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to date, there have been a total of 1040 citations for that article (Source: PubMed).
Introduction

The first step towards treating a disease is accurate and efficient detection. Paper-based analytical devices (PADs) can provide a quick, inexpensive, and simple detection method for biological and chemical analysis, which can easily be employed in medical diagnostics. Currently, applications, such as blood typing and pathogenic detection, require a technical infrastructure operated by qualified staff for accurate interpretation. Paper diagnostics could offer a cheap, simple, and convenient method for the user to directly test and interpret results, such as the pregnancy test and electronic blood glucose tests. The past 5 years has seen the rise of a strong interest in paper-based diagnostic methods. Although PADs hold great promise as a bio-diagnostic platform, there are still many limitations preventing its commercial applicability, as evidenced by the lack of new products on the market after 5 years of intense research and development.

Paper diagnostics are devices made of paper, cellulosic films, cardboard, and fabrics to recognize and quantify biomolecules and chemical agents affecting health. While paper-based analysis has been readily used in biomedicine since the 19th century, its potential in modern medicine has yet to be fully realized (Li et al. 2009a, Pelton 2009, Martinez et al. 2010a). The past 5 years has seen a rapid increase in interest, due to the compelling advantages of using paper-based diagnostic methods. A key advantage is its ubiquitous nature. Paper is a staple in everyday life all over the globe, which is manufactured on a large-scale into a plethora of different structures and properties. Paper is cheap, easily engineered, biodegradable, combustible, biocompatible, sterilizable, hydrophilic, and easy to functionalize and process into diagnostic devices. Paper is a very attractive substrate to develop as a low-cost diagnostic platform (Table 1).

With these attributes, paper can become an ideal platform for point-of-care (POC) diagnostic devices and eliminate or restrict the need for external equipment and specialized technical personnel; this would be ideal in developing countries, and military, emergency, and humanitarian field operations (Pelton 2009). As defined by the World Health Organization, diagnostics in developing countries should be ASSURED i.e., affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, and deliverable (Table 1) (Lee et al. 2010). Additionally, POC diagnostics need to be disposable, portable, sterilized, and rugged (Lee et al. 2010). These requirements help to overcome the unpredictability exhibited during in-the-field testing caused by the surrounding environment (Lee et al. 2010). This includes limited clean water, unreliable electricity, high temperatures, and humidity (Lee et al. 2010). The versatility of paper and its many advantages make it more desirable as a POC platform than other technologies, such as thread and magnetic levitation (Vella et al. 2012). Another requirement of modern paper diagnostics is ease of use and the direct testing and interpretation by users.

Paper can serve four functions in diagnostics: 1) transport and measurement of samples and analytes; 2) reaction support; 3) separation of reactants from products; and 4) communication of results. When an aqueous analyte, such as blood, saliva, urine or feces is tested using a PAD, the fluid is driven by capillary flow induced by the porous and hydrophilic structure of the cellulose fibers, wicking through the paper inter-fiber space. Should hydrophobic barriers be formed onto or within paper, microfluidic PADs (µPADs) can be created (Martinez et al. 2007), allowing for the passive transport of the analyte without any further equipment required (Rivet et al. 2011). This represents a major advantage over traditional microfluidic materials, such as glass, silicone, polydimethylsiloxane (PDMS),

Table 1  The desirable affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, deliverable (ASSURED) properties of paper as a medical diagnostic.

| Requirement          | Paper is:                                                                 |
|----------------------|---------------------------------------------------------------------------|
| Affordable           | Cheap and cost effective                                                  |
| Sensitive            | White-background                                                          |
| Specific             | Dried reagents                                                            |
| User-friendly        | Single use autonomous                                                     |
| Rapid and robust     | Fast acting                                                               |
| Deliverable          | Portable                                                                  |
| Other                | Disposable                                                                |
|                      | Particulate filter                                                        |

Inexpensive manufacturing process
Minimal use of reagents and analytes
A good contrast medium for colorimetric tests
Reagents can be dried and stored within paper fibers
Does not require external power sources or equipment
To eliminate as many unnecessary steps (e.g., washing) as possible
Generally faster analysis compared to conventional testing methods
Easy to manufacture, stack, store, and transport
To protect end-users from exposure to bio hazardous wastes
Fibers can trap unwanted particulates in loading zone, preventing movement to detection zone
and other polymers, which require external pumping (Martinez et al. 2010b, Rivet et al. 2011). Paper microfluidics are achieved by patterning hydrophilic channels that are defined by hydrophobic barriers; the micro-channels allow the testing of small samples with minimal reagents, thus reducing cost (Martinez et al. 2010b, Rivet et al. 2011).

Even though paper has many of the desirable attributes of a diagnostic platform, the current commercial PADs, such as immunochromatographic strips, have limited quantitative capabilities (Rivet et al. 2011). This has generated interest in coupling paper devices with electrical reporting methods, from basic telemedicine (Martinez et al. 2008a) to electrochemical sensing platforms (Dungchai et al. 2009). Another disadvantage is the simplistic nature of the paper device, which has restricted multiple-sample, multiple-analysis or multi-step assays (Rivet et al. 2011).

Despite its recent inception in 2007, the growing interest in paper-based bio-diagnostics has already resulted in several review papers, each detailing the desired properties, patterning methods, and potential applications in health, food, and the environment (Martinez et al. 2007, Pelton 2009, Di Risio and Yan 2010, Martinez et al. 2010a, Ballerini et al. 2012), as well as reviews in microfluidics, in which paper is included (Haeberle and Zengerle 2007, Lee et al. 2010, Rivet et al. 2011). However, few have critically analyzed the potential and limitations of paper as an engineered material to manufacture low-cost biomedical diagnostics.

While medical diagnostics have a single beneficiary, they are used in widely different contexts of applications, each with their specific requirements. Three broad categories can be mapped. The first is generic high throughput diagnostics for routine tests. Cholesterol analysis, basic ABO blood typing, and blood sugar measurement are examples. These generic tests are performed by specialized scientists operating well-automated analytical instruments in dedicated laboratories. Speed of analysis, high throughput, and low cost are the main drivers. The second category of diagnostics regroups the series of patient specific analyses required for a particular diagnostic. Examples are specific antibody identification, complete blood phenotyping for a transfusion, and elemental blood analysis. The main requirements are flexibility and speed. These tests require laboratories with efficient management, a wide analytical expertise, and instrumentation, exclusive to modern medical laboratories. Sample preparation and chemical/biochemical reactions are often required. The last category consists of diagnostics for stand-alone remote testing. These tests are often performed either in the privacy of home or in difficult conditions, either by the patient himself or by untrained personnel in absence of technical or information support. A single specific test is usually performed. Robustness, ease of use, direct quantification/interpretation of results, and speed are four important requirements.

Paper diagnostics cannot pretend to address all medical analytical needs. With the explosion in research development and the strong media interest surrounding paper tests, the medical world and community at large are often left in confusion as to the state of development and potential paper diagnostics offered for medical applications. This article attempts to address these issues and provide an analytic roadmap; it is critical to distinguish between elegant prototypes and research prowess from promising technologies able to impact the medical community. An engineering approach is adopted here, as it best segregates science from development and clearly highlights the strengths and limitations of paper technology in medical diagnostics.

This manuscript consists of four sections. The first presents paper as an engineered composite and highlights its properties and attributes with respect to paper diagnostics needs. Functional printing is introduced as a complementary technology for paper in the manufacturing of paper diagnostics. The second section analyzes paper diagnostic design. PADs based on one dimensional (1D), 2D, and 3D flow are analyzed, followed by a review of the methods of reporting and the principles of detection. The third section reviews high impact applications in health and medicine for paper diagnostics. Applications are studied in terms of clinical diagnostics, physiological disorders, and pathogenic diseases. The last section presents a critical perspective of the current developments, the missing links, and the potential of paper diagnostics. It is the objective of this article to analyze paper as a viable technology for producing low-cost medical analysis and to delimit the range of applications and potential best suited to paper diagnostics.

Paper as diagnostic substrate

Paper is a porous and flexible composite made from cellulosic fibers and functional colloids that can be tailor-made for a plethora of applications (Figure 1). While the near totality of paper diagnostics has been made using filter paper, this substrate represents a negligible fraction of the paper production, and of the range of structures and properties achievable. Furthermore, filter paper is among the most expensive grade of paper. The choice of filter paper
has been dictated by the quest for a standard to avoid unknown variables from cellulosic materials engineering. Most filter papers are made of a single type of fiber, mostly cotton, uniformly distributed in all directions. However, bioactive paper engineering relies not only on the fibrous structure, but also on the structure of the void fraction; fibers control the reactants and biomolecules distribution, while the void fraction dictates liquid transport.

