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Droplet-based microfluidic lab-on-a-chip for glucose detection

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Abstract

A microfluidic lab-on-a-chip (LoC) platform for in vitro measurement of glucose for clinical diagnostic applications is presented in this paper. The LoC uses a discrete droplet format in contrast to conventional continuous flow microfluidic systems. The droplets act as solution-phase reaction chambers and are manipulated using the electrowetting effect. Glucose is measured using a colorimetric enzyme-kinetic method based on Trinder's reaction. The color change is detected using an absorbance measurement system consisting of a light emitting diode and a photodiode. The linear range of the assay is 9–100 mg/dl using a sample dilution factor of 2 and 15–300 mg/dl using a sample dilution factor of 3. The results obtained on the electrowetting system compare favorably with conventional measurements done on a spectrophotometer, indicating that there is no change in enzyme activity under electrowetting conditions.

Keywords: Electrowetting; Droplet; Glucose; Lab-on-a-chip; Microfluidics

1. Introduction

The in vitro measurement of glucose in human physiological fluids is of great importance in clinical diagnosis of metabolic disorders. The most important of these disorders is diabetes mellitus (hyperglycemia), which is characterized by high levels of glucose in human physiological fluids. Diabetes is the most common metabolic disorder in the world with more than 150 million affected people, and the number expected to double over the next two decades [1]. The assessment of glucose levels in body fluids is also useful in the diagnosis of hypoglycemia (low levels of blood glucose).

In recent years, there has been considerable interest in miniaturized analysis systems for chemical and biological applications [2]. These devices, also known as lab-on-a-chip (LoC), offer several distinctive advantages over macroscopic systems, such as reduced sample and reagent consumption, faster analysis, and higher levels of throughput and automation. In the microfluidic domain, glucose analyzers are usually based on continuous flow, using syringe pumps [3] or electroosmosis [4] for pumping. Microdialysis sampling has also been coupled with continuous flow microfluidic devices for real-time monitoring of glucose [5]. The most commonly used biorecognition element is immobilized glucose oxidase [6] though enzymatic assays in solution phase coupled with electrophoretic separation have also been reported [7]. Detection is typically done using electrochemical methods such as amperometry [3–5], and optical methods such as absorbance [6] or chemiluminescence [8].

An alternative paradigm towards microfluidic systems is to manipulate the liquid as discretized microdroplets. This approach has several advantages over continuous-flow systems, the most important being the ease of fabrication, and reconfigurability and the scalability of architecture [9]. Due to the architectural similarities with digital microelectronic systems, we have often referred to our approach as "digital microfluidics". Electrowetting is one of several techniques that have been proposed to actuate microdroplets. Electrowetting refers to the modulation of the interfacial tension between a conducting liquid phase and an insulated solid electrode, by the application of an electric potential between the two. The use of electrowetting for droplet dispensing, transport, merging, mixing, and splitting, has been demonstrated previously [9–12]. Based on our earlier work [13], in this paper we present and characterize our electrowetting-based digital microfluidics platform for an eventual real world application; to assay glucose in physiological samples.

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2. Glucose assay

A colorimetric enzyme-kinetic method based on the Trinder's reaction [14] is used for the determination of glucose concentration in our system. Glucose is enzymatically oxidized to gluconic acid and hydrogen peroxide in the presence of glucose oxidase (EC 1.1.3.4). The hydrogen peroxide reacts with 4-amino antipyrine (4-AAP) and *N*-ethyl-*N*-sulfopropyl-*m*-toluidine (TOPS) in the presence of peroxidase (EC 1.11.1.7) to form violet colored quinoneimine, which has an absorbance peak at 545 nm:

$$\text{glucose} + H_2O + O_2 \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + H_2O_2$$

$$2H_2O_2 + \text{4-AAP} + \text{TOPS} \xrightarrow{\text{peroxidase}} \text{quinoneimine} + 4H_2O$$

