A DIGITAL MICROFLUIDIC BIOSENSOR FOR MULTIANALYTE DETECTION

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ABSTRACT

A microfluidic lab-on-chip (LoC) for measuring the concentration of human body metabolites, using sub-microliter droplets as reaction chambers, is presented in this paper. The device is based on the manipulation of droplets using the principle of electrowetting and is integrated with an optical absorbance measurement system for detection. We have demonstrated the detection of glucose using a colorimetric enzyme-kinetic assay in less than 40 seconds. The sensor response is linear in the range of 25mg/dl to 300mg/dl, with less than 5% deviation from linearity at the upper limit. In addition to glucose, we have also demonstrated the feasibility of lactate, glutamate, and pyruvate assays using our system.

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 is expected to double over the next two decades [1]. The assessment of glucose levels in body fluids is also used in the diagnosis of hypoglycemia (low levels of blood glucose).

In recent years, there has been considerable interest in miniaturizing conventional glucose sensors, especially for use in a clinical point-of-care setting. Microfluidics has been the enabling technology behind such miniaturized analysis systems for chemical and biological applications [2]. These devices, also known as Lab-on-Chip (LoC), offer several distinctive advantages over their macroscopic counterparts, such as reduced reagent consumption, smaller analysis volumes, faster analysis times, and higher levels of throughput and automation. Currently most microfluidic glucose sensors are enzyme based and use continuous flow, using either mechanical actuators [3,4] or electrokinetic phenomena [5] for pumping liquids. In most of these devices the enzyme is immobilized on the sensing surface [3,4] and the detection is done using amperometric techniques [3-5]. The most obvious disadvantage of the existing sensors is the rigidity and specificity of the architecture to the application.

An alternative approach towards microfluidic systems is to manipulate the liquid as discretize microdroplets. This approach, which we refer to as "digital microfluidics", has several advantages over continuous-flow systems, the most important being the ease of fabrication, and reconfigurability and the scalability of architecture [6].

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 a solid electrode, by the application of an electric field. The use of electrowetting for droplet dispensing, transport, merging, mixing, and splitting, has been demonstrated earlier [6,7]. In this paper we demonstrate a complete Lab-on-Chip, which integrates all the fundamental digital microfluidic operations, along with detection, for a biological application. The proposed device is used to measure the glucose concentration in a sample droplet, using a colorimetric enzyme-kinetic assay. The device uses enzymes in solution instead of immobilizing them, and a non-invasive optical detection technique, which can be easily integrated with electrowetting chips.

LAB-ON-CHIP ARCHITECTURE

The schematic of the fabricated device is shown in figure 1. The device consists of the electrowetting chip on which the sample and the reagent droplets are dispensed, transported and mixed for chemical reactions to occur, and an integrated optical absorbance measurement system.

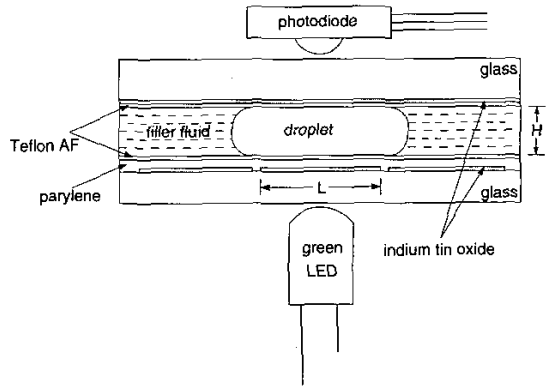

Fig 1 - Schematic of device (vertical cross section)

Electrowetting actuator

The electrowetting system consists of two parallel electrode plates, a continuous ground plate on top and an addressable electrode array as the bottom plate. The droplet is sandwiched between the two plates, and is surrounded

by immiscible silicone oil. The electrode array is insulated from the liquid and both surfaces are hydrophobized. The fabrication details of the electrowetting chip can be found in [6]. Instead of Chrome electrodes as described in [6], Indium Tin Oxide (ITO) is used as the material for the control electrodes. The transparent nature of ITO enables the easy integration of optical measurement techniques with the electrowetting system. In the experiments reported in this paper, we have used electrowetting chips with an electrode pitch of L=1.5mm and a gap spacing H=0.475mm.