Paper is made by uniformly distributing and draining a suspension of cellulosic fibers onto a moving wire passing over a series of drainage elements, and then into a series of three presses, and finally through a long dryer. Paper is a specific type of non-woven material, manufactured by a wet laid process, which involves four steps: 1) fiber preparation, 2) forming, 3) bonding, and 4) surface treatment. Fiber preparation consists of selecting and preparing cellulosic fibers to optimize the properties of the paper, the selection determining whether recycled or virgin fibers are used. Recycled fibers are avoided for bioactive paper and paper diagnostics, due to the high risk of contaminants associated (dirt, oils, polymers, ink and organic residue, microbial growth). For wood-based paper, the fibers can be long softwood or short hardwood fibers. They can also be made by a chemical or by a mechanical pulping process. Chemical processes dissolve most of the extractives (fatty, resin acids, tannin, lignin, and hemicellulose), basically producing almost pure cellulose fibers. Mechanical pulping processes rely on a combination of shear, heat, and plasticization by water to separate fibers from wood by fracturing the middle lamellae; the resulting mechanical fibers are made of cellulose, hemicellulose, and lignin. The fibers are then lightly refined to provide the optimal surface area and bonding ability. Plant fibers from cotton, linen, flax, and hemp are sometimes used for specialty papers. Additives, such as polymers, microparticles (calcium carbonate, clay, kaolin, titanium dioxide), and dyes, are often added to the fibrous suspension to control the strength and the optical properties of paper. A cationic hydrophobic colloid, referred to as internal size, is often adsorbed onto pulp fibers prior to papermaking, to control paper wettability and ensure sharp printability. The typical internal sizes are: alkyl ketene dimers (AKD), alkenyl succinic anhydride (ASA), and rosin. Sizing agents are mostly used in office paper and packaging paper. Forming transforms the pulp suspension into paper, a uniform and continuous network of cellulosic fibers and functional colloids. The fiber suspension is impinged onto a moving wire, where it is drained and pressed to give fiber orientation, thickness, and the basic structure of paper. Fiber orientation in paper is affected by the difference of velocity between the moving forming wire and the impinging jet of pulp fibers. Bonding links the discontinuous fibers into a continuous non-woven paper network, which is achieved by pressing and drying. The “hydrogen bond” between hydroxyl groups forms a network between the cellulosic surfaces and provides the bonding between fibers. Polymeric strength agents can increase the paper’s wet and dry strength. Surface treatments allow paper specific properties. This is achieved by calendering, surface sizing, coating, spraying or other processes. Calendering passes the paper through a series of roll nips of increasing pressure to decrease paper porosity, and increase thickness uniformity and surface smoothness. Surface sizing applies a polymer solution, usually starch with a surface active polymer or latex, onto both surfaces of paper, which creates an interphase when the fluid polymer solution penetrates a few μm within the surfaces of paper. Coating applies a viscous solution of inorganic (filler) and organic (latex) colloids, which smooth the surfaces of paper and provide a surface of controlled microporosity, surface energy, and wettability (Kendel et al. 2008).

The paper properties are in large part defined by the choice of fibers and the papermaking process. Fiber dimension and chemistry depend on the type of lignocellulosic material pulped and the pulping process. The fiber orientation in the X-Y plane and in the paper thickness (Z) is controlled by the process. Process and fiber selection affect the porosity (1/paper density), and size and orientation of pores in paper. This means that the fiber orientation in the machine direction (MD – long axis of the paper sheet) differs from those of the cross machine direction (short dimension of the paper sheet). Basic properties for important grades of paper are presented in Table 2.

Many different structures and interphase morphologies of paper can be achieved using conventional paper technology (Figure 2). Refining and blending fibers, such as nanocellulose crystals or microfibrillated cellulose fibers, can increase the internal surface area, bonding, and density, while reducing internal pore sizes of paper (Figure 2A). Functional colloids, such as gold nanoparticles (AuNP), can be adsorbed either as individual particles (Figure 2A) or as aggregates of controlled size (Figure 2B) (Ngo et al. 2011). By controlling the conditions of the process at the size press and the properties of the solution, polymer layers of different thickness and concentration gradients can be deposited and pressed into both surfaces of the paper (Figure 2C) (Cho and Garnier 2000, Shirazi et al. 2003, Shirazi et al. 2005). The color contrast by scanning electron microscopy (Figure 2C) was achieved using a marker (KI) (Shirazi et al. 2003). The polymer sizing solution can contain a hydrophobic surface active polymer, such as styrene maleic anhydride polymer derivatives, or
| Type of paper   | Basis weight (g/m²) | Structure                                              | Fibers                                                                 | Additives                                                                 |
|----------------|---------------------|--------------------------------------------------------|------------------------------------------------------------------------|---------------------------------------------------------------------------|
| Filter paper   | 90–200              | Random fiber orientation, variable porosity,           | Mostly cotton; sometimes bleach kraft                                    | Wet strength agent [such as polyamide epichlorohydride (PAE)]               |
|                |                     | and controlled pore size (1–20 μm)                    | softwood                                                               | High concentration of wet strength agent                                    |
| Tea bags       | 15–30               | High porosity, low density, high strength              | Bleach kraft softwood                                                 | Filler (10–20% precipitated calcium carbonate (PCC), clay, or titanium     |
| Uncoated       | 80–120              | Dense, low porosity                                   | Blend of bleach chemical softwood (spruce, pine, fir) hardwood fibers   | dioxide (TiO₂)]. Retention aid, internal size, surface size starch, and     |
| fine paper     |                     |                                                        | (eucalyptus, maple, aspen), and sometime recycled fibers | latex or polymer                                                            |
| Coated fine    | 80–120              | High machine direction (MD) fiber orientation         | Blend of mechanical, chemical, and recycled fibers. Mostly long softwood | Same as above, plus latex and PCC for coating                               |
| paper          |                     | Dense, low porosity                                   | fibers                                                                 |                                                                           |
| Newsprint      | 42–48               | High MD fiber orientation                             | Blend of mechanical, softwood, and recycled fibers. Resin and fatty acid | Filler from recycled paper, ultraviolet (UV) brighteners, yellowing         |
|                |                     |                                                        | impurities from wood                                                  | inhibitors, retention aids                                                  |
| Facial         | 15–20 per ply,      | Low density/high porosity,                            | Layered eucalyptus/softwood/fibers structured                          | Wet (PAE) strength agents, softener.                                       |
| tissue         | 2–3 ply per         | dense formation, molded or creped structure. 1, 2 or   |                                                                        | Sometimes silicone (polydimethylessiloxane, PDMS) applied as surface       |
|                | tissue              | 3 layers ply per, 1, 2, or 3 plies                    |                                                                        | treatment                                                                  |
| Bath tissue    | 15–32 per ply       | Low density/high porosity,                           | Layered eucalyptus/softwood/fibers structured. Recycled fibers         | Temporary wet strength agents,                                             |
|                | 30–40 single ply    | high porosity, excellent formation, molded or creped  | sometimes used                                                         | (glyoxylated polyacrylamide) softener                                      |
| Towels         | 30–50               | structure                                             | Blended or layered eucalyptus/softwood/fibers structured. Recycled      | Wet (PAE) and dry carboxymethylcellulose strength agents, softener.         |
|                |                     |                                                        | fibers sometimes used                                                  | Sometimes PDMS applied as surface treatment.                                |
| Packaging      | 80–200              | Dense, fibers aligned                                 | Recycled and high yield chemical softwood fibers (high lignin content) | Wet and dry strength agents, sizing agents                                 |
| Liner board    | 200–600             | Dense, fiber aligned. 2 or 3 layers can be wet        | Recycled and high yield chemical softwood fibers (high lignin content) |                                                                           |
|                |                     | pressed into a single layer                           |                                                                        |                                                                           |
Figure 1  Schematic roadmap of paper and cellulosic diagnostics in biomedicine.

Figure 2  Paper as an advanced material: (A) cellulosic fiber bonding and microfibrillation; (B) retention of aggregated gold nanoparticles on paper; (C) creation of an inter-region on paper: surface sizing a starch-polymer solution; and (D) creation of surface active assembled alternating copolymers.
latex that can migrate to the surface of paper to minimize differences in surface energies. The polymer can assemble into polymer domains, as for styrene-maleic anhydride copolymers (Figure 2D) (Duskova-Smrckova et al. 1999, Garnier et al. 2000). Therefore, controlled paper structures having a complementarity of length scales in the nm, the μm, and the mm ranges can be engineered with the current paper technology.