The glucose concentration is measured using a rate-kinetic method. Using the kinetic method, the entire assay can be done in less than 1 min, as compared to the end-point method which typically takes more than 5 min, depending on the reagent composition and the temperature. Kinetic methods do not require a blank reading and also require significantly lower concentrations of enzyme when compared to end-point methods. The rate equation for the glucose assay, assuming Michaelis–Menten kinetics, can be written as

$$V_i = V_{\max}\frac{[\text{glucose}]/\text{DF}}{K_M + [\text{glucose}]/\text{DF}} = V_{\max}\frac{[\text{glucose}]}{K_M \times \text{DF}} \quad \text{if } \frac{[\text{glucose}]}{\text{DF}} \lll K_M \tag{1}$$

where V_i is the initial rate of the reaction, [glucose] the glucose concentration in the sample, DF the dilution factor of the sample in the assay mixture, K_M the Michaelis constant, and $V_{\max}$ is the limiting maximum reaction rate, which is proportional to the enzyme activity. The initial rate is proportional to the concentration of glucose if $[\text{glucose}]/\text{DF} \lll K_M$. Therefore, increasing the dilution factor of the sample improves the linear range of the assay.

The initial rate is measured as the rate of formation of the colored product quinoneimine, which is related to the rate of change of absorbance by Beer's law:

$$A(t) - A_{\text{blank}} = \varepsilon L[\text{quinoneimine}] \Rightarrow \frac{dA(t)}{dt} = \varepsilon L V_i \tag{2}$$

where $A(t)$ is the absorbance at time t, A_{blank} the absorbance of the blank solution, L the optical path length and ε is the extinction coefficient of quinoneimine under the assay conditions.

Combining Eqs. (1) and (2) we get the following relation between the glucose concentration and the (initial) rate of change of absorbance:

$$\frac{dA(t)}{dt} = \varepsilon L V_{\max}\frac{[\text{glucose}]/\text{DF}}{K_M + [\text{glucose}]/\text{DF}} = \frac{\varepsilon L V_{\max}}{K_M \times \text{DF}}[\text{glucose}] \quad \text{if } \frac{[\text{glucose}]}{\text{DF}} \lll K_M \tag{3}$$

The analytical sensitivity of the method is the slope of the calibration curve in the linear range, and quantifies the change in measurable signal relative to the change in the concentration of analyte. For the glucose assay, Eq. (3) represents the calibration curve and the sensitivity can therefore be written as

$$S = \frac{\varepsilon L V_{\max}}{K_M \times \text{DF}} \tag{4}$$

From Eq. (4), it is evident that the sensitivity is a measure of the enzyme activity ($V_{\max}$). The sensitivity is also inversely proportional to the dilution factor if the other assay conditions (ε, L, $V_{\max}$, and K_M) are kept constant. The enzyme activity (and therefore the sensitivity), in addition to being directly related to the protein content in the reagent, is a function of other external factors such as temperature, pH, and ionic concentration. Each of these external factors has an optimum point at which the enzyme exhibits its maximum activity.

3. Experimental

3.1. Chip fabrication

The schematic of the LoC device is shown in Fig. 1. The LoC consists of the electrowetting chip on which the fluidic operations and the chemical processes occur, and a non-invasive optical absorbance measurement system. The electrowetting system consists of two parallel glass plates separated by a spacer of known thickness. The bottom glass plate consists of an array of independently addressable electrodes patterned in a 200 nm thick layer of optically transparent indium tin oxide (ITO). The top glass plate is coated with a continuous layer of ITO to form the ground electrode. The droplet is sandwiched between the two plates and is surrounded by air or any other immiscible liquid. The droplet is insulated from the electrode array by Parylene C (~800 nm) and both the top and bottom plates are hydrophobized with a thin layer of Teflon AF 1600 (~50 nm). The fabrication of the electrowetting chip and experimental setup are described in detail in [9]. In the experiments reported in this paper,

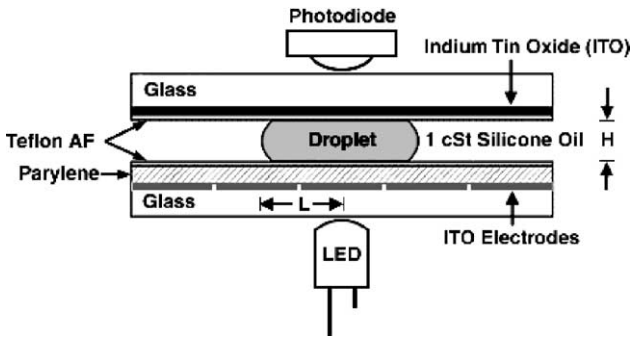

Fig. 1. Vertical cross-section of the electrowetting chip along with the optical detection instrumentation.

we have used electrowetting chips with an electrode pitch of $L = 1.5$ mm and a nominal gap spacing of $H = 0.475$ mm. 1 cSt silicone oil (DMS-T01, Gelest, Morrisville, PA, USA) was used as the filler medium in all experiments, to prevent evaporation and contamination of the sample and the reagent [13].

