Paper-Based Analytical Device for Quantitative Urinalysis

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Paper-based analytical devices are fluidic chips fabricated with extremely inexpensive materials, namely paper, thereby allowing their use as a zero-cost analytical device in third-world countries that lack access to expensive diagnostic infrastructures. The aim of this review is to discuss: (1) microfluidic paper-based analytical devices (µPADs) for quantitative analysis, (2) fabrication of two- or three-dimensional µPADs, (3) analytical methods of µPADs, and (4) our opinions regarding the future applications of µPADs for quantitative urinalysis.

Keywords: Paper-based analytical device; Zero-cost analytical device; Urinalysis; Quantitative detection

INTRODUCTION

Limitations of Conventional Analytical Devices
The conventional analytical device for urinalysis is a dipstick. The first dipstick was introduced in 1957 to detect glucose [1]. Today, urinalysis dipsticks can detect a number of analytes [2-7]. The measurements with dipsticks are typically carried out using the naked eye to evaluate the color change of the indicator when analytes present in urine are captured. However, dipsticks based on colorimetric detection for quantitative analysis require an expensive electrical reader, which is not appropriate for resource-limited environments such as third-world countries that lack access to electrical sources.

An Ideal Analytical Device
The World Health Organization has listed seven guidelines for ideal analytical tests. Specifically, tests must be affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, and deliverable to end-users [8]. According to these criteria, the conventional analytical device is not adequate as an ideal analytical tool, because it is neither equipment-free, nor affordable.

Microfluidic Analytical Devices for Quantitative Analysis
A microfluidic paper-based analytical device (µPAD) is fabricated with an extremely inexpensive material, paper. This allows it to be used as a zero-cost analytical device [9]. µPADs, first introduced by Martinez et al. [10] in 2007, are microfluidic platforms that use cellulose paper to generate flow by the capillary action. Unlike the conventional dipstick, µPADs can enable fluid control including injection, transportation, and flow rate on the paper. These characteristics are key for the use of µPADs and their potential applications in zero-cost quantitative urinalysis.

TWO-DIMENSIONAL µPADs
µPADs can be fabricated in two-dimensions (2D) or three-dimensions (3D) to transport fluids in the vertical or horizontal dimensions depending on the diagnostic application.

Fabrication of 2D µPADs
Fabrication of µPADs can be divided into two types of patterning: chemical patterning [11-19] and physical shaping [20-23]. In this review, we focus on chemical patterning. The chemical treatment changes specific areas (or lines) of cellulose paper from hydrophilic to hydrophobic. The two parallel hydrophobic lines act as a channel, because the hydrophilic sample solution cannot penetrate the hydrophobic line or barrier. Consequently,
the liquid flows in the channels owing to capillary action.

**Photolithography**

The chemical fabrication of µPADs using photolithography was first introduced by Martinez et al. [10,13]. They demonstrated a simple fabrication of patterned paper to create channels comprising hydrophilic cellulose paper combined by the hydrophobic polymer, SU-8 2010 photoresist (Fig. 1A). They soaked a piece of chromatography paper in SU-8 2010 photoresist, spun it, and then baked it to remove the cyclopentanone in the SU-8 formula. After removing cyclopentanone, they exposed the photoresist and paper to UV light through a photomask. After the exposure, the paper was baked to cross-link the exposed portions of the resist. To remove unpolymerized photoresist from the paper, it was soaked in propylene glycol monomethyl ether acetate and the pattern was washed with propan-2-ol. After it was patterned, the paper was more hydrophobic, including the area which was supposed to be the hydrophilic channel, because of residual resist bound to the paper. Accordingly, the hydrophilicity of the paper was increased by exposing the entire surface to oxygen plasma. The patterned paper could transport biological liquids to a specific position. For the glucose and protein assay, the patterned paper was functionalized by the addition of reagents, which changed color upon reaction with glucose or albumin (Fig. 1B).