**Functional printing**

Printing is a process which transfers controlled patterns of liquids or dye onto a surface: traditionally this has been ink on paper. Ink is made of dyes and particles dispersed in water or an organic solvent, containing additives to control viscosity and surface tension, to give required optical properties. Contact printing, such as flexography, lithography and rotogravure, and non-contact printing, like ink jet or laser jet, allow a printing resolution better than 20 μm. If dye/nanoparticles are replaced by a hydrophobic wax, such as AKD, ASA or wax, hydrophobic barriers can be printed to produce microfluidic systems on paper (Khan et al. 2010a). Biomolecules, such as enzymes, antibody molecules, or cells in an aqueous media form a bio-ink which can be injected in the microfluidic system previously printed on paper. Paper diagnostic prototypes have been entirely manufactured by printing (Li et al. 2010a).

**Paper diagnostics design**

Optimization of the structures and fabrication methods for microfluidic paper devices has focused on a perceived limitation of the current μPADs: performing multiplex assays. While a simple, single step assay is invaluable in many circumstances, in some instances, multi-test ability is required. Research interest has shifted to the actual design structure of the devices, expanding from single sheets of paper to 2D networks and 3D designs, for creating diagnostics able to test multiple analytes or samples simultaneously. While more complex designs might add functionality where single-step procedures are inadequate, a balance with simplicity is required in PAD design. This section analyzes PAD design.

A simple differentiation between device designs is the directional flow of fluid. For instance, 1D indicates the flow of liquid in a single direction, while 2D describes lateral flow in multiple directions on the horizontal plane (e.g., the spread of liquid from a single corner to multiple detection zones), and 3D designs add a vertical component (Figure 3).

**1D paper diagnostics**

1D lateral flow paper diagnostics, commonly known as dipstick tests, have been used for decades. These simple dipstick tests were first used to detect urinary glucose levels (Comer 1956, Free et al. 1957). Dipsticks are made of stiff paper and used by dipping one end of the strip into the sample, allowing fluids to be transported passively through the cellulose fibers towards the reagent zone (Haeblerle and Zengerle 2007). In the early 1960s, testing evolved to a triple analyte test, adding the ability to detect protein albumin and pH levels; it has since expanded to a 10-type multi-analyte test which can assay for additional biomarkers, such as leukocytes, nitrite, ketones, bilirubin, and urobilirubin. Some assays can also measure and report the sample specific gravity (Fenton et al. 2008).

In the 1980s, the applicability of dipsticks was increased to include immunorecognition. The spotting and immobilization of antibodies on nitrocellulose led to the development of a wider range of PADs, which are now found on the market. Urine analysis tests include the take-home pregnancy tests, testing for human chorionic gonadotropin, and in drug testing, such as the 9-tetrahydrocannabinol agent to detect marijuana users. Immune-based PADs can also test blood analytes for cholesterol levels, diabetes, and pathological diseases, such as hepatitis C and human immunodeficiency virus type 1 (HIV-1), as well as autoimmune screening (Hawkes et al. 1982, Dineva et al. 2005, Binder 2006). In 1989, the need

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**Figure 3** Schematic representation of the different types of paper diagnostics in: (A) 1D; (B) 2D; and (C) 3D design showing separate testing zones (T) connected to a single sample zone (S).
for incubation and wash steps was eliminated with the integration of capillary-driven lateral fluid transport with the dipstick technology. This amplified the total number of captured and detected bioanalytes, thus improving the lower limits of detection (LOD) (Rosenstein and Bloomster 1989, Gordon et al. 1990).

Typically, the reagents are dried and stored in the fleece sections of the assay during fabrication. The fleece sections allowed for different detection zones to be present on a single stick. The fluid solution dissolves the dried reagents, allowing a reaction to occur. Figure 4 illustrates a typical dipstick assay. The 1D flow of liquid through the stick from one end to the other was integrated into a 1D paper diagnostic. While useful, 1D paper diagnostics are limited to single-step tests and lack the ability to perform multiple-step assays, often required for techniques, such as paper-based enzyme-linked immunosorbent assay (ELISA). However, these tests are cheap, reliable, and easy to use.

2D paper diagnostics

Simple 2D PADs

The advent of patterned paper to create micro-channel designs has enabled multidirectional flow paper diagnostics. Martinez et al. (2007) patterned a simple device with three detection zones comprising indicators for glucose and protein, as well as a control zone (Figure 5). The “walls”, emplaced by hydrophobic boundaries patterned using photolithography, allowed fluid to be directed into the three different zones without cross-contamination, thus adding a spatial advantage against the conventional 1D-μPAD dipsticks.

Previous reviews have summarized patterning methods for diagnostic design. Such methods include: photolithography, plotting, inkjet or plasma etching, cutting, and wax printing (Martinez et al. 2010a). A summary of the patterning techniques analysis is presented in Table 3. The concept is to create hydrophobic barriers onto or within the paper structure and to rely on paper capillarity for liquid flow. While the original techniques created rigid and brittle barriers, technology has nicely progressed, allowing channels as narrow as 250 μm width to be created (Khan et al. 2010a). Common 2D-PAD test designs still lack the ability to perform multi-step assays for more complex applications and remain prone to contamination and fluid evaporation. However, 2D-PADs are very cheap (especially when manufactured by printing), easy to use, versatile, and robust.

Partially and fully enclosed PADs

A major disadvantage of the basic 2D-μPAD design is its exposed nature. Both sides of the paper devices are uncovered, resulting in the evaporation of reagents and samples, and risking contamination from the support beneath. Contact with the support can also result in loss of reagent and sample fluids. Early attempts to avoid such loss and contamination investigated adding samples and solutions onto the device held in mid-air, which is simply impractical (Schilling et al. 2012).

Fenton et al. (2008) proposed a method to avoid imbibing paper with hydrophobic/hydrophilic patterns. Instead, the paper was shaped into one of three desired 2D designs using a computer-controlled x-y knife plotter. Type 1 consisted of a single sheet of paper cut into the desired shape; for type 2, paper was mated with one layer of polyester cover tape before being cut; and for type 3, pre-cut paper was sandwiched between two layers of cover tape. Type 3 was the first example of a fully enclosed μPAD. Partially or fully enclosed PADs are reported to decrease the rates of operator error; however, this may simply be that paper cut into clearly labeled sections leaves no room for error interpretation (Fenton et al.
**Table 3** Analysis of microfluidic paper-based analytical device (μPAD) fabrication by functional printing.

| Paper patterning techniques | Description: Patterned using chromatography paper soaked in SU-8 photo resist polymer solution before being selectively exposed to ultraviolet (UV) radiation using a patterned mask to shield desired pathways. Shielded regions remain hydrophilic and the unreacted SU-8 is washed away. Unshielded regions become hydrophobic after undergoing polymerization. | Advantages: Convenient, useful. Disadvantages: Expensive chemicals and equipment, multiple steps, time consuming, reduced paper flexibility. Examples: Martinez et al. (2007, 2008b) |
|-----------------------------|-------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------|
| Photolithography            | **“FLASH” printing** Description: Fast Lithographic Activation of Sheets (FLASH); Much like photolithography, except the paper is laminated between a transparent film and a black paper sheet. A standard ink-jet printer is then used to print a black ink mask onto the film. After polymerization the black paper and film is removed. Advantages: Faster, customized masks. Disadvantages: Expensive, multiple steps, reduced paper flexibility. Examples: Martinez et al. (2008b). |
| Etch printing               | Description: Completely hydrophobized paper, using a polystyrene toluene solution, is “etched” using a toluene solvent printed on the surface which dissolves the solution to allow for the hydrophilic channels to form. Advantages: Custom designs, faster. Disadvantages: Corrosive/flammable, chemicals. Examples: Abe et al. (2008, 2010). |
| Polydimethylsiloxane (PDMS) printing | Description: PDMS is dissolved in hexane and printed onto filter paper using a modified x-y plotter. To form the hydrophobic barriers, the PDMS solution penetrates through the paper thickness. Advantages: Enhanced flexibility. Disadvantages: Reduced channel resolution due to “creeping” solution. Examples: Bruzewicz et al. (2008). |
| Plasma printing             | Description: Paper previously hydrophobized using the cellulose reactive compound, alkyl-ketene-dimer (AKD) is patterned using metal masks that are clamped to the paper before being placed in a plasma asher. The AKD hydrocarbon chains are then oxidized by the plasma, leaving the hydrophilic channels. Advantages: Flexible. Disadvantages: Expensive, slow manufacturing rate. Examples: Li et al. (2008, 2010b). |
| Wax printing                | Description: Multiple techniques. The simplest involves patterning both sides of filter paper with a wax crayon before heating it. It was then melted into the substrate to form hydrophobic barriers. Also, it can be extended to inkjet printing for more complicated designs with higher resolution, but at an increased cost. Advantages: Good in resource limited settings. Disadvantages: Low resolution. Examples: Li et al. (2008), Carrilho et al. (2009a). |
| Laser cutting               | Description: Uses a computer-controlled x-y knife plotter to cut the paper into the desired design with very high detail. Does not utilize imbibing techniques. Advantages: Cheaper fabrication costs, detailed design, clear labeling, can be fully or partially enclosed. Disadvantages: experimental technique, heating. Examples: Fenton et al. (2008). |
| Inkjet                      | Description: Office or specialized inkjet printer receives hydrophobic and bioactive ink cartridges. Resolution of 20 μm or better determined by the diameter of the ink droplet. Advantages: low cost and flexibility of digital printing, established technology. Disadvantages: interaction ink-paper critical, plugging of nozzles. Examples: Khan et al. (2010a) |
| Lithography, flexography, silk screening | Description: contact printing techniques. Advantages: fast, cheap, established technology. Disadvantages: require a mold, blanket or negative. |

The design is cheaper to fabricate and impervious to external contaminants.

