3

Fabrication and Applications of Paper-Based Microfluidics

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3.1 Introduction

Microfluidics, or lab-on-a-chip, has been developed for nearly 30 years, providing a versatile toolbox with strong capability and huge potential to push forward the boundary of many disciplines, with particular applications in biomedical research (Whitesides 2006, Mu et al. 2013, Sackmann et al. 2014). In recent years, a new trend has emerged, which is to integrate microfluidic concepts and techniques into a form of paper-based devices, that is, paper-based microfluidics (Figure 3.1).

On the one hand, paper as a ubiquitous microfibrous material (Pelton 2009, Ren et al. 2013, 2014, Mahadeva et al. 2015), would offer unique structure-relevant merits in microfluidics other than conventional materials such as silicone, plastics, and glass. The advantages mainly include: (1) the cost of paper

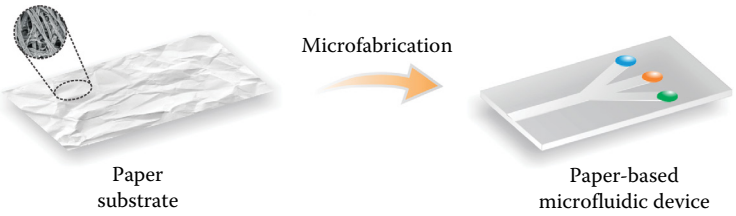

FIGURE 3.1
Paper-based microfluidic device is made of various paper substrates via a range of microfabrication strategies. The reconciliation of material advantages of paper and engineering advances of microfluidics shows huge benefits in developing novel clinical diagnostic techniques.

is much less expensive than others; (2) the fibrous nature of paper would lead to high surface-to-volume ratio and thus enhance a variety of size-relevant processes; and (3) the spontaneous wicking flow on paper is highly beneficial to the transport of liquid in the absence of external power sources. All of these advantages would render microfluidics a detection method much more accessible and affordable than conventional microfluidics.

On the other hand, the emerging paper-based microfluidics relies on the concept and techniques of microfluidics, especially surface patterning and vertical stacking (Webster and Kumar 2012, Cunningham et al. 2016). For example, by adopting microfluidic techniques, a colorimetric assay on paper not only allows for automated fluid distribution but also achieves as high as a throughput of 1024 reactions per test (Martinez et al. 2008). In another example, a four-step assay could be established on paper simply by a single activating procedure (Fu et al. 2012).

In fact, paper has for long been utilized in a myriad of commercial assays such as dot-immunoassay (Pappas et al. 1983, Coelho et al. 2007, Rodkvamtook et al. 2015), dried blood spotting (Spooner et al. 2009, Smit et al. 2014), Western/Northern blotting (Streit et al. 2008, MacPhee 2010), urine dipstick tests, and pregnant lateral flow tests (Wong and Tse 2009). Nevertheless, over these conventional diagnostic assays, the reconciliation of the unique characteristics of paper and the established microfluidic methods represents a great opportunity to forge new analytical and diagnostic strategies, demonstrating unprecedented and much more desired analytical functions. In our opinion, paper-based microfluidics is highly promising in developing functional analytical assays yet in a low-cost, less laborious, and easily accessible manner.

Although paper-based microfluidics is useful in a wide range of applications including environment monitoring (Ma et al. 2012), portable energy (Esquivel et al. 2014), screening of drugs (Weaver et al. 2013, Koesdjojo et al. 2014, He et al. 2016), and cell culture (Derda et al. 2009, Deiss et al. 2014, Kim et al. 2015b), its potential is fully demonstrated when it comes to the field of

clinical diagnosis and point-of-care tests, especially in developing countries and resource-limited settings (Martinez et al. 2010, Mu et al. 2014, 2015).

The significance of clinical diagnosis doubtlessly soars in the landscape of globalization and population aging. It is a prerequisite to the downstream healthcare intervention and treatment. Although diagnosis itself only accounts for less than 5% of overall healthcare cost, it can influence as much as 60%–70% of healthcare decision-making (The Lewin Group 2005). However, most clinical diagnostic tests are instrument based, expensive, complicated, and thus limited in centralized medical centers and hospitals. This barrier, in fact, poses an elusive challenge of how to make these technically sophisticated diagnostic tests ultimately available to patients. Paper-based microfluidics, as mentioned earlier, is of great potential to address this challenge and may revolutionize the way of delivering diagnosis to patients.

