Microfluidic Paper-Based Analytical Devices (µPADs): Miniaturization and Enzyme Storage Studies

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Abstract

This paper describes the design and development of miniaturized microfluidic paper-based analytical devices (µPADs) for biological assays and enzyme storage instruments. Here, a glucose assay utilizing glucose oxidase (GOx), horseradish peroxidase (HRP), and potassium iodide (KI) is used as the model system. The efficacy of the miniaturized devices is further examined by assessing the activity of acetylcholinesterase (AChE). Two types of µPADs were developed: one, “strip” chips of detection zones of area 0.5 cm$^2$, 0.1 cm$^2$ and, two, “grid” chips of detection zone 0.05 cm$^2$. The devices are easily fabricated via a wax printing process whereby lines of wax are deposited onto chromatographic paper and heated to create rows of hydrophobic barriers. The “strip” chips were subjected to three different temperature environments (-20°C, 0°C, and 20°C) over 30 days and glucose assays conducted at intermittent times yielding a correlation between corrected average inverse yellow intensity, days, and glucose concentration. Calculated and experimentally derived color intensity values for 1, 4, and 9 mM glucose concentrations after a 7-day storage study showed a good correlation (0.89% - 15.76% error). Both types of µPADs are effective platforms as potential point-of-care (POC) diagnostic devices and display minimal enzyme denaturation. µPADs of this size show promise as alternative devices for resource-limited regions and especially those areas where materials and instrumentation are not always available.

Keywords: miniaturization, enzyme storage, diagnostic device, glucose, point-of-care
Introduction

Since the first reports of microfluidic paper-based analytical devices (µPADs) by Whitesides et al., their potential as biosensors has yielded an array of applications not foreseen in these original papers. Paper is low-cost, easy to fabricate, thin, available in a variety of thickness and lightness, easy to stack, store and transport, compatible with biological samples, easy to chemically modify for functionalization, and available in many forms with a wide range of properties. µPADs are appealing especially in resource-limited setting as they typically do not require an external power source and fabrication techniques and instrumentation for production are usually less costly than those required for other devices.

Depending on the application, sample volumes in µPADs are typically in the microliter range (10^-12 M protein). Assuming detection limits have yet been realized, there is still opportunity to develop smaller paper-based devices that utilize smaller amounts of sample and subsequently less reagents thereby lowering the cost for the consumer.

Investigations of protein folding, unfolding and stability are important for understanding the molecular basis of biological structure and function. Biomolecular stability plays a critical role in every biological process within living systems. Proteins can be denatured by changing their physical or chemical environments, the pH of the solution, pressure, and temperature. µPADs have their greatest potential in underdeveloped regions of the world where medical facilities, state-of-the-art healthcare, and medicines are not always available to the poorest populations. Given the ease of which µPADs can be fabricated and the amounts of biological materials needed in a given assay, to date, they are the best option for people in need of healthcare and especially for what first-world countries consider mundane illnesses. Two barriers to realizing good healthcare for resource-challenged regions is one: minimizing the detection footprint vis-à-vis reducing the amount of reagents required in an assay, and; two: ensuring the biological reagents (e.g. protein) retain activity and do not denature while in storage.

Although a number of properties utilizing paper for enzyme activity on microfluidic devices studies have been reported, storage and shelf life studies have yet been examined on µPADs. Traditionally, enzyme-based assays performed on µPADs utilize enzymes that are spotted on the device or the µPAD is submerged into a solution. Enzyme activity is dependent on temperature, hence, storage of the µPADs is critical for later use as a diagnostic device.

Herein, we describe the design and development of three miniaturized µPADs as enzyme storage instruments using as model systems the enzyme assay of glucose and activity assay of acetylcholinesterase. Termed “strip” and “grid” chips, the devices are smaller in area than typical µPADs. The µPADs were subjected to three different temperature environments and the effect of these conditions on a colorimetric glucose assay assessed. Minimal denaturation is observed for the chip platforms. The devices are simple to fabricate, yield reproducible results, and have potential as point-of-care (POC) diagnostic devices.

Experimental

Materials

Glucose oxidase (GOx) from Asperillus niger was a gift from Fitzgerald Industries International (Acton, MA, USA). D-(+)-glucose, acetylcholinesterase (AChE) from Electrophorous electricus (electric eel) (14.6 mg solid, 137 U/mg), acetylthiocholine iodide (ATC), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), and sodium phosphate monobasic were purchased from Sigma Aldrich Co. (Saint Louis, MO, USA). Sodium phosphate dibasic and KI were purchased from Fischer Scientific Co. (Hampton, NH, USA). Whatman Grade 1 CHR chromatography paper was purchased from GE Healthcare Life Sciences (Pittsburgh, PA, USA).