Glucose Assay

Trinder's reaction, a colorimetric enzyme-based method, is used to detect glucose in our system. The enzymatic reactions involved in the assay are

$$\begin{aligned} &\text{Glucose} + H_2O + O_2 \xrightarrow{\text{Glucose Oxidase}} \text{Gluconic Acid} + H_2O_2 \\ &2H_2O_2 + 4\text{-AAP} + \text{TOPS} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + 4H_2O \end{aligned} \quad (1)$$

Glucose is enzymatically oxidized to gluconic acid and hydrogen peroxide in the presence of glucose oxidase. The hydrogen peroxide formed reacts with 4-amino antipyrine (4-AAP) and TOPS (N-ethyl-N-sulfopropyl-m-toluidine), to form violet colored quinoneimine, which has an absorbance peak at 545nm. The absorbance is related to the concentration of quinoneimine by Beer's law,

$$A = \varepsilon \times l \times C$$

where A is the absorbance, ε is the extinction coefficient of quinoneimine under the assay conditions, l is the optical path length, and C is the concentration of quinoneimine.

The glucose concentration can be calculated from the absorbance (or the concentration of quinoneimine) using either an end-point method or a kinetic method. In an end-point method the concentration of glucose is directly proportional to the absorbance at the end of the reaction. In the kinetic method the concentration of glucose is calculated from the rate of the reaction, which is equivalent to the rate of change of absorbance. Trinder's reaction obeys Michaelis-menten kinetics and therefore the effective rate equation can be written as

$$\text{Rate of Reaction} = \frac{dA(t)}{dt} = \frac{V_{MAX} \times [\text{Glucose}]}{K_M + [\text{Glucose}]} \quad (2)$$

where A(t) is absorbance as a function of time, V_{MAX} is the maximum rate of the reaction, [Glucose] is the effective concentration of glucose (including dilution factors), and K_M is the effective Michaelis-Menten constant for the reaction. From the above equation, we can infer that, the reaction rate is directly proportional to the glucose concentration only if $[\text{Glucose}] <<< K_M$. Therefore diluting the sample improves the linear range of operation of the device.

Equation (2) can be rewritten as

$$\frac{1}{\text{Rate}} = \frac{K_M}{V_{MAX}[\text{Glucose}]} + \frac{1}{V_{MAX}}$$

and the effective K_M of the reaction can be obtained by plotting 1/Rate as a function of 1/[Glucose] (Linweaver-Burke plot). The effective K_M is calculated as the slope of the Linweaver-Burke plot divided by the intercept on the y-axis.

Using the kinetic method, the entire assay can be done in less than a minute, as compared to the end-point method which typically takes more than 5 minutes, depending on the reagent composition and the temperature. Kinetic methods also require significantly lower concentrations of enzyme as compared to end-point methods. We have used the kinetic method in our system for these advantages.

Chemicals

Glucose oxidase (G-6125), peroxidase (P-8125), 4-aminoantipyrine (A-4382) and TOPS (E-8506) were purchased from Sigma Chemicals (St Louis, Missouri, USA). The Trinder's reagent constitutes of glucose oxidase (3 U/ml), peroxidase (3 U/ml), aminoantipyrine (3 mM) and TOPS (5 mM) in 0.1M phosphate buffered saline (0.1M Sigma PBS, pH 7.0). Glucose solutions of various concentrations were prepared by diluting 100mg/dl, 300mg/dl and 800mg/dl standards obtained from Sigma (16-11).

Optical detection

The optical detection is performed in a plane perpendicular to that of the device, and the setup consists of a green LED and a photodiode, as shown in the schematic in figure 1. The photodiode (TSL257, obtained from Texas Advanced Optoelectronic Solutions) is a light to voltage converter that combines a photodiode and an amplifier on the same monolithic device. The output of the photodiode is externally amplified 10 times using a non-inverting op-amp configuration and the amplified signal V(t) is logged by the computer through a data acquisition board. The voltage V(t) is directly proportional to the light intensity incident on the photodiode. The absorbance is calculated from V(t), using the equation

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

where V_0 corresponds to zero absorbance (or 100% transmittance), and V_{dark} corresponds to the dark voltage of the photodiode amplified 10 times. The rate of the reaction is computed as dA/dt, which in turn is proportional to the glucose concentration. Note that dA/dt is independent of V_0 and V_{dark}, which are constants for a given experiment.