The field has been developing rapidly, on the topic of which several extensive reviews have been previously published (Li et al. 2012b, Yetisen et al. 2013, Cate et al. 2014, Gomez 2014, Phillips and Lewis 2014, Chen et al. 2015, Su et al. 2015, Cheng et al. 2016). On the basis of these works, we further discuss up-to-date trends and recent achievements in this chapter. First, we will give a brief introduction regarding newly developed fabrication methods, and the choice of fabrication methods depends on specific contexts and paper used. Second, we will focus on the applications that are of the most clinical relevance, presenting the bottlenecks in current clinical diagnosis. We hope that this chapter would inspire more technological endeavors on continuously developing paper-based microfluidics devices.

3.2 Microfabrication Techniques

Microfabrication in paper-based microfluidics relies on the engineering techniques to pattern paper with hydrophobic areas, which are crucial features and prerequisites to achieve sophisticated analytical functions on paper. It also well distinguishes paper-based microfluidics from conventional paper-based tests. The microfabrication methods can be roughly divided into different categories from the technical perspective (Figure 3.2).

3.2.1 Photolithography

In the early stages, paper-based microfluidics is largely compliant with the conventional fabrication protocols in microfluidics. Photolithography is widely employed to pattern chromatographic paper and generate structures in a well-controlled and high-precision manner (Martinez et al. 2007, Carrilho et al. 2009b; Figure 3.2a). In a typical process, paper is first impregnated with the precursor of photoresist; upon the exposure of UV in a certain pattern, the photoresist can be selectively cross-linked in the paper and

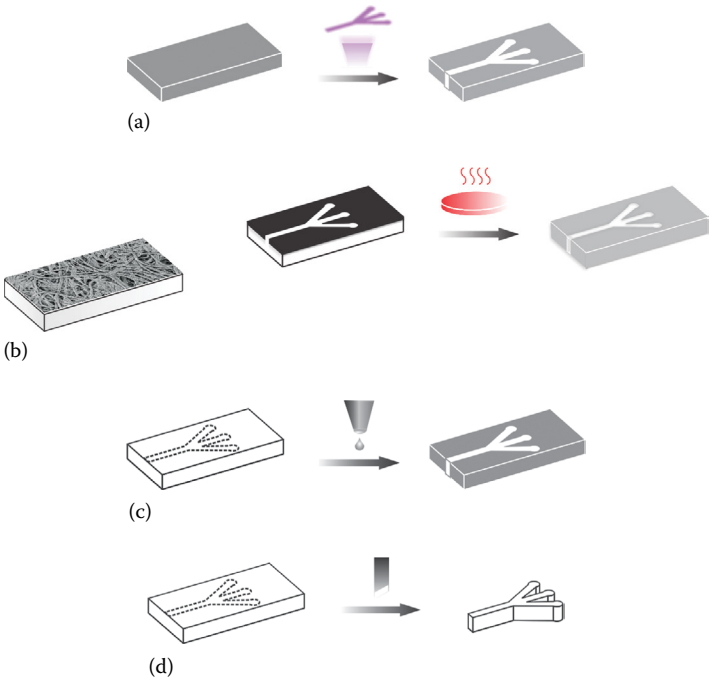

FIGURE 3.2
Microfabrication of paper-based microfluidics. (a) Photolithography. (b) Wax printing. (c) Inkjet printing. (d) Mechanical cutting.

the uncured monomers will be subsequently washed away. As such, the photoresist-occupied areas become hydrophobic, while the rest of the areas still remain hydrophilic and allow for transport of liquids.

Besides two-dimensional (2D) patterning, multilayers of patterned paper can be vertically aligned together to form a three-dimensional (3D) interconnected network, leveraging analytical functions and throughput (Martinez et al. 2008). Such a 3D network can be alternatively achieved by paper origami (Liu and Crooks 2011, Kalish and Tsutsui 2014).

The photolithography-based fabrication proves the concept and the value of paper-based microfluidics. However, the costly photoresist and instrument are hardly affordable to most research labs and, in particular, remote regions, which seems incompatible with the original intention of using paper to develop affordable analytical devices.

