FABRICATION OF LAMINATED PAPER-BASED ANALYTICAL DEVICES (LPAD) FOR COTININE DETECTION

Z. Hugh Fan[1,2,3,*], Christopher L. Cassano[1], Wei Liu[1,4]

[1]Department of Mechanical & Aerospace Engineering, [2]Department of Biomedical Engineering,
[3]Department of Chemistry, University of Florida, PO Box 116250, Gainesville, FL 32611, USA
[4]Department of Chemistry, Shaanxi Normal University, Xi'an, Shaanxi 710062, P.R. China

ABSTRACT

The majority of paper-based microfluidic devices were produced by patterning hydrophobic zones via photoresist or wax. Others were created by cutting paper using a laser. We present a fabrication method by craft-cutting and lamination, in a way similar to making an identification card. The method employs a digital craft cutter and roll laminator to produce laminated paper-based analytical devices (LPAD). Lamination with a plastic backing provides the mechanical strength for a paper device. The devices have been exploited for the detection of glucose in urine with colorimetric assays and for the measurement of cotinine in mouse serum using chemiluminescence immunoassay.

KEYWORDS: Paper, Microfluidics, Lamination, Glucose, Cotinine

INTRODUCTION

Paper-based microfluidic devices have gained significant interest primarily due to their low cost, flexibility, and ease of use. They are often fabricated by patterning hydrophobic zones using SU-8 or wax [1]. Some of them are made by cutting paper with a laser [2]. Here we report a method by cutting paper using a digital craft cutter, followed by lamination. Laminated paper-based analytical devices (LPAD) are mechanically sturdier than a paper device without a plastic backing. The approach of using a craft cutter and roller laminator makes it possible to prototype LPAD with no more difficulty than producing a typical identification card.

The functions of LPAD have been confirmed by using colorimetric assay for the measurement of glucose at clinically relevant concentrations. In addition, we applied LPAD for analysis of cotinine, the primary biomarker for exposure to secondhand smoke (SHS). SHS is a mixture of side stream smoke from cigarettes and the smoke exhaled by smokers [3]. It has been associated with a variety of adverse health outcomes in nonsmokers, including lung cancer, respiratory illness and cardiovascular diseases. The most commonly used biomarker for detecting exposure to SHS is cotinine, the primary metabolite of nicotine [4].

EXPERIMENTAL

Materials and Reagents. Whatman chromatography paper (2 cm wide roll) was obtained from Fisher Scientific (Pittsburgh, PA) while rolls of 75-µm-thick polyester thermal bonding lamination films were from Lamination Plus (Kaysville, UT). Potassium iodide, glucose, and horseradish peroxidase was obtained from Fisher Scientific (Pittsburgh, PA) while glucose oxidase was from MP Biomedical (Solon, OH). Synthetic urine was obtained from Ricca Chemicals (Arlington, TX). Luminol (3-aminophthalhydrazide), H_2O_2, chitosan, acetic acid, glutaraldehyde and phosphate buffered saline (PBS, 1X) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Cotinine was obtained from Alfa Aesar (Ward Hill, MA) while HRP conjugated cotinine (cotinine-HRP) was from CalBioreagents (San Mateo, CA). Mouse monoclonal cotinine antibody (anti-cotinine) was bought from Abcam (Cambridge, MA) and bovine serum albumin (BSA) was from Sigma-Aldrich (St. Louis, MO). Mouse serum samples were purchased from Equitech Bio (Kerrville, TX).

Fabrication of Paper Devices. As illustrated in **Figure 1**, the process to fabricate LPAD devices is very similar to making typical ID card. To make a device, the pattern of LPAD was designed first using AutoCAD (Autodesk Inc.). The design was then exported as a DXF file into the controller software of a craft cutter, ROBO Master-Pro (Graphtec Corporation). A section of chromatography paper was affixed to an adhesive carrier sheet and cut using the cutting plotter. Paper strips were obtained after removing the unwanted edges. One difference from an ID card is that the cover sheet of LPAD needs to be open for reagent/sample accesses. We also used AutoCAD to design a cutout pattern

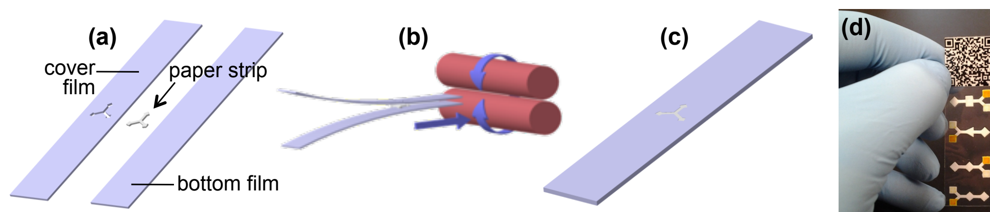

***Figure 1.** The fabrication process for LPAD. **(a)** A cover film, paper strips, and a bottom layer are cut according to the design. **(b)** The film layers and paper strips are properly aligned, and then passed through a heated roll laminator. **(c)** An LPAD device is obtained after lamination. **(d)** Picture of a device containing four test strips and a QR (quick response) code. Each test strip consists of a sample pad in middle, detection zones for two samples on one side, and a negative control on the other side.*

for the cover sheet. After cutting, the cover, paper strip and bottom sheet were aligned and assembled together. The assembly was passed through a heated laminator (Catena 35, GBC), which was set at 220°F.