![Fig. 1. Fabrication of two-dimensional microfluidic paper-based analytical devices (µPADs). (A) Schematic diagram of the fabrication of µPADs by patterning hydrophobic photoresist onto paper by photolithography. (B) Actual image of µPAD functionalized by wicking two reagents, which are sensitive to glucose and proteins, respectively. (C) The three steps for fabricating µPADs by wax patterning. (D) Schematic illustration of the spreading of molten wax during printing or the thermal process on paper, and the variables for the design of µPADs: \( W_p \) is the printed width of the wax, \( W_g \) is the gap between the edge of the parallel printed wax lines before melting, \( W_b \) is the thickness of the hydrophobic barrier, \( W_c \) is the width of the channel after thermal treatment, \( L \) is the length of the spread wax. (E) Actual image of µPAD functionalized by individually wicking three different reagents including albumin, glucose, and cholesterol.](image-url)
**Wax Patterning**

One simple chemical patterning is wax patterning on the paper (Fig. 1C). The process comprises three steps including (1) designing the shape of the pattern, (2) printing the wax-pattern on the paper using a wax printer, and (3) heating the patterned paper on hot plate [16]. The first step is to design patterns as hydrophobic barriers on the chromatography paper using softwares such as Clewin or Adobe Illustrator. The designed patterns are printed using the solid-wax printer. After printing patterns on the paper, the printed paper is placed on a hot plate and the wax on the paper melts and spreads through the thickness of the paper, thereby forming hydrophobic barriers (Fig. 1D). The distance of the spread molten wax on paper can be described by Washburn’s equation [24]. Carrilho et al. [16] suggested the following equation to predict the width of the hydrophobic barriers of wax after the thermal process:

\[
W_b = W_t + 2L
\]

Where \( W_b \) is the width of the wax barrier, \( W_t \) is the width of the printed line, and \( L \) is the distance of the width of the spread wax after heating (\( 2L = \text{L}_1 + \text{L}_2 \)) from the edge of the printed line in the direction vertical to the line. To predict the width of the hydrophilic path that acts as a channel determined by two parallel hydrophobic barriers of the wax, Carrilho et al. [16] also suggested equation:

\[
W_c = W_t - 2L
\]

Where \( W_c \) is the width of the channel between two parallel hydrophobic barriers and \( W_t \) is the gap between the two printed lines. It is easy to predict the width of the designed pattern on the paper after printing and the thermal process with these two equations. Therefore, the fabrication of µPADs using wax patterning is relatively simple to design and the formation of the channels on the paper is relatively facile. After wax patterning, the device was functionalized by depositing three reagents to detect albumin, glucose, and cholesterol (Fig. 1E).

**Quantitative Analysis With 2D µPADs**

The measurements of conventional analytical devices, such as dipsticks, are carried out using the naked eye and a calibration chart to determine the color change of the dye absorbed in the device when it binds to analytes. This colorimetric determination is required for quantitative measurements. However, these measurements can be expensive and might pose an issue for use in developing, third world countries. Recently, Cate et al. [25] demonstrated the ability of a µPAD to detect glucose quantitatively by measuring the color change of an absorbed dye on the paper upon reaction between glucose and the dye. The length of the color change was determined by the concentration of glucose in the sample solution. They called this concept distance-based detection. The distance-based detection represents a simple quantitative detection using µPADs without the requirement of an electric reader. However, there were issues with the reproducibility of assay, because the distance could be changed depending on the volume of the sample solution. Therefore, a relatively expensive micropipette was required.

We demonstrated the quantitative detection of albumin using µPADs and retention factors [26]. A retention factor (\( R_f \)) is the ratio of the distance traveled by albumin to that by the solvent in the sample solution in the sample solution. The µPAD was fabricated by printing wax-patterns on chromatography paper using a wax-printer. The shape of the wax-pattern was designed using Adobe illustrator CS5. The design of the pattern comprised two components: the sample pad to absorb the sample solution and the assay site to measure the distances traveled by the albumin and solvent in the sample (Fig. 2A). We spotted a 0–8 mg/mL sample solution of albumin on the sample pad. The assay method is very simple; the level of albumin was

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**Fig. 2.** Two-dimensional microfluidic paper-based analytical devices (µPADs) for quantitative urinalysis without requiring an external electrical equipment. (A) Schematic diagram of the assay. The assay is carried out by an analysis of the retention factor (\( R_f \)). \( R_f \) is the ratio of the distances traveled by albumin and the solvent in the sample. The \( R_f \) indicates the concentration of albumin in the sample. (B) Actual image of the device after the injection of the sample. In the image, the distance traveled by the solvent is 4.5 cm and the distance traveled by albumin (green) is 1.9 cm.
quantified by measuring the ratio of the distance traveled by the sample solvent versus the distance traveled by the albumin developed by the transfer of the solvent. The ratio indicates the concentration of albumin in the sample.