3.2.2 Wax Printing

Wax instead of photoresist has been employed to generate controlled hydrophobic areas (Carrilho et al. 2009a, Lu et al. 2009). Since both the wax and wax printer are relatively inexpensive and can be easily adopted, wax printing has

soon become the most popular technique in fabricating paper-based microfluidics (Kurdekar et al. 2016, Li et al. 2016). The general process contains two steps: (1) printing the wax on the surface of paper; and (2) melting the wax on a heating device for it to penetrate the full thickness of the paper (Figure 3.2b). The second step inevitably reduces the fabricating resolution because of the wicking of the molten wax. Of note, even though the penetration of wax is troublesome in most cases, it can still be harnessed to achieve sophisticated functions, such as generating 3D and multilayer structures inside one single layer of paper (Li and Liu 2014, Renault et al. 2014).

3.2.3 Inkjet Printing

Inkjet printer, a common office instrument, is applicable to manufacturing paper-based microfluidics (Liao et al. 2014, Sun et al. 2015, Yamada et al. 2015b, Wang et al. 2016). To achieve this purpose, several hydrophobic materials in solutions with optimized viscosity have been developed to replace common printing ink, including alkyl ketene dimer (Li et al. 2010), resin (Xu et al. 2015), polystyrene (Abe et al. 2008), and polyacrylate (Apilux et al. 2013, Maejima et al. 2013). The printing or deposition of these hydrophobic materials on paper can form well-controlled hydrophobic patterns (Figure 3.2c). Besides printing the barriers for the channels, the printer may be equipped with a multicartridge system containing different inks, which is very useful to deposit chemical reagents necessary in the subsequent assay at the same time with the fabrication of the device.

More recently, the resistance to solutions containing surfactants is increasingly emphasized because of the potential of direct analysis of cell lysates using paper-based devices. To achieve this goal, improved hydrophobic barrier materials have been developed, including hydrophobic sol-gel-derived methylsilsesquioxane (Wang et al. 2014b), silicone resin (Rajendra et al. 2014), fluoropolymer (Chen et al. 2013), and Teflon (Deiss et al. 2014).

3.2.4 Cutting

The fabricating methods, mentioned earlier, always use hydrophobic materials to pattern papers. However, it is equally feasible to simply cut though the paper, allowing the geometric shape of the paper to guide the liquid flow (Figure 3.2d). Therefore, a myriad of cutting approaches have been developed and adopted on the basis of different cutting mechanisms and devices, including laser cutting (Nie et al. 2013, Spicar-Mihalic et al. 2013, Arrastia et al. 2015), plotter (Chen et al. 2016), mechanical cutting (Mu et al. 2014, 2015, Feng et al. 2015), and any combination of these techniques (Li et al. 2013b, Cai et al. 2014, Song et al. 2015).

Notably, some cutting methods provide an excellent alternative for patterning under room temperature. For example, nitrocellulose (NC) is a very useful paper substrate for constructing paper-based tests (Fridley et al. 2013, Arrastia et al. 2015). However, it is extremely vulnerable to high temperature over 100°C since it will decompose at 55°C and undergo autoignition

at 130°C (Credou et al. 2013). Therefore, photolithography, wax printing, and even laser cutting is incompatible to pattern NC, while the mechanical cutting that avoids the generation of heat would be advantageous for this purpose. Furthermore, in some occasions, manual cutting, while seemingly less controlled, would provide necessary active intervention and maximally costumed flexibility (Fang et al. 2011, Nie et al. 2012).

3.3 Representative Applications of Paper-Based Microfluidics in Clinical Diagnosis

In this section, we will further discuss three specific applications of paper-based microfluidics in clinical diagnosis, that are, blood typing, ELISA, and sickle cell disease detection. A brief summary of broader applications of paper-based microfluidics is also provided in Table 3.1 but will not be discussed in details.

3.3.1 Blood Typing

Blood typing is of great clinical significance in blood transfusion and transplantation (Daniels and Bromilow 2014). The accurate and rapid detection of blood groups is imperative to prevent hemolytic transfusion reactions and other fatal consequences. Conventional tests heavily rely on laboratory-based instruments (e.g., centrifuges), requiring intensive and skilled manual operations, and thus limit the efficiency and accessibility of the assay. To develop an alternative blood grouping method, Gil, Shen, and colleagues harnessed the different transport behaviors on paper between agglutinated and non-agglutinated red blood cells (Khan et al. 2010; Figure 3.3a). The agglutinated red blood cells would form a spot with high optical density, allowing visual identification, while non-agglutinated ones would show nearly no visual trace of the spot. The paper-based assay not only enables streamlined operations with only one step of pipetting a drop of blood but also shows advantages on the aspects of assay time (several minutes) and cost (a few cents). It was further demonstrated that a step of elution could improve assay reliability (Al-Tamimi et al. 2012).