PBS buffer (0.1 M, pH = 6.0) was prepared by addition of 87.7 mL of sodium phosphate monobasic (0.2 M) and 12.3 mL of sodium phosphate dibasic (0.2 M). GOx (120 U/mL) was prepared with phosphate-buffered saline (PBS) (pH = 6.0). A HRP solution was prepared with PBS at 30 U/mL. KI (1.0 M) was prepared with DI water. Various concentrations
of glucose were made in DI water (0.5, 1-14 mM). DTNB (5 mg/mL), various concentrations of ATC (0, 0.5, 1-14 mM) and AChE (0.18 U/mL) were prepared in PBS buffer (pH = 6.0). A solution of AChE:DTNB (1:1) was prepared fresh prior to the ATC assays.

**Microfluidic Analytical Device Fabrication**

µPADs were fabricated using Inkscape 0.91 software (Boston, MA, USA) and printed on Whatman Grade 1 CHR chromatography paper using a Xerox ColorQube 8580 (Norwalk, CT, USA). To ensure the hydrophobic wax barrier prevented leaking of solution from outside the boundaries of the µPADs, the chips were heat pressed on both sides to melt the wax through the pores of the paper using a Hotronix Auto Open Clam Heat Press at 176 °C for two min. For precise quantification of substrate and enzyme interaction, µPADs were designed with wax expansion taken into consideration. Parameters are based on previous studies measuring time of heating of the chips vs. wax expansion. Miniaturization optimization of the µPAD strip chips, 0.1 cm² detection zone, and grid chips, 0.05 cm² detection zones, were designed and examined (Fig. 1). µPADs were cut (80 mm x 30 mm) and a solution (500 µL) of a 1:1 enzyme cocktail of GOx (120 U/mL) and HRP (30 U/mL) added. The µPADs were dried and cut into strips (0.5 cm width) creating detection areas of 0.5 cm². Individual strip/grid chips were used to run a glucose assay with increasing glucose concentration solutions (0, 0.5, 1-14 mM) prepared in DI water and KI (1 M) (5 µL) pipetted into each detection area). Chips were run for 10 min followed by air drying for 1 min before analysis. A similar protocol was followed for the 0.1 cm² and 0.05 cm² detection zone/grid chips with adjustments to time and volume. The 0.1 cm² µPAD strip chips were treated with 0.5 µL glucose solutions and run for 5 min then air dried for 1 min. The 0.05 cm² grid chips were treated with 0.25 µL glucose solutions, run for 1 min., and air dried for 1 min.

**Microfluidic Device Analysis**

Colorimetric detection was conducted by scanning the chips using an Epson Perfection V600 Photo at 1600 dpi high resolution. Photoshop CS2 was used to determine the mean and mean inverse (yellow and blue) intensity of each analysis site using the marquee tool. For example, the corrected average inverse yellow intensity value was obtained by subtracting the experimental intensity value of a chip containing no glucose from the experimental intensity value of a chip containing a given concentration of glucose.

**Results and Discussion**

**Glucose Assay Storage and Stability**

Storage and stability experiments were performed on the 0.5 cm² strip chip to obtain normalized values by first examining the efficacy of drying and storing (-20 °C, 0 °C, and 20 °C) the enzyme cocktail (GOx:HRP) (120 U/mL:30 U/mL) for 30 days and subsequently assessing its use in a glucose assay. Fig. 2(A) shows the initial chip with increasing concentrations of glucose. An increase in the intensity of the brown color was observed as the glucose concentration increased. A graph of corrected average yellow color intensity vs. glucose concentration showed a Michaelis-Menten enzyme kinetics relationship (Fig. 2(B)). This initial run was set as the control for storage experiments.

Glucose assays were performed on 0.5 cm² strip chips in triplicate for a total of 30 days with storage of the chips at the specified temperatures (-20 °C, 0 °C, and 20 °C). From these assays, 3D graphical representations were plotted for 20 °C (Fig. 3(A)), 0 °C (Fig. 3(B)), and -20 °C (Fig. 3(C)). The 0.5 cm² strip chips stored under room temperature (20 °C) conditions (Fig. 3(A)) showed a slight decrease in activity reflected in a decrease in color intensity (indicated by the red peaks for 0–3 storage days). However, when compared to refrigerator temperature conditions (0 °C) (Fig. 3(B)), several chips displayed a color intensity value as intense as assays performed on the first day (initial strip chip used for normalization) as indicated by the four red peaks from 0–5, 15, 19, and 29-30 storage days (Fig. 3(B)). For the strip chips stored at -20 °C, broader red peaks were observed throughout the storage study (Fig. 3(C)). This increase in peak
range correlates with enzyme activity, hence, a trend was observed where color intensity increased as storage temperature decreased. For all storage temperatures there was precise and sensitive glucose detection readings throughout the 30-day storage trials.