3.2. Chemicals

Glucose oxidase (G-6125), peroxidase (P-8125), 4-aminoantipyrine (A-4382) and TOPS (*N*-ethyl-*N*-sulfopropyl-*m*-toluidine) (E-8506) were purchased from Sigma (St. Louis, MO, USA). The reagent was constituted such that the assay mixture consists of 3 U/ml glucose oxidase, 3 U/ml peroxidase, 3 mM 4-aminoantipyrine, and 5 mM TOPS in 0.1 M phosphate buffered saline (pH 7.0). Reference glucose solutions of different concentrations were prepared by diluting 100, 300 and 800 mg/dl standards (Sigma, 16-11) in deionized water.

3.3. Optical detection

The optical detection is performed in a plane perpendicular to that of the microfluidic device, and the setup consists of a green light emitting diode (LED) and a photodiode, as shown in Fig. 1. The photodiode (TSL257, Texas Advanced Optoelectronic Solutions, TX, USA) is a light to voltage converter that combines a photodiode and an amplifier on the same monolithic device. The voltage output of the photodiode $V(t)$ is optionally amplified and logged on a computer, using a 12-bit analog data acquisition board (PCI-DAS08, Measurement Computing, Middleboro, MA, USA), with custom written software. The analog data acquisition board has a measurable range of −5 to 5 V and an analog-to-digital (A/D) resolution of 2.44 mV. Post-processing of the collected data was done using Microsoft Excel. The voltage $V(t)$ is directly proportional to the light intensity incident on the photodiode and is related to the absorbance by the following equation:

$$A(t) = \ln\left(\frac{V_0 - V_{\text{dark}}}{V(t) - V_{\text{dark}}}\right) \quad (5)$$

V_0 corresponds to zero absorbance (or 100% transmittance), and V_{dark} corresponds to the voltage output of the photodiode under dark conditions. The rate of the change of absorbance $dA(t)/dt$ is equivalent to the reaction rate, and is related to the glucose concentration by Eq. (3).

3.4. Experimental procedure

The on-chip glucose assay is performed in three steps—dispensing, mixing and detection. Droplets of the glucose sample and the reagent are first pipetted manually onto the electrowetting chip. They are then merged and physically mixed by shuttling the coalesced droplet across three electrodes for 15 s, at a switching rate of 8 Hz, and an actuation voltage of 50 V. The time for mixing protocol is higher than what is required and can be reduced to less than 5 s [11,12]. At the end of the mixing phase, the absorbance is measured for at least 30 s, using the LED-photodiode setup described earlier. This measurement time can be reduced by increasing the sensitivity of the assay, since we can now achieve the same change in the measured signal (absorbance) in lesser time (from Eq. (3)). The mixed droplet is held stationary by electrowetting forces, during the absorbance measurement step. Since the absorbance measurements begin 15 s after the droplets are merged, the measured reaction rate may not be exactly equal to the initial rate. This difference between the measured and the actual initial rate may be noticeable for high glucose concentrations. All experiments were performed at room temperature. An increase in the reaction temperature will increase the sensitivity till an optimal value is reached.

The volumes of glucose sample and reagent were chosen to result in dilution factors (DF) of 2 and 3, which are

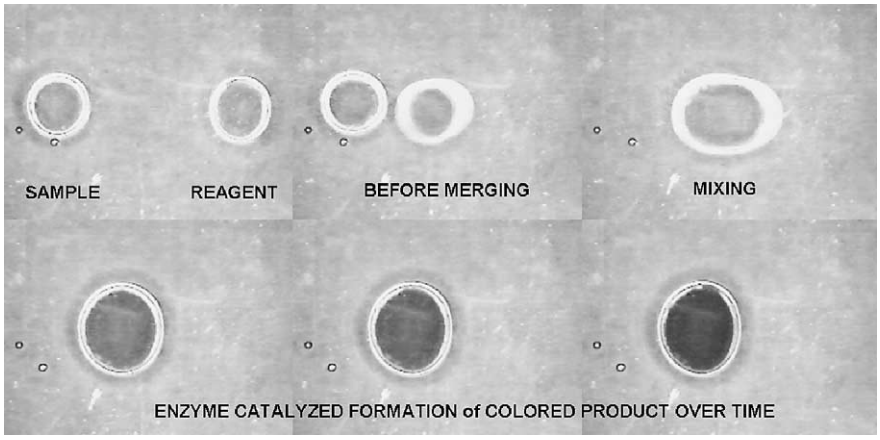

Fig. 2. Snapshots of the sample and reagent droplet from the top during a glucose assay. The concentration of the sample droplet is 800 mg/dl.