Shen and colleagues also made an effort to investigate the underlying mechanism of the agglutination of red blood cells on a piece of antibody-treated paper (Jarujamrus et al. 2012, Li et al. 2013a). It turns out that the antibodies desorbed from cellulose fibers, instead of the absorbed ones, played a more critical role in generating a large lump of agglutinated cells that are entangled in the network of paper fibers. The factors that may influence assay performance have been exploited, including paper structure (Su et al. 2012, Li et al. 2014a), papermaking additives (McLiesh et al. 2015,

TABLE 3.1

A Brief Summarization of Paper-Based Microfluidic Devices for Clinical Diagnosis

Clinical Samples	Disease	Detection Method	Analytical Target	Limit of Detection	References
Tear	Ocular disease and corneal epithelium disorders	Separation-based detection	Lactoferrin	0.1 mg/mL	Yamada et al. (2014), (2015a)
		Electrochemistry	Na^+ K^+	4.9 μM 6.8 μM	Chagas et al. (2015)
Sweat	Cystic fibrosis	Colorimetry	Anions	10 mM	Mu et al. (2015)
Saliva	Hemodialysis	Colorimetry	Nitrite	5 μM	Klasner et al. (2010)
	Tobacco smoke exposure	Distance-based detection	Thiocyanate	0.06 mM	Pena-Pereira et al. (2016)
Blood	Anemia	Colorimetry	Hemoglobin	1 g/L	Yang et al. (2013b)
Blister fluids	Bullous pemphigoid	Colorimetry	NC16A autoimmune antibody	NA	Hsu et al. (2014a)
Aqueous humor	Retinal ischemic condition	Colorimetry	Vascular endothelial growth factor (VEGF)	33.7 fg/mL	Hsu et al. (2014b)
Spiked Plasma	Phenylketonuria	Colorimetry	Phenylalanine	0.5 mg/L	Thiessen et al. (2015)
Semen	Infertility	Colorimetry	Live sperm Motile sperm	8.46 million/mL 15.18 million/mL	Nosrati et al. (2016)

Guan et al. 2016), and antibody stability (Guan et al. 2014a). The thin, porous, and lightweight paper is better to construct the blood group typing assay. Such paper could facilitate the elution of non-agglutinated red blood cells from the paper, and leads to a lower background and an improved signal-to-noise ratio. Papermaking additives have the potential to enhance the performance as well as accelerate the commercialization of the paper-based blood typing device.

Besides the agglutinated spot, other methods to enhance the visual detection has also been developed. Inspired by the magic paper in the movie of "Harry Potter," Shen and colleagues proposed a method to present the