Flow Characterization. Flow rates in LPAD devices were determined by applying Coomassie Brilliant Blue R-250 to the end of a 1-mm-wide paper strip and measuring the time for the flow front to pass from the sample pad to a given location on the test strip. For flow rate comparisons between bonded and unbonded strips a linear strip with a test section length of 45 mm was used. In the case of the unbonded strips, a single unbonded strip was placed on a glass microscope slide. No extra backing material was required for the bonded strips. For both types of paper strips the flow front was measured in 2.5 mm increments from 5 mm to 25 mm. An average linear velocity was obtained from multiple experiments.

Glucose Assay. For urine samples spiked with a certain concentration of glucose, a drop (18 μL) of samples was dispensed on the sample pad of one paper strip. The samples were allowed to flow through the paper strips, waiting for 15 minutes to ensure sufficient time for interactions with potassium iodide. At the end of the experiments, one can observe the color changes if analytes are present in samples. To quantify the results, the images of the paper devices were acquired using a scanner (Epson 300). The color images of 1200 dpi were analyzed using ImageJ.

Cotinine Detection. The immobilization steps were carried out before immunoassay. The detection zone of a paper device was coated with chitosan, followed by cross-linking using an amine-reactive bifunctional molecule (glutaraldehyde). Anti-cotinine in PBS was then immobilized onto the detection zone, followed by applying 1% BSA in PBS to block possible non-specific binding sites.

Competitive immunoassay format was used because cotinine is a small molecule and it does not have multiple epitopes that can be used for binding with both capture antibody and detection antibody. To implement it on LPAD, a sample solution containing cotinine and cotinine-HRP was applied to the detection zone. Luminol and hydrogen peroxide was then dispensed onto the reagent pad that was connected to the detection zone via a paper channel. The luminescence was measured in a microplate reader (Mithras LB 940, Berthold Technologies).

RESULTS AND DISCUSSION

Flow Rate in LPAD. We studied the effects of the several fabrication conditions on the flow rate in LPAD. In particular, we compared an unbonded plain paper strip and a bonded strip (**Figure 2**). The relationship between the flow rate and the paper strip length can be explained theoretically by Washburn's equation [5]. In the equation, $L^2 = \frac{\gamma D t}{4\eta}$, L is the length of the paper strip, γ is the effective surface tension, D is the pore diameter, t is the time, and η is the dynamic viscosity of the fluid. Washburn equation can be rearranged to obtain this relationship, $\frac{L}{t} = \frac{\gamma D}{4\eta}\frac{1}{L}$. This equation explains the inverse linear relationship between the flow velocity (L/t) and the length of the paper strip (L).

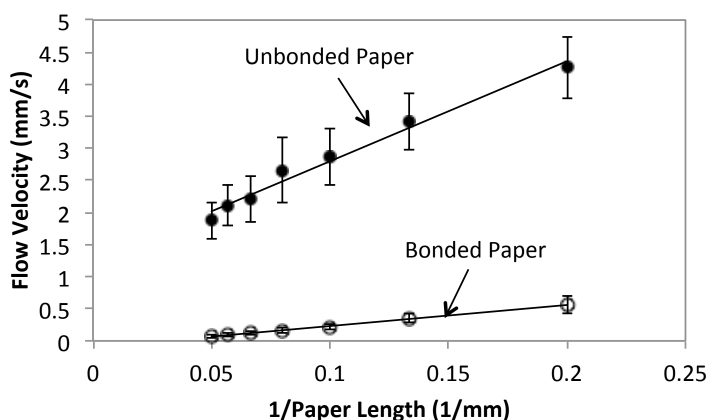

Figure 2. Comparison between bonded paper in LPAD and unbonded plain paper. The flow velocities were plotted as a function of the inverse of the paper length. Each data point is an average of four experiments and the error bars represent one standard deviation. The lines are the theoretical best-fit linear regression according to Washburn equation.

The results in Figure 2 indicate that the flow rate in the bonded strip in LPAD is slower than an unbonded plain paper strip. Since flow rate is directly related to pore size in paper strips, the decrease in flow rate from unbonded strips to bonded strips can be partially explained by the compression of paper by the rollers during the lamination process. In addition, the encapsulation of the paper strip increased "back pressure" by not allowing air to escape from the paper matrix, thus leading to a decrease in flow rate.