orders of magnitude smaller than those used in conventional colorimetric glucose sensors (typically >100). We have not used larger dilution factors due to chip real-estate concerns and reduced mixing efficiency. For the low mixing ratios used in our system, we have also observed dissolved oxygen to be the limiting reactant. The reaction slows down once the dissolved oxygen is exhausted, and takes a long time to proceed to completion, making end-point methods unfeasible. Fig. 2 shows the various steps involved in a typical on-chip glucose assay.

4. Results and discussion

4.1. Sample dilution factor of 2 (DF = 2)

Identical volumes of the sample and reagent are mixed to result in a dilution factor of 2 for both the liquids. The constitution of the reagent, taking the dilution into account, is; glucose oxidase 6 U/ml, peroxidase 6 U/ml, AAP 6 mM, and TOPS 10 mM. The volume of the sample and reagent droplets is 1.0 μl each. The absorbance $A(t)$ as a function of time for various concentrations from 25 to 400 mg/dl is shown in Fig. 3. Time $t = 0$ in the graph corresponds to the time instant at the end of the mixing protocol (15 s after merging). The slope of these curves (obtained by least-squares linear regression) gives the initial rate of reaction corresponding to each concentration. Fig. 4 plots this reaction rate as a function of the sample glucose concentration. The solid line indicates the linear region of the assay, obtained by least squares linear regression. The plot shows excellent linearity up to a glucose concentration of 100 mg/dl with an R^2 (measure of goodness of fit) of 99.89%. The sensitivity of the method (slope of Fig. 4) is 27.2 uAU dl/s mg (AU stands for absorbance units).

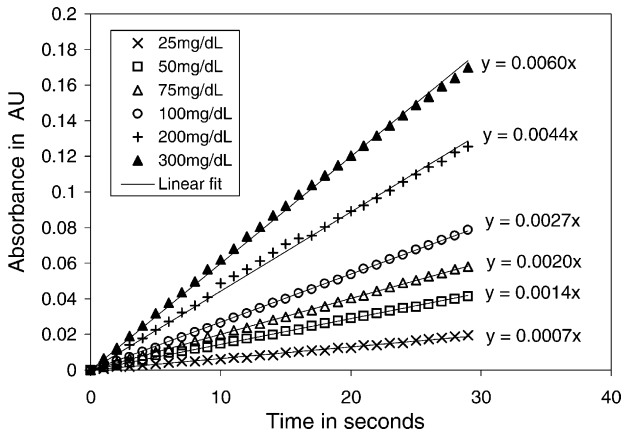

Fig. 3. Optical absorbance as a function of time for the assay for glucose concentrations from 25 up to 300 mg/dl for a sample dilution factor of 2. Time $t = 0$ corresponds to the time instant at the end of the mixing protocol. The solid line is the least-squares linear fit to the data.

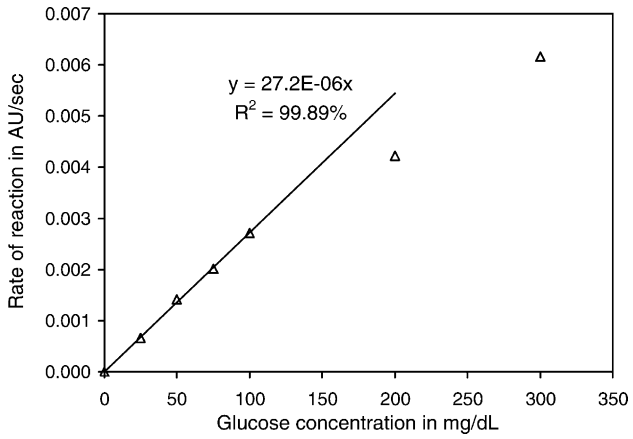

Fig. 4. Calibration curve for the glucose assay for a sample dilution factor of 2. The solid line shows the linear region of the calibration curve, obtained by least-squares regression.