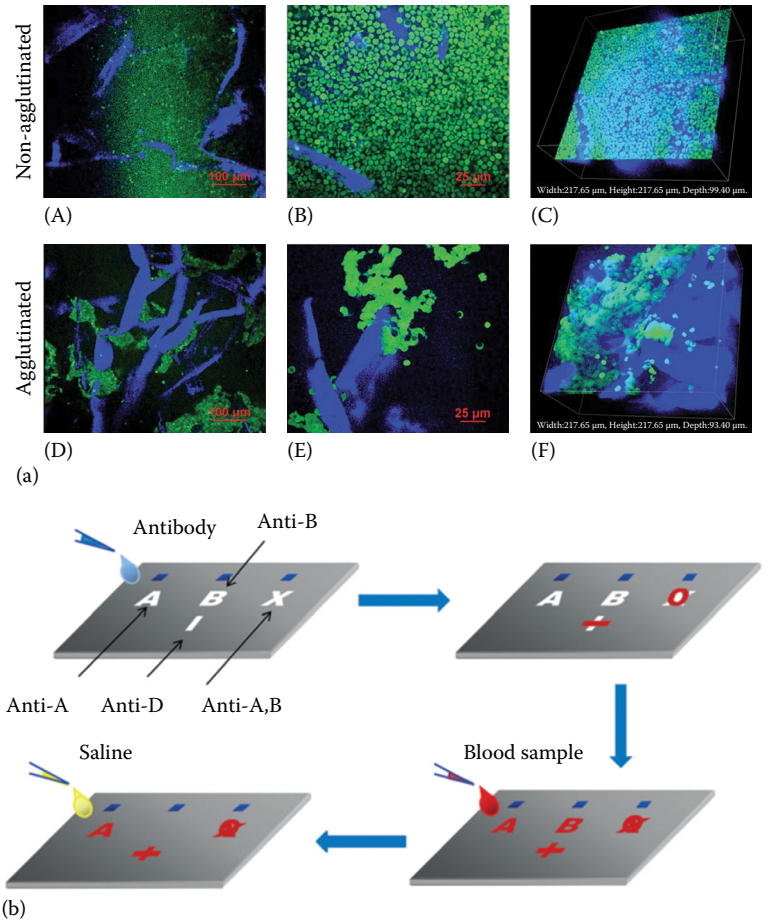

FIGURE 3.3
Clinical applications of paper-based microfluidics. (a) Blood typing based on agglutination of erythrocytes entangled in the fiber network of paper. (b) Display detection of blood types on paper. (*Continued*)

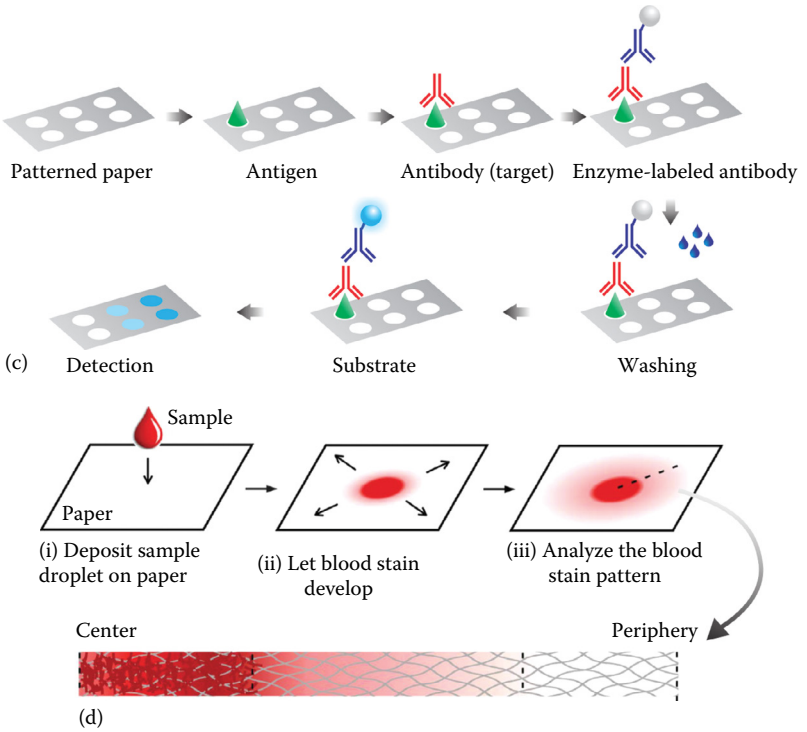

FIGURE 3.3 (*Continued*)
Clinical applications of paper-based microfluidics. (c) Paper-based ELISA procedure for the detection of autoimmune antibodies. (d) Paper-based diagnosis of sickle cell disease. ([a]: From Li, L.Z. et al., *Analyst*, 138(17), 4933, Copyright 2013a; [b]: From Li, M.S. et al., *Angew. Chem. Int. Ed.*, 51(22), 5497, Copyright 2012a; [d]: From Yang, X. et al., *Lab Chip*, 13(8), 1464, Copyright 2013a.)

detection results in written text (Li et al. 2012a). For example, a capital letter B, instead a red spot, would display on the paper when tested positive (Figure 3.3b), which is greatly beneficial for end users to interpret the assay results. In another case, a barcode-like format was proposed to take the advantages of smartphone readings (Guan et al. 2014b).

The utilization of paper-based assays has expanded to detect secondary blood groups (Li et al. 2014, Then et al. 2015), transform Indirect Antiglobulin Test (IAT) on paper (Yeow et al. 2015), and achieve reverse grouping (Noiphung et al. 2015). Although rare, minor, or secondary blood groups are of significant clinical importance.