A major problem in storing enzymes on µPADs is denaturation further exacerbated by the presence of water in and around the surface of the µPAD. The apparent random onset of decreased activity in the strip chips is attributed to a lack of consistent fully dried µPADs after spotting the GOx:HRP solution. There was no quantitative determination of the ‘dryness’ of the µPADs prior to storage. Strip chips placed in storage under varying temperature conditions would be affected by the retained moisture as the enzyme activity in the paper fibers would be reduced resulting in less glucose conversion.

Using Origin8 software, equations were derived to correlate storage time (days), glucose concentration (mM), and corrected average inverse yellow color intensity units at the three storage temperatures (supporting information). A study was conducted to validate the equations. Specifically, color intensity values were calculated for storage of 7 days at three glucose concentrations (1, 4, and 9 mM) at the three temperature variants and compared to experimentally derived values (Table 1).

An average percent error of 11.24 % for room temperature storage, 6.21% for refrigerator storage, and 9.93% for freezer storage was obtained. The higher percentage in error for storage under room temperature conditions is attributed to the increased temperature conditions the enzyme-saturated strip chips were subjected to, hence, denaturing the enzymes and decreasing the accuracy in the predictions. As seen in Fig. 3[B], refrigerator temperature storage conditions exhibited a constant enzyme activity throughout the 30-day storage study (R² = 0.9486). Moisture within the µPADs was not quantitatively analyzed which may be rationale for the freezer stored strip chips displaying a lower accuracy –moisture affects the accuracy of freezer stored µPADs and studies did not correct for any variations in humidity.

**µPAD Miniaturization**

Special training and accessibility to reagents or instrumentation is often required to make complex modifications of µPADs. A goal was to ensure that miniaturization of the µPADs would not affect the accuracy of a given assay. The previous chips were miniaturized to assess how further miniaturization affected their accuracy and sensitivity. Specifically, 0.1 cm² strip chip and a 0.05 cm² grid chip designs were developed (Fig. 1[B],C). The protocol followed the schematic as described in the supplementary information, however, 1 µL and 0.25 µL of various glucose substrate solutions were pipetted into each section of the 0.1 cm² strip chips and grid chips, respectively. Analysis of the chips was accomplished as described in the experimental methods section.

For the first miniaturization study, 0.1 cm² strip chips underwent analysis of glucose detection and results were graphed (Fig. 4[A], bottom). As visually observed, the 0.1 cm² strip chip clearly produced a trend of increasing color intensity as glucose concentration increased. Graphical analysis displayed a Michaelis-Menten enzyme-kinetics relationship as an initial onset of color intensity increased almost linearly followed by a plateau in color intensity, indicative of enzyme saturation past 8 mM glucose (Fig. 4[A], top). To further support for the validity of the 0.1 cm² miniaturization, similar conditions were run utilizing the colorimetric detection of ATC. The ATC detection assay is carried out with the colorimetric conversion of DNTB through the enzyme catalyst AChE (0.18 U/mL). Visual and graphical representation of ATC detection assays are seen in Fig. 4[B] top and bottom, respectively. Similar to results seen for glucose detection, it was observed that color intensity increased as ATC concentrations increased.

The second miniaturization study involved a further decrease in reaction time and analysis surface area. A 4x4 grid comprised of 0.05 cm² reaction and detection zone µPADs was designed and produced as seen in Fig. 1[C]. These miniature grids (grid chips) were used to run both glucose and ATC detection assays, similarly to the 0.1 cm² strip chips. Protocol was followed as schematically outlined in the supplementary information with a difference in
substrate volume pipetted onto the detection zones decreasing from 0.5 µL to 0.25 µL.

It was observed that as glucose concentrations increased, so too did color intensity (Fig. 5(A)). Graphical analysis of the glucose assay strip chips (Fig. 5(C)) produced a trend less accurate than previously seen (Figs. 2 and 4(A)), however, there is an observed relationship correlating the increase of color intensity with an increase of glucose, such is validated with a Michaelis-Menten enzyme kinetics curve. This trend is not as fitted as the original 0.5 cm² strip chips (Fig. 2), which decreases the overall accuracy of the grid chips. However, having a trend throughout each detection area supports sensitivity of glucose detection on the grid chips. It was determined that the amount of enzyme used for each detection zone of the 0.5 cm², 0.1 cm² and 0.05 cm² chips was 11.3, 2.2, and 1.1 pm, respectively. Overall it was found that the 0.5 cm² and 0.1 cm² are comparable in sensitivity and precision whereas the 0.05 cm² chips are poorer.

