, a 3D printed or a plastic attachment have to be used to place the test strip and then focus the smartphone camera onto the detection zone in order to get optimal results. In case of smartphones having a side camera, the 3D printed dark box should be designed in such a way that the hole should allow flashlight to fall directly on the strip, then the app would automatically detect the detection zone on the strip and glucose concentration is determined. Apart from smartphones discussed above, any smartphone

with a minimum requirement of 5 megapixel camera can be used for glucose estimation.

### 3.4. Measurement with clinical samples

Clinical validation of the biosensor was carried out on 91 subjects, healthy as well as diabetic and correlation between blood glucose levels (BGL) obtained using Accuchek active blood glucometer and salivary glucose (SGL) using our biosensor has been carried out using *t*-test in Microsoft excel. Subjects were divided into several categories based on age, gender, diabeticstatus etc. and correlation coefficient was obtained for each. The results are depicted in Table 1 and Supplementary Table S1. In case of few patient samples, apart from obtaining correlation between BGL and SGL, correlation between SGL obtained using our biosensor and SGL obtained by DNS based spectroscopic method has been carried out and comparison of glucose values obtained between all three cases has been carried out (Supplement Fig. S10). As expected, SGL values obtained with developed biosensor and DNS based gold standard for estimation of glucose had close correlations, though, in this result we did not factor in the diabetic condition of individuals.

A very good correlation between BGL and SGL of 0.94 (n = 35, Slope = 0.97) was obtained in case of diabetic subjects with p value of 0.16 indicating nearly identical levels of BGL and SGL; in prediabetic subjects correlation obtained was 0.64 (n = 18, Slope = 0.85) with p value of 0.001 and in case of healthy subjects the correlation is not so significant with $R^2$ value of 0.44 (n = 38, Slope = 1.66) and p value of 9.78 $E^{-09}$ indicating significant difference between values of SGL and BGL. Higher correlation of 0.96 was obtained in case of post breakfast samples (n = 9, Slope = 0.97) as compared to pre-breakfast samples (n = 82, Slope = 1.12) where $R^2$ value has been reported as 0.90.Among pre-breakfast samples, higher correlation of 0.89 (n = 31, p = 0.09, Slope = 0.99) was obtained among diabetics as compared to healthy individuals with a $R^2$ value of 0.36 (n = 36, p = 4.35 $E^{-09}$, Slope = 1.69).The results have been depicted in Fig. 4 and also in Table 1.

With respect to gender wise distribution, males as well as females shared almost similar correlation between BGL and SGL of 0.93 (Males n = 48, Slope = 1.13, p value = 0.010; Females n = 43, Slope = 1.03, p value = 0.11) indicating insignificant difference between BGL and SGL. Among them diabetic males (n = 18, Slope = 1.12) and diabetic females (n = 18, Slope = 0.94) showed a correlation of 0.92 and 0.96 respectively with p values of 0.13 and 0.38 indicating almost similar levels. In case of prediabetic males (n = 10, Slope = 0.92) the correlation obtained was 0.67 which was quite less as compared to prediabetic females (n = 8, Slope = 0.79) with a correlation of 0.71 with p values of 0.01 in both cases indicating significant difference between BGL and SGL. In case of healthy males (n = 20, Slope = 1.67) and healthy females (n = 17, Slope = 1.60) correlation obtained was almost same with $R^2$ values of 0.45 and 0.40 respectively and p values of 7.11 $E^{-07}$ and 0.001