4.2. Sample dilution factor of 3 (DF = 3)

The dilution factor for the reagent, in this case, is 3/2 and the constitution of the glucose reagent is: glucose oxidase 4.5 U/ml, peroxidase 4.5 U/ml, AAP 4.5 mM, and TOPS 7.5 mM. The volume of the sample droplet is 0.7 μl and the volume of the reagent droplet is 1.4 μl. The initial rate of reaction is plotted as a function of concentration in Fig. 5. From the figure, we can see that the reaction is linear up to concentrations of 300 mg/dl with an R^2 of 99.76%. The sensitivity of the method is 16.6 uAU dl/s mg.

Since the assay conditions are the same for both experiments (DF = 2 and 3), the sensitivities are inversely related to the dilution factors (from Eq. (4)). The ratio of the measured sensitivities is

$$\frac{S(\mathrm{DF}=2)}{S(\mathrm{DF}=3)} = \frac{27.2}{16.6} = \frac{3}{1.83}$$

which is very close to the expected value of 3/2.

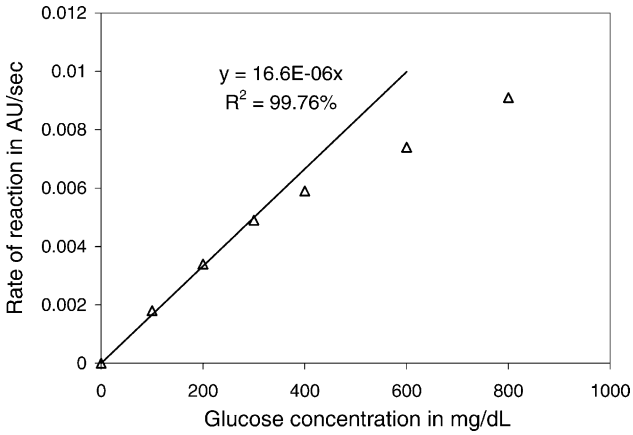

Fig. 5. Calibration curve for the glucose assay for a sample dilution factor of 3. The solid line shows the linear region of the calibration curve obtained by least-squares regression.

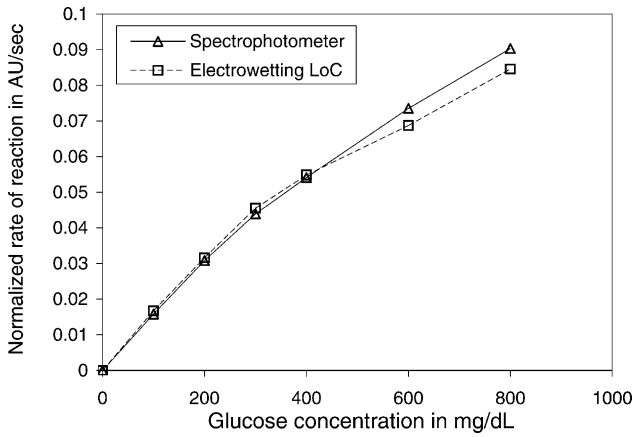

Fig. 6. Comparison between results obtained on the electrowetting device and identical assays on the spectrophotometer for different concentrations of glucose. The sample dilution factor is 3.

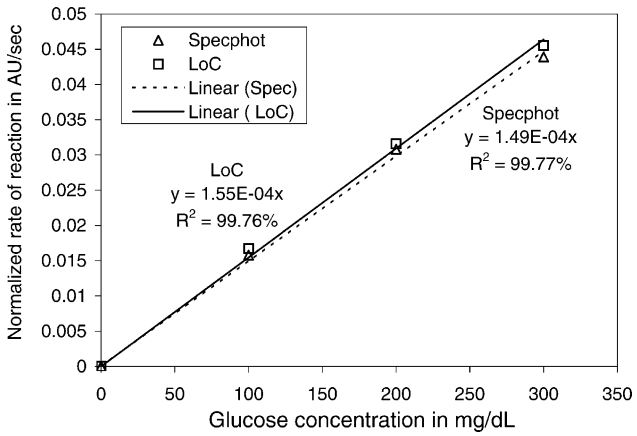

Fig. 7. Comparison between results obtained on the electrowetting device and identical assays on the spectrophotometer in the linear range of operation of the method.