ATC detection was also carried out on the grid chips as visually seen in Fig. 5(B). Visual analysis of the grid chips supports a relationship positively correlating ATC concentration and color intensity (Fig. 5(B)). Graphical representation of the ATC detection on the grid chips showed an expected relationship, however unlike the glucose grid chips detection seen in Fig. 5(C), there is less of a relationship when fitted with a Michaelis-Menten enzyme kinetics curve. The graph observed a lower V_max plateau when compared to the glucose detection assay curve. This observation is explained through the lower concentration of AChE (0.18 Units/mL) which in turn required having a longer reaction and development assay time allotment, saturating the enzyme at a lower V_max, ultimately affecting standard deviation.

Conclusions

The feasibility of using miniaturized µPADs in biological assays and examining one size device for its stability under various temperature conditions and time domains were conducted and validated through glucose detection assays. Equations mathematically derived from graphical analysis of three temperature conditions produced accurate predictions for short-term (7 day) storage tests within an error of 0.89% - 15.76%. Future studies will encompass several other enzyme-substrate colorimetric assays, such as bovine serum albumin, and equations for storage predictions based on enzyme activity will be similarly produced. Future work is also directed at quantitative solutions, such as vacuum chambers, to quantitatively remove all moisture and dry µPADs before storing, hypothetically increasing the storage abilities in a myriad of temperature environments.

Miniaturization of paper devices was conducted utilizing GOx and acetylcholinesterase (AChE) enzyme based colorimetric substrate detection of glucose and acetylthiocholine iodide (ATC), respectively. The decrease of reaction and development surface area provided accurate and sensitive results for the first miniaturization study testing 0.1 cm² strip chips. The second miniaturization study, 0.05 cm² grid chips, produced sensitive albeit inaccurate results for both glucose and ATC detection; however, glucose detection on the grid chips produced a trend capable of predicting the general range of an unknown glucose concentration. Future work for µPAD miniaturization will focus on applying the miniaturization technology to novel platforms, further decreasing the need for materials used to run the devices.

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Supporting Information

Detailed fabrication of chips and Origin analysis of 3D surface plots including derived equations and respective parameters. This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.
Financial & competing interests disclosure

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Fig. 1. Fabricated µPADs in comparison to a quarter. µPADs shown: 0.5 cm² (A), 0.1 cm² (B) and 0.05 cm² (C).

Fig. 2. (A) Enzyme saturated 0.5 cm² strip chip run with glucose (0, 0.5, 1-14 mM) prepared in 1 M KI. (B) Quantitative analysis and graphical representation of 2A.

Fig. 3. 3D surface plots displaying relation between corrected average inverse yellow color intensity (y-axis), time (x-axis) and glucose concentration (z-axis) of 0.5 cm² storage strip chips stored for 30 days in (A) room temperature (20 °C) (B) refrigerator temperature (0 °C) and (C) freezer temperature (-20 °C).

Fig. 4. Visual and graphical representation of 0.1 cm² strip chip µPAD miniaturization running (A) glucose detection assays and (B) ATC detection assays.

Fig. 5. Digital microscope captured image of 0.05 cm² grid chip µPADs used for (A) glucose detection and (B) ATC detection. (C) Graphical representation of (A) and (B) displaying relationship of normalized color intensity with varying substrate (glucose and ATC) concentrations.
Table 1. Calculated and experimentally derived color intensity values for 1, 4, and 9 mM glucose concentrations after a 7-day storage study with varying temperature conditions.

| Temperature | Experimental Color Intensity Values | 1 mM glucose | 4 mM glucose | 9 mM glucose |
|-------------|-------------------------------------|--------------|--------------|--------------|
|             | % Error                             | % Error      | % Error      | % Error      |
| 23 °C       | Experimental                        | 24.47        | 98.63        | 123.92       |
|             | Calculated                          | 25.66        | 85.20        | 142.98       |
| 0 °C        | Experimental                        | 25.43        | 69.46        | 119.77       |
|             | Calculated                          | 23.38        | 76.70        | 130.59       |
| -20°C       | Experimental                        | 21.42        | 101.89       | 135.55       |
|             | Calculated                          | 23.74        | 113.72       | 150.00       |
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