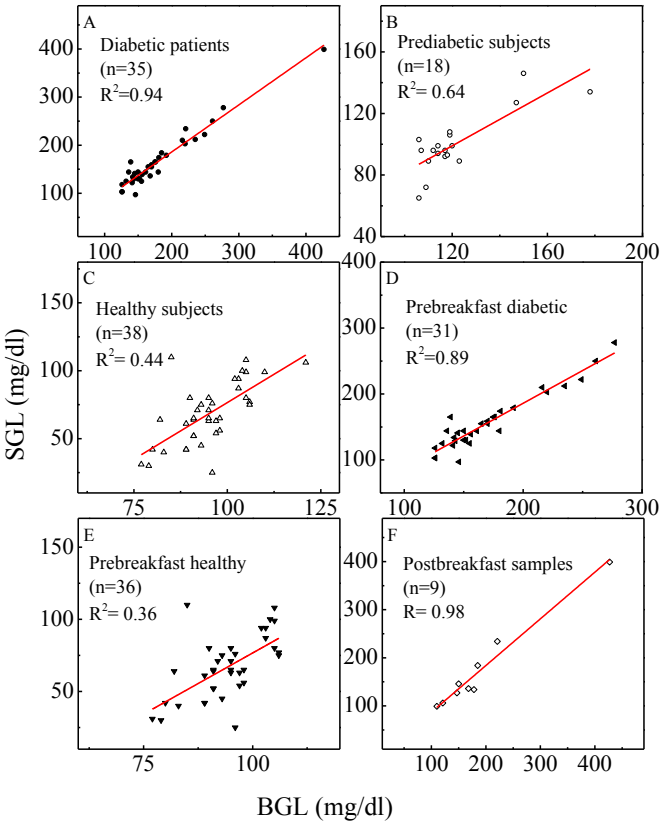


**Fig. 4.** Correlation between blood and salivary glucose levels in case of (A) diabetic (-□-), (B) pre diabetic (-○-) and (C) healthy (-Δ-) subjects. Correlation curves obtained between BGL and SGL for (D) pre breakfast diabetic (-◂-), (E) pre breakfast healthy (-▼-) and (F) post breakfast samples (-◊-).

indicating significant difference between BGL and SGL (Supplement Fig. S11 A and Supplement Table S1A).

With respect to subject's age, correlation between BGL and SGL was reported to be higher in case of subjects within the age group of 41–50 years ($R^2$ = 0.97, n = 23, Slope = 1.05) followed by subjects within the age groups of 61–70 years ($R^2$ = 0.94, n = 11, Slope = 0.22) and 51–60 years ($R^2$ = 0.93, n = 28, Slope = 1.13). This was expected as parotid gland damage in diabetic or aged individuals are high compared to non-diabetic or low age group individuals, thus the release of glucose in saliva is more prominent in these individuals. The correlation obtained was comparatively less in case of subjects within the age group 31–40 years with $R^2$ value of 0.76 (n = 17, Slope = 1.11) and was least in case of age group 71–80 years with $R^2$ value of 0.39 (n = 11, Slope = 0.69) (Supplement Fig. S11 B and Supplement Table S1B). With respect to age as well as gender, almost similar correlation has been obtained

**Table 1**
Correlation between BGL and SGL in clinical samples.

| Category | N | $R^2$ | Slope | *t*-test p values | Inference |
|---|---|---|---|---|---|
| Diabetic | 35 | 0.94 | 0.97 | 0.16 | Near identical levels |
| Pre diabetic | 18 | 0.64 | 0.85 | 0.001 | Significant difference in levels |
| Healthy | 38 | 0.44 | 1.66 | 9.78$E^{-09}$ | Significant difference in levels |
| Pre breakfast | 82 | 0.90 | 1.12 | 0.002 | Significant difference in levels |
| Post breakfast | 9 | 0.96 | 0.97 | 0.36 | Insignificant difference in levels |
| Pre breakfast diabetic | 31 | 0.89 | 0.99 | 0.09 | Insignificant difference in levels |
| Pre breakfast healthy | 36 | 0.36 | 1.69 | 4.35$E^{-09}$ | Significant difference in levels |
| Post breakfast diabetic | 6 | 0.94 | 0.96 | 0.39 | Insignificant difference in levels |
| Post breakfast healthy | 3 | 0.14 | 1.27 | 0.04 | Significant difference in levels |

between BGL and SGL in males as well as females for all age-groups with $R^2$values > 0.9 in the age groups 41–50, 51–60 and 61–70 years, in case of males and females between age group of 71–80 years, the correlation coefficient was reported to be quite less (0.53 in case of males and 0.62 in case of females respectively (Supplement Fig. S11C and Supplement Table S1 C).