Olkkonen et al. (2010) presented a partially enclosed device which flexographically printed a polystyrene xylene/toluene ink onto the back of the device, while the front was printed with the microfluidic channel design. The hydrophobic back layer provided a protective layer that prevented fluid from escaping through
the back via contact with the underlying support and protected against contamination from the support. This method allows direct roll-to-roll production well suited for high throughput manufacture. The enclosed backing contributed to full penetration of the hydrophobic barrier, while protecting from contaminants and loss of fluids.

More recently, Schilling et al. (2012) investigated printing toner to produce fully enclosed μPADs. A thermally bonded thin plastic protective layer was printed onto paper. Similar laser printers and toners are commonly purchased for offices, and have been used extensively in the fabrication of μPADs. It is a cheap and convenient method of manufacturing μPADs. The toner had no effect on the microfluidic channels, since it does not diffuse into paper, nor does it come off when wetted. Printing four layers of toner could enclose the μPADs and resulted in faster wicking rates.

However, the heat required (180°C) during the laser printing method can affect the biological reagents that are affixed prior to printing and enclosed beneath the toner layer (Figure 6). At extreme heats, proteins undergo denaturation, lose their ability to bind to other molecules, and become inactive. Schilling et al. addressed this problem and reported a 90% decrease in enzymatic function using glucose oxidase for testing glucose concentration (Schilling et al. 2012). The decreased function was reported not to affect test sensitivity and to allow detection at concentrations as low as 1 mm. However, this raises serious concerns on optimization and economics, and questions as to why a 90% decrease in enzyme activity did not affect sensitivity. An alternative was explored where a 1 mm diameter hole was designed in the surface to act as a reagent addition port, therefore allowing the addition of reagents after enclosing the μPAD, allowing wicking to the reagent-storage zone (Figure 6). This resulted in no loss of reagent or enzyme function, and with the exception of the area of the port, the reagents were protected from the surrounding environment.

**PAD networks**

A disadvantage of the “traditional” single sheet μPADs is their limitation to single-step processes. A single-step process is ideal for the user, but restricts its applicability, since most laboratory-based diagnostic assays involve multi-step processes. This is the case for multi-step assays, such as ELISA, which improve sensitivity and specificity with signal amplification and washing steps. 2D paper networks (2D-PNs) attempt to emulate multi-step assays, while using a single activation step, thus retaining the simplicity and affordability of the single-step μPADs.

Fu et al. (2010) developed a 2D-PN that retains the autonomous nature of μPADs, while allowing the complexity of multiple reagents to be delivered sequentially to a detection zone (Figure 7). The first reported 2D-PN used three methods that allowed multiple inlets to simultaneously converge toward a single point (Fu et al. 2010). All methods focused on varying the delivery time of the fluid by: 1) varying the length of paper, 2) varying its width, or 3) creating a dissolvable barrier (trehalose) to slow the liquid in its tracks. Factors, such as paper composition, pore size, and surface chemistry can also affect the fluid flow rate, but were not explored. Paper was patterned using a laser cutter to fabricate the device, much like Fenton et al. (2008), and was supported by double-sided tape on a glass substrate (Fenton et al. 2008, Fu et al. 2010). Absorbent pads were used at the inlets for reagent application, allowing the solutions to wick to the detection zone. Using dye and pH as examples, the 2D-PN design staggered the delivery of each component to a common site. Later studies used the same design for chemical signal amplification (Fu et al. 2010, Fu et al. 2011a). The transport mechanisms of fluid through the paper networks were also explored (Fu et al. 2011a). However, the effects of paper composition, pore size, and surface chemistry remained unaddressed. The added complexity of the 2D-PN design also increases the possibility of errors.

A method using a single fluid source, rather than an individual source for each of the reagents, was explored Fu et al. (2011b). It involved “programming” the device to disconnect each reagent in a particular order. The design
used a single source well and shaped the 2D-PN by varying the length of each reagent segment. Isolated strips of different lengths were used to show the overall method. The paper strips were housed in a poly (methyl methacrylate) (PMMA) plastic casing to reduce evaporation and provide a support from which to mount the device into the source well. Plastic cartridges and wells were designed to receive a paper cartridge/strip inserted by the user. The paper strips are immersed at different depths into the fluid. The well depletes as fluid wicks paper, thereby disconnecting strips from the source well. This was controlled by: 1) the fluid depletion rate, 2) the immersion depths, and 3) the cross-sectional area of the fluid source. The design also relies on a large and thick paper strip used as a regulator; without it, the lengths of paper varied only slightly. The test is very sensitive to the cross-sectional area of paper. Thin paper strips of small cross-sections are often used, which increases variability due to the high heterogeneity of paper structure at different cross sections. Using these basic principles of managing fluid flow, the 2D-PNs can be programmed to deliver fluids to a common point and disconnect from the source well in a timed, sequential manner. This series of experiments used colored dyes, previously dried onto paper.

### 3D Paper diagnostics

The desire or need to increase the density of circuitry and testing has led to the development of 3D paper devices with often complex fluidic systems. Improving the multi-analyte capabilities of μPADs has been a major focus of development. It has culminated with the advance of multiplex 3D-μPADs prototypes, often consisting of multiple 2D layers. 3D paper diagnostics certainly offer advantages; however, it sacrifices simplicity, cost, and practicality for increased functionality. Other technologies such as threads (Li et al. 2009b, Reches et al. 2010) and traditional microfluidics (Haeberle and Zengerle 2007) devices provide competitive alternatives at this high end market.

The design alternates layers of patterned paper and perforated, double-sided tape stacked upon each other (Figure 8). This allows not only lateral flow, but also vertical flow. The perforated holes were filled with cellulose powder to connect each layer of paper, creating vertical micro-channels through which the analyte solution can wick. This design can create interweaving pathways through the device and allows the analyte to reach multiple detection zones without cross-contamination. Martinez et al. (2008c) designed a four channel device, where each channel underwent eight connections as fluid travelled through the layers at the top to the detection zones at the bottom. The purpose of each layer could vary throughout the device, including fluid distribution, filtration, or combination with other reagents (Martinez et al. 2008c).

Martinez et al. (2010a) later modified the 3D-μPAD design to allow a choice of specific applications. Usually, the function of each test is predetermined during fabrication. However, designing a single platform to test multiple analytes enables a certain level of specificity for each patient. The testing function can be configured at the site, allowing the user to program the device by choosing only the channels needed. This might be useful when
a limited sample quantity is available and only specific analytes are desired. Using a single platform allows for mass production of a “universal device” to test a wide range of samples, and eliminates the need to design each test separately. The gaps that were once filled with cellulose powder are left empty, and are instead labeled as buttons. When pushed, the “on” buttons form a bridge in the required channels through which the fluid can travel. When the button is left un-pushed, the bridge is not connected and liquid will only flow to the desired tests. Once a button has been turned “on” it cannot be turned “off” afterwards, as paper deforms inelastically and remains compressed. However, ease of use and reliability tend to decrease exponentially with test complexity. While an elegant study, it is questionable whether the user might be able to identify the needs and modify the test in a time of stress or illness.

Origami

3D-µPADs can be constructed using origami, the art of paper folding. Liu and Crooks (2011) report a method using a single sheet of folded paper to design a 3D-µPAD (Figure 9). A piece of chromatography paper is patterned using a single photolithographic step. The design consists of micro-channels, reservoirs, and a frame. The frame provides the template for the paper folding and ensures correct alignment. When folded in a particular sequence, the micro-channels are matched to allow flow in both the lateral and vertical directions. The four corners are trimmed and fit in an aluminum clamp. Samples and solutions are injected through one of the four holes drilled into the clamp’s top plate. An advantage origami-PADs (o-PADs) have over the previous designs is the elimination of double-sided tape, which can diffuse into the paper over long periods of time and decrease the hydrophilicity of the micro-channels, along with assembly tools, such as the laser cutter. However, despite these advantages, origami PADS require an aluminum clamp to ensure alignment, which increases the cost of the device and decreases disposability.