\[ R = \frac{\text{The distance traveled by albumin}}{\text{The distance traveled by solvent}} = \text{The concentration of albumin} \]

The distance traveled by albumin in the sample strongly depends on the interactions among albumin, solvent, and the adsorbent (tetrabromophenol blue, TBPB) in the paper. There is a difference between the distance traveled by albumin and water because albumin interacts more with TBPB which is absorbed in the assay site within the channel, as compared to water. Additionally, the color of TBPB changes from yellow to blue when bound to albumins. The assay results can be quantified by calculating the ratio of the length of the color change of TBPB (from yellow to blue; distance traveled by albumin) versus the distance traveled by water (Fig. 2B).

In conclusion, we confirmed that the ratio was constant in samples with the same concentration of albumin regardless of the volume of the sample. This µPAD could be utilized without an external electronic reader. This µPAD also provided an equipment-free and inexpensive urinalysis to third world countries that lack access to external electronic equipment.

**THREE-DIMENSIONAL µPADs**

A 3D µPAD can run fluid horizontally and vertically; therefore, it has potential application in quantitative analysis and multiple assays within a compact space [27,28].

**Fabrication of 3D µPADs**

The original fabrication involves stacking papers to form vertical channels in the bundle of papers.

**Stacking Papers With Tape**

The 3D µPADs are fabricated by stacking layers of patterned paper with double-sided tape [27]. After stacking the layers of paper and tape, there are gaps between the layers of paper because of the thickness of the tape. Therefore, fluid cannot flow vertically across the hydrophobic tape and the gap between the layers of paper. The gap is closed by punching holes in the tape and filling it with hydrophilic materials such as cellulose powder [29], or by compressing the two layers of paper via a hole in the paper.
the tape between the two layers (Fig. 3). The gaps filled with hydrophilic materials play a similar role to vertical channels.

**Lamination With Toner**

The fabrication of 3D µPADs uses toner as a thermal adhesive to bind multiple layers of patterned paper together. The toner can be used to print patterns with hydrophilic areas that act as ports, designed by an imaging software. Therefore, this method can be viewed as an alternative to the fabrication of 3D µPADs using tape that requires punching holes in the tape to form the vertical channels [30]. The fabrication of 3D µPADs using toner starts with printing wax-patterns on layers of paper followed by printing one of the layers with toner. The printed paper with wax and toner is folded to assemble the layered 3D device and the folded paper is passed through a laminator to melt the toner, thereby binding each layer together with toner that serves as a thermal adhesive. This method is a simplified version of the original method for preparing 3D µPADs using toner.

**Quantitative Analysis With 3D µPADs**

Lewis et al. [31] introduced 3D µPADs for quantitative detection based on counting the number of bars dependent on the concentration of analytes. The 3D µPAD was fabricated by assembling patterned chromatography papers and tapes (Fig. 4). Samples were injected onto the layer 1 on the top of the device; then, the liquid flowed through the device vertically to layer 7 in the bottom of the device. In layer 7, the sample separated into the 16 surrounding conduits (Fig. 4A). These conduits transported the sample to the bars of layer 1 that were visible on the top of the device. Layer 8 was a single-sided tape to prevent contact with other surfaces when the user placed the device on a surface. The 16 conduits, surrounding the central conduit of this device, were composed of paper disks containing hydroxyethyl-piperazineethane-sulfonic acid (HEPES) buffer salts, followed by two layers containing hydrophobic compound 1 and a paper disk containing a dried green dye. An assay with samples containing different amounts of hydrogen peroxide was performed. After the injection of the sample, the liquid was separated in layer 7 into the 16 conduits and moved through layers 6, buffering the sample with the HEPES. At the time, the sample arrived in layer 5 and hydrogen peroxide in the sample reacted with compound 1 to yield hydrophilic products [31]. The reaction changed the wetting properties of the paper to let the sample flow through the device to layer 2, where it dissolved the green dye in the paper disk (Fig. 4B, C). The liquid and the dye flowed into layer 1, where the green-colored liquid was revealed as green bars.