When combining subjects age with their diabetic status, 21–40 years healthy subjects had lowest correlation between BGL and SGL with $R^2$ value of 0.24 (n = 9, Slope = 1.58, p = 0.02) indicating significant difference between levels whereas in case of prediabetic and diabetic subjects there was an insignificant difference between BGL and SGL with $R^2$ values of 0.82 (n = 3, Slope = 1.27, p = 0.28) and 0.81 (n = 6, Slope = 1.04, p = 0.05). In 41–60 years healthy as well as prediabetic subjects, significant difference between BGL and SGL was reported with correlation coefficient of 0.57 (n = 18, Slope = 1.72, p = 6.55 $E^{-06}$)and 0.68 (n = 11, Slope = 1.21, p = 0.0002) respectively, in case of diabetics however insignificant difference was obtained with $R^2$ = 0.96 (n = 22, Slope = 0.97, p = 0.25). In case of subjects between age group of 61–80 years, significant difference was obtained in case of healthy subjects with $R^2$ = 0.48 (n = 9, Slope = 2.08, p = 0.003) whereas in case of prediabetic and diabetic subjects insignificant difference between BGL and SGL has been reported with $R^2$ values of 0.98 (n = 4, Slope = 0.44, p = 0.17) and 0.81 (n = 8, Slope = 1.05, p = 0.30) respectively (Supplement Fig. S11 D and Supplement Table S1 D).

These results reiterated our previous claim of having a significant correlation between SGL and BGL levels [13] and rebukes older studies by several groups which did not find significant correlation between BGL and SGL perhaps due to lack of technology during the era [33–36]. Thus it enables the developed sensor to be ready for commercialization, especially for the purpose of mass screening of diabetes in resource limited areas.

Finally, tests for reproducibility were also carried out on the biosensor. The sensor was found to be around 90% reproducible when readings were obtained for five different samples collected from healthy individuals on three consecutive days (Supplement Fig. S12). Repeatability test was also carried out by obtaining glucose levels through our biosensor for a healthy individual's saliva sample eight times (n = 8), results revealed a mean salivary glucose level of 45 mg/dL with SD of 6.16. The biosensor also met the ISO standards in terms of accuracy as the readings obtained for healthy as well as diabetic subjects stood within the zones A and B of the Clarke's error grid (Supplement Fig. S13). The analytical performance of our sensor with respect to other reported systems for glucose sensing is described in Supplement Table S2. The cost of the developed strip was also less (about 1$) as compared to other commercially available glucometer strips and can be reduced even further during mass production. Biosensing can be performed using any smartphone models (including cheaper ones which cost around 77$ to 120$ from the perspective of mass healthcare) with minimum requirement of 5 megapixel camera or above and an inbuilt flash light, while minor tweak in dark box attachment shall be needed according to the smartphone model.

## 4. Conclusion

In the present study, a smartphone based non-invasive optical biosensor has been developed for the estimation of glucose levels in saliva samples. The sensor was fabricated using a simple methodology by immobilization of *Glucose oxidase* enzyme along with a pH indicator bromocresol purple on a filter paper based strip. The color change with respect to glucose concentration was determined using RGB profiling with our developed smartphone application based on slope method. Slope method has been chosen against differential method of obtaining response to increase the sensitivity and reduce ambient light interferences and also for the reason that no baseline correction was needed. Different modes of obtaining response were tested, such as effect of different dyes and smartphone models so as to get maximum response and increase accuracy of results. Calibration curve with (R + G + B) Slope was found to be most sensitive with sensitivity of 0.0012 pixels $sec^{-1}$/mg $dL^{-1}$ within the linear detection range of 50–540 mg/dL and LOD of 24.6 mg/dL within a response time of 20 s. Clinical validation of the biosensor has been carried out on both diabetic and healthy subjects where correlation was established between their salivary glucose (SGL) obtained through our biosensor and blood glucose (BGL) obtained using commercial Accuchek active blood glucometer. A very good correlation of 0.94 has been established in case of diabetic patients while in healthy subjects it was not so significant. For better standardization of the biosensor, subjects were grouped into several categories based on age, sex, status etc. and correlation between BGL and SGL in all these cases has been carried out. The sensor was found to be free of interferents such as ascorbic, lactic acid and lactose within the clinically relevant range of these acids reported in human mouth. Other advantage of the developed sensor is that the procedure involved is simple where a person just has to obtain saliva in an ear bud and apply it on the strip; the procedure is painless and can be performed even by a layman with limited training. Moreover, results are obtained in less time (just 20 s) and screening is done through a smartphone which has become a common gadget nowadays in almost everybody's homes, thus eliminating the need for procuring any specialized instrument for analysis. The sensor also satisfies the ISO standards in terms of accuracy as the readings obtained were in zones A and B of Clarke's error grid. The overall analytical performance and sensor test strip cost compared well or better than those reported in literature. In the future course of actions, we shall further develop this sensor by automating strip manufacturing to enhance reproducibility and look towards commercializing the product.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.aca.2017.10.003.

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