Govindarajan et al. (2011, 2012) described a type of 3D-µPAD which utilizes the origami principles (Figure 9) (Govindarajan et al. 2011, Govindarajan et al. 2012). However, it is used functionally rather than to simplify fabrication. The origami serves to sequence the complex processing steps, by folding the layers in succession to prepare collected DNA samples for molecular diagnosis. The design uses stacked layers of cellulose paper and double-sided tape for assembly, but also uses single-sided tape, a repositionable adhesive layer, and card paper to allow for bidirectional folding. Another difference is the way in which the device is constructed. Rather than shaping each layer individually before stacking, a laser cutter is used after stacking instead. The cutting step during fabrication serves as a method for patterning the fluid channels. This is achieved by a combination of full-pass and partial cuts in the device, before peeling away the adhesive layer, leaving fluid channels on a non-wicking substrate.

Detection principles in paper diagnostics

Using PADs for the detection of a disease is a two-fold process that relies on the ability to report a specific recognition event. Both the reporting and recognition methods differ for various applications, and are discussed below.

Methods of reporting

Detecting the presence of an analyte is half the challenge. Accurate and successful diagnosis also requires a reliable and simple method of relaying the result to the user. Currently, most PADs report results visually; some have attempted to incorporate electronics into the reporting process. This section provides an overview of the development and issues of the main technologies.
Colorimetry and visual signals
Most PAD analyses rely on a visual change within the device detection zone to communicate results. Colorimetric analysis is by far the most common reporting technique described in PAD literature. It has also been a common approach in many laboratory-based testing procedures for decades. Colorimetric reporting consists of a visual color change that occurs after the addition of the sample, usually in the presence of the desired target. For example, the ELISA uses an immobilized biosensor on the substrate surface to capture a desired analyte, which induces a color change after binding with a second reporting enzyme, thus showing a positive result.

The appeal for colorimetric analysis on PADs is simplicity and applicability to POC situations. Other visual reporting methods, such as florescence or absorbance, require analytical instrumentation for accurate interpretation. Colorimetric assays in their simplest form can provide a qualitative binary result: whether or not a color change is observed. Paper is an ideal substrate for colorimetric analysis, due to the excellent contrast provided by the white background. Conversely, this white background and the ultraviolet (UV) brighteners present in paper (lignin residue and dyes) can interfere with fluorescence and absorbance reading methods, further supporting the use of colorimetric reporting. Smart phones with applications can also process data directly.

While colorimetric reporting is reliable and easy to implement on PADs, there are, however, a few drawbacks. Accurate interpretation, especially for quantitative analysis, may require trained personnel or analytical instruments. An alternative is telemedicine – using a communication device to interpret or transmit results to a specialized center for analysis before reporting the result back (Martinez et al. 2008a, Martinez et al. 2010a).

The concept of printing symbols or text has been demonstrated for qualitative results to contour user barriers. The concept behind the “writing” technique is a refinement and simplification of the previous method by Bodenhamer et al. (2000) which described a displacement assay for transparent packaging. A printed pattern of antigens is immobilized on the substrate and saturated by the corresponding antibody with a dye attached. The dye is then released from the surface when interacting with the antigenic target. The resultant disappearance of the printed pattern indicates the presence of the target, in this case a pathogenic antigen, which results from an undesired exposure event.

Reporting with electronics
While stand-alone paper diagnostics are ideal, many reporting methods rely heavily on colorimetric results. This requires interpretation by trained medical personnel. It has been suggested that μPADs be combined with mobile phones with camera capabilities for rapid and accurate interpretation. This is a field which has been dubbed telemedicine (Martinez et al. 2008a). It involves the image capture of the PAD, either by a scanner or camera, and then sending it via satellite for expert analysis. The expert would not be required on-site for interpretation, and the delay between when the test was taken and the time it takes for the results to reach the intended party would be minimalized. An alternative is to rely on downloadable applications for direct analysis by intelligent phones.

While colorimetric reporting is the most prevalent method for PAD analysis, electrochemical reporting methods have been explored (Liu and Crooks 2012). Colorimetric detection is adequate, but to discern an accurate result often requires trained personnel for interpretation. The use of telemedicine improves the situation; however it still relies on a person specifically qualified to correctly identify and diagnose the results. Using a metal/air battery powered biosensing platform could be promising to eliminate the need for a trained professional. The microfluidic paper electrochemical devices method could provide an easily readable result for non-trained users, by adding a user-friendly reporting method to paper devices.

Biorecognition in paper diagnostics
Accurate diagnosis of a disease is not solely reliant on the reporting method, but also the ability to selectively detect the presence of specific biomolecules, also known as biorecognition. The human body has an abundance of distinct analytes which can be used for the diagnosis of not only pathogenic diseases, but also physiological disorders. It all hinges on finding the correct biomolecular target and developing methodologies to detect its presence. Biomolecules, such as nucleic acids, enzyme proteins, and antibodies, can be immobilized onto paper, and can reportedly be dried without denaturation for this purpose. This process allows for storage and use in remote areas, without access to laboratory facilities, while retaining the thermal stability that is often lost with analyte solutions not properly stored at low temperature. This ability to immobilize biomolecular targets upon paper thus provides the other crucial half of the detection process. There are four main methods of biomolecule immobilization on paper: 1) physical, 2) chemical, 3) biochemical
couple, and 4) bioactive pigments; Pelton provided a detailed description of these techniques (Pelton 2009). A summary is provided in Table 4.

Perhaps the greatest challenge of achieving biorecognition is choosing the correct target that will detect a specific marker in the body. The job of this marker is to unequivocally detect the presence of a disease to avoid misdiagnosis of a patient. Whilst the principles of detection have been modeled after current diagnostic methods, the behavior of biomolecules on paper is not always congruent with the behavior exhibited in current laboratory methods.

There is no distinct advantage of a specific biomolecule over another, but is rather a function of the current methods of detection available. For example, utilizing the specificity of antibody-antigen interactions is desired for applications, such as blood typing, while recognition of a specific protein is required when diagnosing malaria. The type of biomolecular target will therefore differ from application to application.

Because of the wide array of types and properties of analytical targets, each must be examined separately to develop a robust detection method for the purpose of each device. The source of the analyte is an important factor in the diagnostic design. Samples can be primarily taken from urine, blood, and saliva for PADs, as they are already present in liquid form, but fecal matter is also a potential analytical source.

### Applications in health and medicine

While some μPADs have been designed for a specific purpose, other 2D and 3D platforms have focused on performing parallel or multiplex analysis for general bioassay applications. 3D systems have been explored for paper-based ELISA tests. Both 2D and 3D array formats have been investigated, including an emphasis on converting well-established techniques from plastic to paper. This section reviews and analyzes the important paper test designs and their specific medical applications.

### Clinical diagnostics

Although conventional 1D dipstick assays have been used for decades and represent a well-established example of a multiplex assay on paper (Section 3.1), the number of potential applications of PADs in biomedicine has grown rapidly in recent years. The following section outlines general applications, as well as some specific examples for physiological and pathological disorders.

#### Paper micro-zone plates

Many medical applications rely on paper micro-zone plates (Carrilho et al. 2009b). Conventional micro-zone plates are usually made from plastic and consist of 96 or 384 wells, in which analytes can be deposited. The result is then determined quantitatively via absorbance or fluorescence measurements using a micro-plate reader. Paper micro-zone plates can be functionally similar to its plastic predecessor, but with all the familiar benefits of using a paper substrate: cheap, easy storage and disposal, small volume requirements, etc. Like plastic plates, the paper version can be designed with 96 or 384 detection zones using photolithography. Many paper types were found suitable for the micro-zone plate format (Carrilho et al. 2009b). The main limiting factor was paper thickness. If the thickness was too great, the entire depth of the paper could not be properly hydrophobized. Paper thickness defines the required volume of samples and the capacity for the hydrophilic zones. It can be improved through plasma oxidation (Carrilho et al. 2009b). In comparison to the plastic micro-zone plates, paper showed a 40-fold increased sensitivity when read using fluorescence. However, under absorbance mode, detection was limited by light scattering caused by paper. The addition of mineral oil or similar liquids matching the refraction index of cellulose allows accurate measurements by absorbance (Carrilho et al. 2009b). The 2D format of the paper micro-zone plates was ideal for telemedicine reporting methods and can perform serial dilutions – a benefit unachievable with the 1D-μPAD design. A unique advantage paper has over plastic is the ability to perform serial concentrations. A sample can be added, and then a known volume of the solution can be

| Technique                  | Description                                                                 |
|----------------------------|-----------------------------------------------------------------------------|
| Physical immobilization    | Relies on van der Waals and electrostatic forces. Polymers can be used for bridging |
| Chemical immobilization    | Relies on covalent bonding                                                  |
| Biochemical coupling       | Relies on cellulose binding modules or bother biochemical binding agents    |
| Bioactive pigments         | Coats colloidal particles that and then printed or coated onto paper         |
evaporated, thus removing the excess liquid and increasing the sample concentration.