EXPERIMENTS

The glucose assay is performed on-chip in three steps - dispensing, mixing and detection. Droplets of the glucose sample and the reagent are first dispensed manually on the electrowetting chip using a pipette. They are then merged and physically mixed by shuttling the coalesced droplet across 3 electrodes for 10 seconds, at a switching rate of 8Hz, and an actuation voltage of 50V. The time for physical mixing (10 seconds) is higher than what is required and can be reduced to less than 5 seconds [9]. At the end of the mixing phase, the absorbance is measured for 30 seconds using the LED-photodiode setup described earlier. 1cSt silicone oil is used as the filler medium for all experiments. The volumes of glucose sample and reagent were chosen to result in dilution factors (DF) of 2 and 3, which are orders of magnitude smaller than those used in conventional colorimetric glucose sensors, which are typically greater than 100. The use of small dilution factors greatly minimizes reagent consumption and also reduces the footprint area for the assay.

For the low mixing ratios used in our system, we have 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.

RESULTS AND DISCUSSION

Dilution factor of 2 (DF=2)

The volumes used were 1.0μl for both the sample and the reagent, resulting in a dilution factor of 2. The absorbance as a function of time for various concentrations from 25mg/dl to 100mg/dl is shown in figure 2. The time t=0 in the graphs corresponds to 10 seconds (mixing time) after the sample and reagent droplets are merged. The slopes of these curves give the rate of reaction corresponding to each concentration. Figure 3 shows the rate of the reaction as a function of concentration. The response is linear up to a concentration of 100mg/dl as can be observed from the graph.

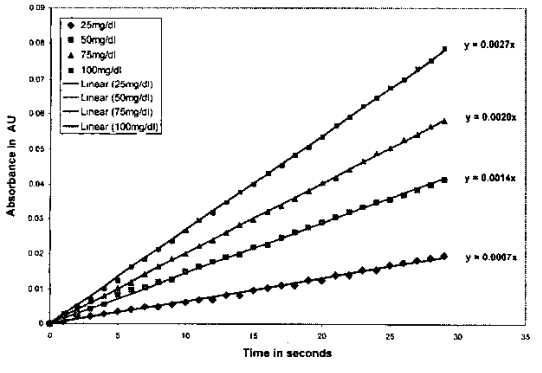

Fig 2 - Absorbance vs Time (DF = 2)

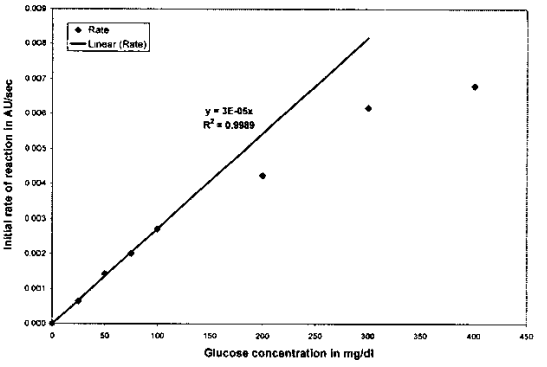

Fig 3 - Rate vs Glucose Concentration in sample (DF = 2)

Dilution factor of 3 (DF=3)

The volume used for the sample and the reagent is 0.7μl and 1.4μl respectively, resulting in a dilution factor of 3. The rate of reaction is plotted as a function of concentration in figure 4, along with the results obtained from a commercial Gensys 20 spectrophotometer. In order for the comparison to be meaningful, the absorbance values from the electrowetting system are multiplied by a scaling factor to account for the difference in path lengths between our system and the spectrophotometer. The scaling factor is determined by measuring the end-point absorb-

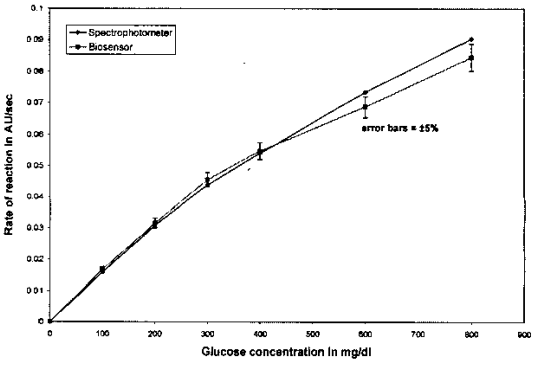

Fig 4 - Rate vs Glucose concentration in sample (DF = 3)