3.3.2 ELISA

ELISA in the form of plastic well plate is one of the most ubiquitous assays performed in routine clinical laboratories. The transition of this assay into

a form of patterned paper would improve its accessibility and efficiency, as well as expand the range of its potential applications (Figure 3.3c). Cheng, Whitesides, and colleagues developed a pioneering paper-based microfluidic ELISA (Cheng et al. 2010), which demonstrated significant advantages over conventional ones (Heller et al. 1998). The paper-based ELISA had the same layout as plastic 96-well plates, allowing high throughput. Each well was surrounded by hydrophobic SU-8 patterns that limited the spreading of liquid on paper. Therefore, each well of 5 mm in diameter only required 3 μL of solution to fill in, compared with 20–200 μL in plastic well-based ELISA. The high surface-to-volume ratio also contributed to the shorter assay time of 51 min, in comparison with the 213 min of conventional ELISA. This pioneering work set the tone for the following research on revolutionizing ELISA on patterned paper.

Currently, the role of paper-based ELISA has been widely expanded into detection of a variety of pathogenic and disease-relevant analytes, including Neuropeptide Y relative to cognitive performance (Murdock et al. 2013), Influenza H1N1 and H3N2 viruses (Lei et al. 2015), NC16A antibody of an autoimmune disease (Hsu et al. 2014a), *Escherichia coli* (Shih et al. 2015), hepatitis C virus (Mu et al. 2014), vascular endothelial growth factor (Hsu et al. 2014b), and Dengue virus serotype-2 envelope proteins (Wang et al. 2014a).

Several strategies have been developed to improve the sensitivity of paper-based ELISA, such as silicon dioxide beads to modify filter paper (Bai et al. 2013), polymerization-based amplification (Badu-Tawiah et al. 2015), poly(oligoethylene glycol methacrylate) (POEGMA)-based blocking agents (Deng et al. 2014), rolling circle amplification (Liu et al. 2016), modifications to monoclonal antibody (Hsu et al. 2014b), and Ring-Oven washing technique (Liu et al. 2015b).

3.3.3 Sickle Cell Disease

Sickle cell disease is a common recessively inherited blood disorder. It results in the altered conformation of hemoglobin and chronic anemia that is associated with life-long morbidity and significantly shortened life span (Yawn et al. 2014). Even though early diagnosis and intervention have been proven effective to control this disease, a rapid, reliable, and inexpensive method to diagnose sickle cell disease patients remains a huge challenge (Archer 2014).

The sick hemoglobin, due to the hydrophobic valine substitution, can be polymerized in a concentrated phosphate buffer solution. This phenomenon has been exploited to establish a liquid turbidity assay (SickleDex). Shevkoplyas and colleagues further developed an on-paper version of this assay mechanism (Yang et al. 2013a). The polymerized hemoglobin would entangle with the fiber network in the paper, and thus lead to differential patterns of blood stain (Figure 3.3d). They successfully employed this paper-based method to distinguish sickle cell trait carriers and sickle cell disease

patients, and used it to quantify the ratio of sickle hemoglobin in blood that is beneficial to monitor the effectiveness of medical therapies (Piety et al. 2015).

3.4 Summary

Over the past several years, the field of paper-based microfluidics has developed rapidly and broadly. Paper-based microfluidics possesses a number of benefits such as reduced cost, speedy assay, increased portability, sensitivity, and multiplicity. Among them, we would like to emphasize the enhanced access for common patients to timely healthcare intervention, which may be the most influential aspect of paper-based microfluidics to clinical diagnosis.

The extraordinary material features of paper reconciled with microfluidic techniques are crucial to constructing a functional paper-based microfluidic device. The study on patterning and modifying paper, however, still requires continuous exploration and optimization.

The rapid growth of paper-based microfluidics has also inspired and echoed with the utilization of many other well-controlled and low-cost substrate materials, including eletrospun nanofibrous membrane (Yang et al. 2008), thread (Zhou et al. 2012, Nilghaz et al. 2014, Kim et al. 2015a), cotton (Lin et al. 2014), cloth (Liu et al. 2015a, Wu and Zhang 2015), and lignocellulose (from bamboo) (Kuan et al. 2015). We believe that the combination of these varieties of materials with paper will likely further enhance the functions of paper-based microfluidics and its applications in medicine.

Last but not least, the rational combination of paper-based microfluidics with other disciplines should not be overlooked. Several disciplines including chemometrics (Jalali-Heravi et al. 2015), nanotechnology (Sun et al. 2014, Warren et al. 2014), small molecular logic system (Ling et al. 2015), and synthetic biology/gene network (Pardee et al. 2014, Slomovic et al. 2015) have already demonstrated a glimpse of augmenting analytical functions on paper. Therefore, it is believed that the continual development of the paper-based microfluidics will ensure its translation into clinical diagnosis that spans across a much wider range of applications than currently available, hopefully in the near future.

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