ELISA
The paper micro-zone plate format was incorporated into a 2D paper-based ELISA design (Cheng et al. 2010). ELISA tests typically use microtiter plates or small vials; it is one of the most commonly used assays for disease marker analysis. This paper-based design uses a 96-microzone paper plate printed with a 12×8 grid of circular detection zones (Cheng et al. 2010). This allows for multiple paper-ELISAs (P-ELISAs) to be run in parallel. Like the micro-zone plates, the P-ELISAs are compatible with existing equipment, such as 8- or 12-channel pipettes and plate readers. The design allows a washing step by leaving the top and bottom faces open to the atmosphere. The buffer is added to the top of the paper and blotted against the bottom. The perpendicular fluid flow removes any unbound reagents through the paper. However, the open bottom surface is impractical, requiring the paper be suspended in mid-air while the reagent solutions are initially added; this prevents the solutions from wicking through the test zones. A colorimetric reporting system is used, enabling the P-ELISA to be coupled with telemedicine if used in remote areas. However, the ability to perform a P-ELISA outside the laboratory environment is dubious.

There are three different methods for ELISAs: 1) indirect, 2) direct, and 3) sandwich ELISA. An indirect P-ELISA was demonstrated to detect rabbit IgG. Indirect P-ELISAs involve five steps (Cheng et al. 2010): 1) immobilization of antigens, 2) blocking of non-specific protein adsorption, 3) labeling the immobilized antigen with enzyme conjugated antibodies, 4) washing of unbound antibodies, and 5) the addition of a substrate solution for the enzyme. The results were proportional to the concentration of the rabbit IgG dilutions; however, the sensitivity decreased 10-fold compared to traditional ELISAs. This could be due to shorter incubation periods for the antibody-antigen interactions, or non-specific interactions between antibodies and the cellulose fibers. Despite this disadvantage, the P-ELISA still has benefits; it requires a shorter completion time, less analytical reagents, and can be quantified using a desktop scanner, thus retaining the appeal of a paper-based device. The test zone concentrations increase as the solution dries, enhancing the binding kinetics involved, however, the rate of evaporation is dependent on the surrounding environment, particularly relative humidity and temperature, and thus could affect the results (Cheng et al. 2010).

The P-ELISA can be integrated with printed electrodes to improve the LOD to a similar sensitivity observed with the colorimetric assays (Li et al. 2010c). Printed electrodes from graphite ink were used for an indirect P-ELISA and the voltage was measured periodically. Using this electrochemical method of detection produced an LOD similar to the conventional ELISA. However, the need for additional equipment and fabrication steps could negate the benefits of a more sensitive LOD.

Liu et al. (2011) also demonstrated an ELISA test using the 3D-µPAD design. The 3D design can test multiple analytes in parallel, which is applicable to ELISA analyses. However, the 3D-µPAD adds another level of complexity to the design. The vertical component allows for the fluid transport within the device to be controlled to fit multi-step assay requirements. The difficulty with duplicating conventional ELISAs on paper is the need for multiple distinct working steps. µPADs are mostly designed for a single-step process. The 3D design adds a movable delivery strip to allow multiple reagent delivery and washing steps without cross-contamination. The strip can be manually operated. Reagents are dried and stored on the paper substrate, removing the need for pipetting reagents and buffers. This eliminates the need for operator training. The test takes around 45 min to complete (Liu et al. 2011). The performance of the 3D-ELISA device was demonstrated using the indirect testing of rabbit IgG. The intensity of the colorimetric signals was proportional to the concentration of rabbit IgG, and had a five-fold decreased sensitivity compared to conventional testing, which is an improvement over the P-ELISA. Some clinical uses could accept the reduced sensitivity of paper ELISA; however, whether the sensitivity improvement over the colorimetric techniques justifies the increased cost and complexity is not clear.

Paper for sample preparation and storage
Rather than testing for a particular disease, paper has also been explored as a platform for preparing samples at POC. While paper for sample preparation has been mostly investigated for urine samples, it is applicable to any biofluid (Parker and Cubitt 1999). Parker and Cubitt (1999) developed a dried blood spot (DBS) device where blood samples could be taken, stored, and transported on paper (Parker and Cubitt 1999). The DBS card is an older concept and can collect blood samples using finger or heel pricks, which can then be reconstituted in the laboratory for examination. This is of interest for epidemiological studies in remote regions. Even when kept in conditions varying from cold storage to humid and tropical conditions, the samples were found to remain stable after many
years on paper. Additional benefits are the significantly reduced infection risk, ease of use with minimal training, the elimination of needles and syringes, and storage at ambient temperature, removing refrigerated storage and transport requirements. This method was investigated for the surveillance and detection method of HIV (Parker and Cubitt 1999, Ayele et al. 2007). The interaction between paper and blood is poorly understood, but is critical for improving blood sample aging and preservation.

Sample separation

Not all μPADs report using colorimetry, one of the simplest methods. An alternative strategy is to separate analytes for detection (Carvalhal et al. 2010a). Carvalhal et al. (2010a) reported an electrochemically-coupled device that separates the analytes, uric acid (UA) and ascorbic acid (AA), and amperometrically detects their presence (Carvalhal et al. 2010a,b). Much like a chromatography column, eluent wicks paper by capillary action to dissolve the sample. The solubility is dependent on ionic strength. At an optimal pH lying between the pKa of AA and UA, the AA becomes ionized and more soluble. The type, thickness, and length of the paper control separation efficiency. Electrodes (gold) can be printed on paper for detection. The paper-based device compared to typical high pressure liquid column (HPLC) instrumentation has a slightly longer run time, 16 min compared to 5 min; however, the benefits of paper could outweigh the extended test time. This method could be developed not only for clinical diagnostics, but also for forensics, agriculture, and environment applications.

A potential μPAD application is the separation of red blood cells (RBC) from untreated whole blood, and integration with a colorimetric diagnostic test (Yang et al. 2012). Yang et al. (2012) described a method utilizing RBC agglutination principles using antibody-A,B to form aggregates too large to flow through the porous structure of the substrate (e.g., chromatography paper). However, the plasma constituents can still flow through the pores to adjacent testing terminals functionalized with reagents. Colorimetric detection for various compositional properties of the plasma can be achieved. This principle was demonstrated for measuring glucose concentration in blood. However, this test did not account for the blood group O population.

Blood group typing

The accurate, rapid and reliable blood typing of human blood, particularly during medical emergencies, is important (Hillyer 2007, Daniels and Bromilow 2007, Contreras and Daniels 2010a,b). Mismatched blood typing can lead to a hemolytic transfusion reaction which can be fatal. Traditionally, an individual’s blood type is determined by detecting the presence or absence of antigens on the surface of their RBCs, as well as the antibodies present within the blood serum, more specifically the blood plasma. The most commonly used methods for blood typing include the slide test, tube test, micro-plate method, gel column agglutination systems, thin layer chromatography (TLC)-immunostaining, fiber optic-microfluidic device, and spin tube method, etc. (Harrenning 1999). The gel column test is the most prevalent in industry; however, it requires centrifugation, and each of these methods requires professionally trained personnel to be reliably analyzed. Developing a low-cost alternative for remote regions or home care which is user-friendly, portable, and equipment free, could provide a great alternative to the blood typing options currently available (Khan et al. 2010b).

In recent years, several studies have explored the possibility of using paper as a blood typing diagnostic tool (Khan et al. 2010b, Al-Tamimi et al. 2011, Li et al. 2012). Much like the traditional blood typing tests, the paper diagnostic also relies on the principles of hemagglutination.

When an aqueous analyte such as blood is tested using paper, the fluid is driven by capillary actions created by the structure of the cellulose fibers, wicking through the inter-fiber space of the paper (Khan et al. 2010b). This is known as capillarity microfluidics and allows for the passive transport of the analyte without any further equipment (Rivet et al. 2011). However, when blood agglutinates due to a specific antibody/antigen interaction, the resultant RBC complexes become too large to adequately transport through the paper structure and retain on paper; in the absence of agglutination, RBCs are easily removed by elution. This contrast in RBC retention or elution allows for the differentiation between when agglutination does occur (specific) and when it does not (non-specific) to directly report typing (Khan et al. 2010b). This has been the primary basis for the investigation of a paper-based diagnostic tool for blood typing.