With an increase in the amount of hydrogen peroxide in the sample, the speed of the liquid flow through the disks containing compound 1 increased. Consequently, the number of green-colored bars resulting from the flow could differ depending on the amount of hydrogen peroxide in the sample at a fixed time during the assay. HEPES, hydroxyethyl-piperazineethane-sulfonic acid.

**Fig. 4.** Three-dimensional (3D) microfluidic paper-based analytical device (µPAD) for quantitative assays. (A) 3D illustration of the movement of sample flow. (B) The reaction mechanism between component 1 and hydrogen peroxide. (C) The layers assembled in a conduit. (D) Actual image of device after assay. The number of green colored bars vary depending on the amount of hydrogen peroxide in the sample at a fixed time during the assay. HEPES, hydroxyethyl-piperazineethane-sulfonic acid.

In conclusion, Lewis et al. [31] described a simple quantitative assay by measuring the number of colored bars at a fixed time during the time. This assay did not require external equipment, and therefore, this 3D µPAD is a useful analytical device in the developing world.
CONCLUSIONS

In this article, µPADs for quantitative analysis were reviewed. µPADs fabricated using extremely inexpensive paper can serve as zero-cost analytical devices for urinalysis. Three-dimensional µPADs can transport liquid vertically and horizontally within the device. Accordingly, this renders the µPADs viable as novel analytical devices for the quantitative detection of target materials in urine without external electrical equipment, a commodity which is difficult to access by users in resource-limited environments such as third world countries. Additionally, 3D µPADs generate multiple flows in a small space to detect multiple targets in urine for the diagnosis of various diseases. Therefore, we assume that 3D µPADs will be developed for quantitative urinalysis. However, the fabrication of 3D µPADs is still time-consuming. Therefore, fabrication of 3D µPADs must be simplified to render zero-cost analytical devices for quantitative urinalysis.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

ACKNOWLEDGEMENTS

The research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Science, ICT & Future Planning (MSIP) (No. NRF-2011-0017322).