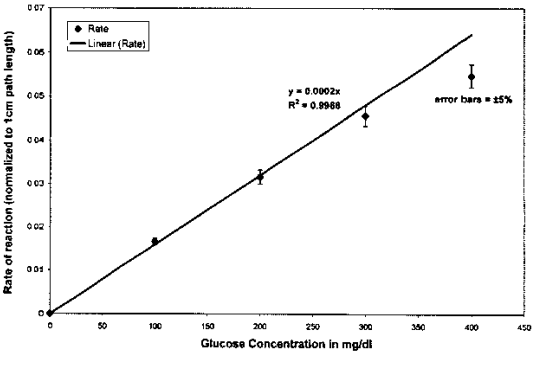

Fig 5 - Rate vs Glucose concentration (Linear Range)

ance of a glucose assay on both the systems. From the figure we can see that the data from the spectrophotometer and from the electrowetting system are in good agreement, implying no change in enzyme activity under electrowetting conditions. The response is linear up to a concentration of 300mg/dl with a deviation of less than 5% at 300mg/dl, as seen in figure 5.

The Linweaver-Burke plot, for the glucose assay using a dilution factor of 3, is shown in figure 6, and the K_M value from the graph is 408 mg/dl. The K_M obtained from the plot is highly sensitive to the value of the reaction rate and in order to minimize the errors only the precise standard concentrations (100mg/dl, 300mg/dl and 800mg/dl) are used in its calculation.

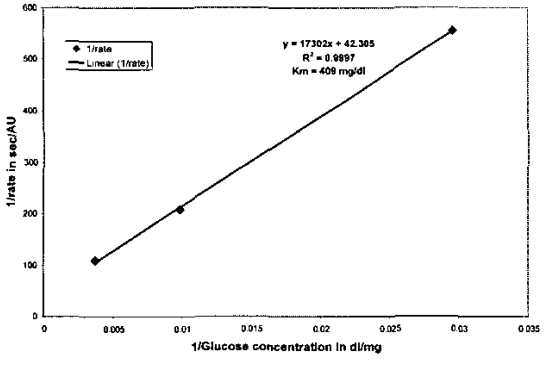

Fig 6 - Linweaver-Burke plots

Filler fluid

The transport of biological fluids containing proteins is not as straightforward as transporting other non-biological electrolytes. This is because most proteins, including enzymes, adsorb irreversibly to hydrophobic surfaces thereby contaminating them. In addition to contaminating the surface, protein adsorption also renders the surface permanently hydrophilic, which is detrimental to transport. Therefore any contact between the reagent droplet and the Teflon surface should be avoided to prevent contamination and loss of activity of the enzymes. As a consequence, air is not a suitable filler medium for protein assays, since the droplet is always in contact with the Teflon surface. Silicone oil with its favorable surface tension properties is an ideal alternative. From visual observations and electrical capacitance measurements, during droplet transport [9], we have inferred the presence of a thin film of oil, encapsulating the droplet at all times. This oil film isolates the droplet from the Teflon surfaces preventing any adsorption.

Other metabolites

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. Lactate oxidase, glutamate oxidase and pyruvate oxidase are used to oxidize their corresponding metabolites to produce hydrogen peroxide. This step is similar to the first reaction in the glucose assay (equation 1). The color producing step is identical to the second reaction in the glucose assay. Further experiments are required to establish the linear range of operation of the device for these other metabolites.

CONCLUSIONS

A complete biological lab-on-chip based on electrowetting microactuation has been presented in this paper. The device demonstrates, for the first time, the transport of droplets containing proteins (enzymes) using electrowetting. Using this device, we have shown the detection of glucose concentrations in the range of 25mg/dl to 300mg/dl, using dilution factors as low as 2 and 3, in less than 40 seconds. The results compare favorably with conventional measurements on a spectrophotometer, implying no significant change in enzyme activity under electrowetting. The use of silicone oil as the filler medium also prevents enzyme adsorption and contamination of the transport surface. We have also demonstrated the feasibility of detecting other human body metabolites, such as lactate, glutamate and pyruvate using our device. Future work would involve integrating a dispensing scheme to generate sample and reagent microdroplets from a reservoir on-chip. On-chip dilution schemes also need to be investigated to improve the linear range of the device.

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