Khan et al. (2010b) developed a method soaking paper strips in antibody solutions of either A, B or D antibodies. Droplets of blood were then added to the center of the paper strip and the resultant wicking behavior of the blood was observed. A distinct difference was discernible between agglutinated and non-agglutinated blood, the former presenting a chromaticographic separation of the RBCs and plasma (Figure 10A). When RBCs agglutinate with the corresponding antibody, very little wicking
is observed, while the plasma does wick along the paper strip. In contrast, non-agglutinated blood forms no separation along the paper strips, but travels uniformly, thus creating a distinction between a positive and negative result.

Al-Tamimi et al. (2011) next developed another paper-based diagnostic for blood typing using the same principles, but presenting two different testing methodologies. The first described an elution-based method using the chromatographic behavior of blood when added to paper (Figure 10B). This was achieved by spotting blood on the surface of the paper where known antibodies had previously been added. A TLC tank was used to elute the blood spot in saline buffer. The capillary action of the paper allowed for the buffer to travel through the paper structure, thus creating a chromatography-like test. Much like Khan et al. (2010b), the fixed agglutinated blood spots showed a positive result, while non-agglutinated elution paths of unbound RBC were negative. Visually, there was a distinct difference between the two results, using both the density of the blood spot and the presence or absence of an elution path to make an informed analysis.

A second method, known as the spot test (Figure 10C), also involved spotting the blood to stock antibodies upon the paper substrate; however they were not eluted in the TLC tank. Instead, saline solution was used to wash the spot directly by pipetting. Separation occurred by filtration through the paper thickness; RBC aggregated by matching antibodies remain on the top of paper, forming an intense red dot, while non-agglutinated cells (non-specific antibody) simply wash through paper and disappear. The results of both tests accurately detected the ABO and RhD blood groups of 100 samples, including four weak AB and four weak RhD samples, thus supporting the use of paper as a robust bio-diagnostic for blood typing. The effect of paper structure on blood typing performance was also investigated in a different study (Su et al. 2012).

Li et al. (2012) recently developed a text-reporting method. The detection principle is an adaptation of Al-Tamimi’s method (Al-Tamimi et al. 2011), relying on filtration through the paper thickness to retain RBC aggregated and coagulated by specific antibody interaction. By patterning the paper substrate with hydrophobic boundaries to surround a hydrophilic channel in the shape of text or symbols, “invisible writing” is present on the paper and dotted with antibodies. The addition of antibodies remains invisible to the naked eye until blood is added, when the shape of the channel is formed, for example an A or a B, and once washed with a saline solution – if a hemagglutination reaction has occurred – the resultant blood type will be directly printed on paper and can be “read” straight from the device (Figure 10D). This test successfully reported the ABO and RhD blood types of 99 samples including weak groups.

DNA extraction and detection

The genetic material of all living organisms is unique. There are well-established DNA detection techniques used for medical diagnosis of pathological diseases, such as the polymerase chain reaction (PCR) and rolling circle amplification (RCA) (Ali et al. 2009). While sensitive, these techniques require complex methodology and elaborate equipment infrastructure. Ali et al. (2009) investigated paper to fabricate a PAD capable of RCA, a technique used for DNA amplification and detection. Despite the well-established PCR technique for DNA amplification, RCA is more appealing as a PAD. It can be carried out under isothermal conditions, at room temperature, or 30°C, using a simple biochemical procedure involving Phi29 DNA polymerase. The Phi29 DNA polymerase enzyme can displace short DNA strands to amplify a DNA primer into long single-stranded DNA products. This increases the detection capabilities. Immobilization of a DNA oligonucleotide on strips of paper containing poly(N-isopropylacrylamide)
microgels creates a platform for which target DNA could ligate and undergo RCA-mediated amplification, enhancing DNA detection. The advantage of identifying a specific DNA sequence, rather than other biomarkers, is the specificity of the DNA itself. It provides an ultrasensitive diagnosis tool for disease. If achievable, it can be applied to any specific pathogen, including bacteria. However, preliminary results have identified a decreased sensitivity compared with other strategies; methods for improving sensitivity are being explored.

Govindarajan et al. (2011) used 2D-oPADs to demonstrate POC cell lysis and bacterial DNA extraction from raw viscous samples of Escherichia coli-spiked pig mucin. The sequential folding of 2D surfaces created temporary circuits, which dictated capillary flow without external pumping or power. The ability to detect DNA using 2D-oPADs can be used for diagnosis of tuberculosis. However, lysis of the Mycobacterium tuberculosis bacteria is difficult and requires further investigation.

Physiological disorders and analytes

The ability to detect a wide variety of physiological biomarkers can be used to develop low-cost bio-diagnostic platforms. Most initial PAD designs used glucose, pH, and protein detection to demonstrate the design’s validity for biomedical testing. However, more recently, specific applications of detecting other biomarkers for disorders, such as cancer and liver function, have also been used to show the functionalization of PDAs. Whilst the current research has only attempted these specific examples, the use of PDAs is not limited to the physiological disorders mentioned below.

Diabetes

One of the most common validation tests used for paper diagnostics is the detection of glucose in urine (Martinez et al. 2007, Fenton et al. 2008, Abe et al. 2008). In fact, dipstick diagnostics to detect glucose were the first functionalization application (Free et al. 1957). While diagnosis and detection of diabetes in patients is well established, paper diagnostics can provide an alternative testing method. The dipstick assay relies on enzymatic reactions of glucose with glucose oxidase to produce gluconic acid and hydrogen peroxide. The hydrogen peroxide would in turn react with orthotolidine which results in a colorimetric change to a deep blue color (Fenton et al. 2008). Later tests involved the addition of red dye and resulted in shades of purple at different intensities to estimate concentration.

Cancer markers

Recently, cancer biomarkers have gained interest as potential analytes for paper diagnostics. An example is a platform for miRNA detection, which does not require the use of equipment for lung cancer diagnosis (Yildiz et al. 2012). miRNA regulates the mRNA function in gene transcription. The miRNA assay detects the formation of duplex and triplex species which show unique and varying optical signals to the naked eye. An miRNA sequence specifically associated with lung cancer is mir21. Analyses showed a linear correlation between concentrations 10 nM to 10 mM. It is selective, displaying a purple color for the duplex and orange color for the triplex. While a good indicative process for lung cancer treatment, further testing is required prior to clinical testing, including an investigation into the sample pretreatment and a sensitivity analysis compared to current standard tests (Yildiz et al. 2012).

Liver function

Paper diagnostics have been considered to monitor liver function (Vella et al. 2012). Overmedication of patients can induce liver toxicity and can also lead to drug resistance, rendering the treatment ineffective. This commonly occurs in developing countries, since tests for monitoring liver function are expensive, and require trained equipment and personnel. Vella et al. (2012) developed a finger-prick paper diagnostic to measure the levels of two enzymatic markers which are indicative of liver function: alkaline phosphatase (ALP) and aspartate aminotransferase. Total serum protein can also be measured. The design comprises a patterned paper-chip, a filter, and self-adhesive laminating sheets. It accomplishes four primary functions: 1) separation of RBC from plasma, 2) distributing the plasma into three regions within the paper, 3) conducting three simultaneous colorimetric assays, and 4) displaying the results for quantitative analysis using tel- emedicine. The results reported were consistent with the colors seen in tests for all three analytes in artificial blood; however, a sensitivity analysis was not provided. Calibration curves were measured, but failed to take into account the background interference from using whole blood. Additionally, further investigation regarding stability in
warmer climates and selectivity from other potential biomarkers are required.

Pathogenic diseases

Paper diagnostics can immobilize analytes which selectively bind to biomarkers for the detection of pathogenic diseases. Such diseases include: malaria, HIV-1, hepatitis B virus (HBV), and hepatitis C virus (HCV). ELISA paper diagnostics, in particular, have great potential applications. The ability to use paper diagnostics for pathogenic detection is ideally suited for diagnosis in developing countries where equipment, resources, and trained personnel are scarce.

Dipstick assays can detect several pathogenic diseases, and these analyses can also be applied to other paper diagnostic designs (Dineva et al. 2005). Dineva et al. (2005) describe a dipstick test combined with multiplex reverse transcription PCR (RT-PCR) for the detection of HIV-1, HBV, and HBC. No 2D or 3D paper diagnostic prototypes for pathogenic detection have yet been commercialized; however, their applicability in the field has been demonstrated.