REFERENCES

1. Free AH, Adams EC, Kercher ML, Free HM, Cook MH. Simple specific test for urine glucose. Clinical chemistry 1957;3:163-8.
2. Chemstrip-Test-Strips [Internet]. Indianapolis (IN): Roche Diagnostics Co.; c2013 [cited 2013 Nov 12]. Available from: https://www.poc.roche.com/poc/rewrite/generalContent/en_US/article/POC_general_article_70.htm.
3. Multistix10-SGReagent-Strips [Internet]. Erlangen (DE): Siemon AG; c2010-2013 [cited 2013 Nov 12]. Available from: http://www.healthcare.siemens.co.uk/point-of-care/urinalysis/multistix-10-sg-reagent-strips.
4. Chemstrip-Micral-Test-Strips [Internet]. Roche Diagnostics Co.; c2013 [cited 2013 Nov 12]. Available from: https://www.poc.roche.com/poc/rewrite/generalContent/en_US/article/POC_general_article_74.htm.
5. Urine Analysis with Test Strips [Internet]. Düren (DE); MACHELEYEY-NAGEL GmbH & Co.; c2013 [cited 2013 Nov 12]. Available from: http://www.nn-net.com/tabid/5526/default.aspx.
6. ARKRAYS-AUTION-Sticks [Internet]. Tokyo (JP): ARKRAY Inc.; c1998-2013 [cited 2013 Nov 12]. Available from: http://www.arkray.co.jp/english/products/urinalysis.html.
7. YD-Diagnostics-UriscAN-Strips [Internet]. Yongin (KR): YD diagnostics Co.; c2013 [cited 2013 Nov 12]. Available from: http://www.yd-diagnostics.com/2012/eng/pdf/uriscan_strip.pdf.
8. Peeling RW, Holmes KK, Mabey D, Ronald A. Rapid tests for sexually transmitted infections (STIs): the way forward. Sex Transm Infect 2006;82 Suppl 5:v1-6.
9. Yetisen AK, Akram MS, Lowe CR. Paper-based microfluidic point-of-care diagnostic devices. Lab Chip 2013;13:2210-51.
10. Martinez AW, Phillips ST, Butte MJ, Whitesides GM. Patterned paper as a platform for inexpensive, low-volume, portable bioassays. Angew Chem Int Ed Engl 2007;46:1318-20.
11. Songjaroen T, Dungchai W, Chailapakul O, Laiwattanapaisal W. Novel, simple and low-cost alternative method for fabrication of paper-based microfluidics by wax dipping. Talanta 2011;85:2587-93.
12. Dungchai W, Chailapakul O, Henry CS. A low-cost, simple, and rapid fabrication method for paper-based microfluidics using wax screen-printing. Analyst 2011;136:77-82.
13. Martinez AW, Phillips ST, Wiley BJ, Gupta M, Whitesides GM. FLASH: a rapid method for prototyping paper-based microfluidic devices. Lab Chip 2008;8:2146-50.
14. Ollkonen J, Lehtinen K, Erho T. Flexographically printed fluidic structures in paper. Anal Chem 2010;82:10246-50.
15. Li X, Tian J, Garnier G, Shen W. Fabrication of paper-based microfluidic sensors by printing. Colloids Surf B Biointerfaces. 2010;76:564-70.
16. Carrilho E, Martinez AW, Whitesides GM. Understanding wax printing: a simple micropatterning process for paper-based microfluidics. Anal Chem 2009;81:7091-5.
17. Songjaroen T, Dungchai W, Chailapakul O, Henry CS, Laiwattanapaisal W. Blood separation on microfluidic paper-based analytical devices. Lab Chip 2012;12:3392-8.
18. Lu Y, Shi W, Jiang L, Qin J, Lin B. Rapid prototyping of paper-based microfluidics with wax for low-cost, portable bioassay. Electrophoresis 2009;30:1497-500.
19. He Q, Ma C, Hu X, Chen H. Method for fabrication of paper-based microfluidic devices by alkylsilane self-assembling and UV/O3-patterning. Anal Chem 2013;85:1327-31.
20. Sibbett SS, Lopez GP, inventors. Multiplex lateral flow devices and
methods. United States patent. US 20080317633 A1. 2008 Dec 25.
21. Chitnis G, Ding Z, Chang CL, Savran CA, Ziaie B. Laser-treated hydrophobic paper: an inexpensive microfluidic platform. Lab Chip 2011;11:1161-5.
22. Fenton EM, Mascarenas MR, Lopez GP, Sibbett SS. Multiplex lateral-flow test strips fabricated by two-dimensional shaping. ACS Appl Mater Interfaces 2009;1:124-9.
23. Fu E, Kaufman P, Lutz B, Yager P. Chemical signal amplification in two-dimensional paper networks. Sens Actuators B Chem 2010;149:325-8.
24. Washburn EW. Note on a method of determining the distribution of pore sizes in a porous material. Proc Natl Acad Sci U S A 1921;7:115-6.
25. Cate DM, Dungchai W, Cunningham JC, Volckens J, Henry CS. Simple, distance-based measurement for paper analytical devices. Lab Chip 2013;13:2397-404.
26. Jeong SG, Lee CS. Diagnostic paper chip for reliable quantitative detection of albumin using retention factor. KSBB J 2013;28:254-9.
27. Martinez AW, Phillips ST, Nie Z, Cheng CM, Carrilho E, Wiley BJ, et al. Programmable diagnostic devices made from paper and tape. Lab Chip 2010;10:2499-504.
28. Lewis GG, DiTucci MJ, Baker MS, Phillips ST. High throughput method for prototyping three-dimensional, paper-based microfluidic devices. Lab Chip 2012;12:2630-3.
29. Martinez AW, Phillips ST, Whitesides GM. Three-dimensional microfluidic devices fabricated in layered paper and tape. Proc Natl Acad Sci U S A 2008;105:19606-11.
30. Schilling KM, Jauregui D, Martinez AW. Paper and toner three-dimensional fluidic devices: programming fluid flow to improve point-of-care diagnostics. Lab Chip 2013;13:628-31.
31. Lewis GG, DiTucci MJ, Phillips ST. Quantifying analytes in paper-based microfluidic devices without using external electronic readers. Angew Chem Int Ed Engl 2012;51:12707-10.

http://dx.doi.org/10.5213/inj.2013.17.4.155