One advantage of a decreased flow rate is an increased residence time of reagents, allowing more time for reactions to take place. In a traditional lateral flow assay the amount of apparent antigen is inversely proportional to the square of the change in linear flow rate. Since the flow velocity decreases with the increasing length of the paper strip, a shorter strip will result in a faster flow rate. As a result, in those situations where a fast flow rate is preferred, the length of the strip can be decreased.

Glucose Assays. We chose two assays to demonstrate the functions of LPAD. One is colorimetric assay for glucose measurement. **Figure 3** shows a series of detection zone images for glucose when its concentration was varied. Glucose

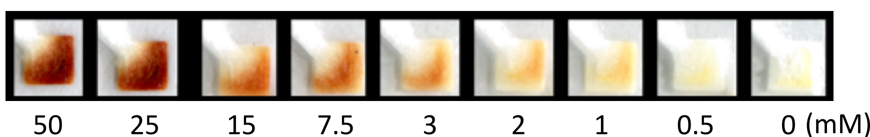

Figure 3. Representative images of the glucose detection zone of LPAD.

was prepared in a synthetic urine solution. The signal in the detection zone was quantified by imaging, resulting in a calibration curve. The results indicated that a linear detection range existed from 0.5 mM to 10 mM. Note that the glucose concentration at greater than 1.4 mM is indicative of diabetes [6], which suggests that LPAD has potential to be used for medical diagnosis.

Cotinine Detection. Schematic of competitive immunoassay for cotinine detection is shown in **Figure 4a**. The format of competitive immunoassay was employed because cotinine is a small molecule and it does not have multiple epitopes that can be used for binding with both capture antibody and detection antibody as required in the sandwich assay format.

We obtained the calibration curve of cotinine detection using chemiluminescence-based competitive immunoassays in LPAD as shown in **Figure 4b**. Since cotinine and cotinine-HRP compete with each other for the fixed amount of anti-cotinine immobilized on the surfaces of the detection zone and the chemiluminescence intensity represents the amount of cotinine-HRP, the decrease in signal from the blank sample indicates the presence of cotinine in a sample and the degree of the decrease is proportional to the concentration of cotinine. The results show that a linear detection range of 0.01 - 1 μg/mL was obtained. The limit of detection was determined to be 5.0 ng/mL using the definition of 3 times the standard deviation of a blank solution. These results are clinically relevant since the concentration of cotinine in SHS samples is typically in the order of 10 ng/mL [7].

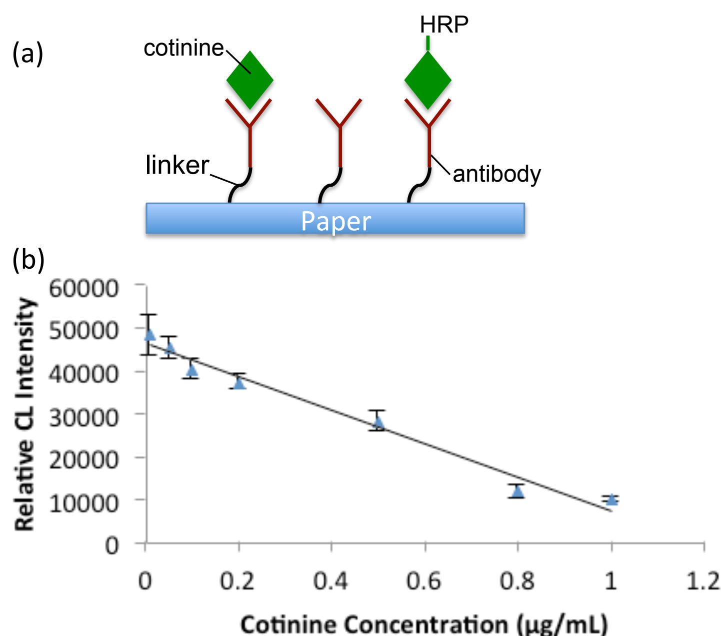

Figure 4. (a) *Schematic of competitive immunoassay for cotinine detection. A sample is mixed with known amount of cotinine tagged with horseradish peroxidase (HRP). The immobilization of anti-cotinine onto paper is based on glutaraldehyde chemistry. Compared to a blank control, the reduction in the chemiluminescent signal indicates the presence of cotinine in the sample.* ***(b)*** *Calibration curve obtained from the competitive immunoassays in LPAD devices. The chemiluminescence (CL) intensity was plotted as a function of the cotinine concentration. Each data point is an average of five repeats and the error bars represent one standard deviation.*

CONCLUSION

We presented a fabrication method for producing laminated paper-based analytical devices in a way similar to making an identification card. We showed functional LPAD and their uses for quantitative colorimetric assays of glucose and for chemiluminescence immunoassay of cotinine. The key advantages of this fabrication method are (1) simplicity, (2) mechanical strength, and (3) low cost.

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CONTACT

*Z. Hugh Fan, tel: +1-352-8463021; hfan@ufl.edu