4.3. Comparison with results from a spectrophotometer

The assay results obtained on the electrowetting chip for a dilution factor of 3 were compared with those obtained on a commercial Gensys 20 spectrophotometer. In order for the comparison to be meaningful, the absorbance measured in the electrowetting system is multiplied by a scaling factor to account for the difference in path lengths between our system and the spectrophotometer (path length = 1 cm). Since the LED light source is not collimated, the path length in the electrowetting system is not exactly equal to the gap spacing. The scaling factor is therefore determined by measuring the end-point absorbance of the same glucose assay on both the systems. Fig. 6 compares the results obtained on both the systems for a sample dilution factor of 3. The solid and the dotted lines are drawn only to guide the eye in visualizing the data and do not represent any fit. From the figure we can see that the data from the spectrophotometer and from the electrowetting system are in good agreement up to 400 mg/dl. We attribute the deviation at the higher concentrations (600 and 800 mg/dl) to the difference in the measured rate and the initial rate. Fig. 7 compares the results in the linear range. The solid and dotted lines correspond to least-squares linear fits to the data points. Since the assay conditions are identical for both the experiments the sensitivities are proportional to V_{max} (from Eq. (4)). The nearly identical sensitivities from the figure indicate that there is no significant change in enzyme activity (V_{max}) under electrowetting conditions.

4.4. Detection limit

The detection limit is defined as the smallest concentration that can be measured using a method, with reasonable certainty. The detection limit and the sensitivity of a method are related, and an ideal device should have a high analytical sensitivity and a low detection limit. The detection limit in our system is limited by the quantization error, the maximum output of the photodiode, and the duration for which the photodiode voltage output is collected. We have assumed that the voltage signal is measured for 30 s, and that at least 15 distinct data points are required to obtain a good linear fit ($R^2 > 99\%$) to the absorbance versus time data. The voltage resolution of the 12-bit A/D converter is 2.44 mV. The minimum voltage change that needs to be measured over 30 s is then $15 \times 2.44\,\text{mV} = 36.6\,\text{mV}$. This corresponds to an absorbance change of $\log_e[5/(5-0.0366)] = 0.0073$ AU (refer Eq. (5)), if the maximum output of the photodiode corresponding to zero absorbance is 5 V. Therefore, at the detection limit (C_{min}), the change in absorption should be at least 0.0073 AU in 30 s. If the sensitivity of the assay is S, then the detection limit can be written as

$$C_{min} = \frac{0.0073}{30S} \quad (6)$$

The theoretical detection limits for the dilution factors of 2 and 3, calculated using Eq. (6), are 9 and 15 mg/dl, respectively. The detection limit is lower for a dilution factor of 2 since the sensitivity is higher.

4.5. Other metabolites

In addition to glucose we have also shown the feasibility of detecting other metabolites such as lactate, glutamate, and pyruvate using our digital microfluidic architecture, by using similar enzymatic reactions and suitably modified reagents. Further experiments are required to establish the linear range of operation of the device for these analytes.

5. Conclusions

A droplet-based microfluidic LoC for measurement of glucose for clinical diagnostics was presented in this paper. The device demonstrates, to our knowledge for the first time, a biochemical assay on an electrowetting-based digital microfluidic platform. Using this device, we have

Table 1
Summary of analytical performance parameters for the electrowetting-based glucose assay

	Dilution factor	
	2	3
Analytical range (mg/dl)	100	300
Analytical sensitivity (uAU dl/s mg)	27.2	16.6
Detection limit (mg/dl)	9	15

shown the detection of glucose concentrations in the range of 25–300 mg/dl, using dilution factors as low as 2 and 3, in less than 60 s. The analysis time can be brought down to less than 30 s by using faster mixing schemes. The results compare favorably with conventional measurements on a spectrophotometer, implying no significant change in enzyme activity under electrowetting. Table 1 summarizes some of the analytical performance parameters of the device for both the dilution factors. In addition to glucose we have also demonstrated the feasibility of detecting other human body metabolites, such as lactate, glutamate and pyruvate using our device. Future work involves integrating an automated dispensing scheme to generate sample and reagent microdroplets from a reservoir on-chip. The clinical applicability of this method also needs to be evaluated using real human physiological samples.

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