Malaria

Malaria is caused by the Plasmodium parasite, which is transmitted by infectious mosquito bites, displaying symptoms of fever, vomiting, and/or headaches (World Health Organization 2013). It is endemic in 104 countries, most of which are remote with humid weather conditions.

Successful identification of the disease can be achieved by detection of the Plasmodium falciparum histidine rich protein 2 found in malaria (Dineva et al. 2005, Fu et al. 2012). The 2D-PN described by Fu et al. (2012) demonstrated a fully automated system for detection using a sandwich format immunoassay. The 2D-PN diagnostic had two additional processing steps for rinsing and signal amplification using a gold enhancement reagent. The LOD for the amplified assay was similar to that reported for ELISA: 2.9±1.9 ng/ml and 4.0 ng/ml, respectively. This demonstrates the applicability of the automated 2D-PNs in a POC setting for malaria.

HIV-1

The retrovirus, HIV, infects cells within the immune system by destroying or impairing their function (World Health Organization 2013). The immune system is progressively weakened over time, until the patient becomes easily susceptible to infections. While there are antiretroviral drugs which slow down the disease, there is currently no cure for HIV-1. Efficient and accurate detection may not stop the illness, but early detection can help slow progression and patient deterioration, while preventing the spread of the disease.

The dipstick assay uses RT-PCR, which tests for specific nucleic acid sequences. For HIV-1, this means detection and identification of the HIV-1 genome (Dineva et al. 2005). However, the HIV-1 RNA must be prepared before testing. While the instrumentation is relatively inexpensive, it still limits application in remote areas and developing countries where equipment-free diagnostics are preferred.

While the dipstick device relied on signal amplification of nucleic acid hybridization, detection of HIV-1 provides an example for the applicability of the P-ELISA (Cheng et al. 2010). The HIV-1 envelope antigen gp41 successfully detected specific antibodies in human serum by using indirect P-ELISA methodology. Serum samples from HIV-1-positive patients were tested at different concentrations against control samples of human serum without anti-gp41. The colorimetric results indicated decreased intensity signals with serum dilution, but could still distinguish a positive result, thereby showing that a complex mixture, such as human serum, could be analyzed. Additionally, Ayele et al. (2007) described a method to collect and store samples for HIV-1 testing in field conditions.

Hepatitis B and C

Both hepatitis B and C (HBV and HCV, respectively) are viral infections transmitted through contact with blood or other bodily fluids, infecting the liver and causing hepatic diseases. Both vary in severity; however, HCV is known to potentially lead to liver cirrhosis or cancer. A vaccine for HBV is currently available but not for HCV (World Health Organization 2013).

Dineva et al. (2005) described a dipstick method to detect HBV DNA and HCV RNA. However, much like with HIV-1, the procedures and equipment used for preparation are not ideal for POC situations.

Successful HBV analysis showed the applicability of the 3D-ELISA paper device to detect HepB surface antigens (HBsAg) in rabbit serum. However, the protocol varied from the indirect ELISA methodology (Liu et al. 2011). A primary antibody was used combined with an ALP-conjugated secondary antibody to label the HBsAg. The additional steps were achievable thanks to the ease
of fabrication and flexibility of the μPADs. Serum samples of HBsAg-positive hosts were compared to serum controls without HBsAg, and showed that a 10-fold dilution was still discernible, thus supporting its potential for detecting hepatitis B and other infectious diseases.

Perspectives

There are a few critical issues to address relating to the development of paper diagnostics for biomedical applications. These include: 1) test sensitivity, 2) test robustness, 3) single versus multiple testing, 4) validity/reproducibility, and 5) simplicity of use.

Test sensitivity

Paper colorimetric tests can directly detect and visually report an analyte down to concentrations of $10^{-6}$ M (Li et al. 2010b). The detection limit is influenced by the wavelength (color) and intensity of the specific dye chemistry, as well as by the lighting conditions, type of paper, and moisture level/relative humidity. However, a concentration of $10^{-4}$ M might be insufficient for certain applications, such as early cancer detection, which might be required to detect analytes at a much lower range, possibly concentrations of $10^{-9}$ M to $10^{-12}$ M. For such applications, an amplification mechanism is required. Two amplification mechanisms have been tested for paper diagnostics. These include: 1) amplification by an enzyme producing a color product upon detection, such as ELISA, and 2) solid-light interaction principles. Mechanisms based on fluorescent dye are possible, but necessitate special instrumentation; quenching and interaction with paper can bring complications. The ELISA paper test, while possible and tested, requires multiple adsorption/washing steps that are cumbersome, time consuming, and delicate operations for standalone paper tests. ELISA for a paper test would only be realistic when a paper insert/test is used in conjunction with a robotized analytical plate reader apparatus.

An optical detection mechanism offers an alternative, with techniques such as surface plasmon resonance (SPR), surface enhanced Raman scattering (SERS), and similar techniques based on solid-light principles. These techniques usually rely on some light-surface interaction. SPR is widely used in biosensors to quantify the adsorption of molecules at the solid-liquid interface. SPR is the collective oscillation of valence electrons that occurs when light irradiates a surface, typically a metal such as gold or silver. SPR is very sensitive to the refractive index at the metal interphase (zone extending around 50 nm from the surface), thus providing a mechanism to measure adsorption of the small molecules widely used in lab-on-chip sensors. SPR of metal nanoparticles, especially gold (AuNP), yields visible color change upon molecule adsorption. Small and well-dispersed AuNPs (diameter of 10 nm–50 nm) show an intense red color with extinction coefficients which are much higher than those of common dyes, due to their localized SPR (Ngo et al. 2012). The surface of AuNPs can be tailored by ligand-functionalization to selectively bind biomarkers. The most general approaches are chemical functionalization and thiol-functionalization to detect specific binding of proteins or antibodies. Upon the addition of analyte, functionalized and well-dispersed AuNPs are induced into aggregates, which show a significant color shift from red to blue (Figure 11). This is due to interparticle plasmon coupling, which occurs as the surface plasmon of the individual AuNPs combine when their interparticle distance is smaller than their diameter. To achieve an efficient SPR performance on paper, a higher coverage of AgNPs is needed on the paper surface to enhance sensitivity of the nanoparticles-functionalized paper. The paper structure must maintain the adsorption state of nanoparticles (e.g., dispersed or aggregated) upon drying. To carry out ideal colorimetric detection, the activity of nanoparticles must be preserved during storage and remain functional upon rehydration for use. Furthermore, careful control of the size of nanoparticles and the porosity of paper is required, so that the nanoparticles will not be entrapped inside the pores within the paper structure, which would restrict the aggregation or dispersion of the nanoparticles and accessibility of target analyte.

Raman scattering is a light scattering technique in which Raman photons are scattered by interaction with vibrational and rotational transitions in a molecule. However, its sensitivity is limited because the Raman signal is very weak. The development of SERS has increased dramatically since Fleischmann et al. (1974)
published their discovery on enhanced Raman signals from pyridine on a rough silver electrode. This discovery of SERS has transformed Raman spectroscopy, from a structural analytical tool, to a sensitive single-molecule detection and nanoscale probe (Freeman et al. 1994). Intense SERS enhancement is often present at the point of contact between two or more metal nanoparticles (Michaels et al. 2000). As the metal nanoparticles are contacted to form aggregates, their transition dipoles couple to each other and the enhanced fields of each nanoparticle coherently interfere at their contact point. When molecules are adsorbed in this contact point – often called the hot spot – their Raman signals can be significantly enhanced \(10^{14} - 10^{15}\) (Toderas et al. 2007). Aggregates of nanoparticles have more efficient SERS properties than individual nanoparticles, because larger enhancements can be achieved at particle junctions of the aggregates.

Since the aggregation of nanoparticles plays an important role in enhancing the SERS signal, their adsorption and aggregation state within the paper structure is critical. Stability of nanoparticles on paper substrates must be preserved, so that they can be stored for long periods between measurements. Reproducibility of their aggregation state is another important factor to achieve accurate SERS results. Since the spot size of Raman’s laser beam is approximately 1 μm, an ideal SERS active substrate needs to have uniform distribution of nanoparticles on a sub-micrometer scale in order to achieve a high degree of reproducibility. This is a critical issue to address when paper is used as a SERS substrate, since it is a challenge to achieve uniform distribution of nanoparticles on paper, due to its high roughness and porosity. In addition, because of the high sensitivity of SERS, the paper substrates must be free of additives or fillers, to avoid any background or fluorescence interference during the analysis. Figure 12 illustrates the different length scales involved in SERS analysis with AuNP-treated paper.

SERS using AuNPs is an exceptional technique for quantifying the adsorption of target molecules on substrates, and allowing different orientations and interactions of the molecules with the substrates to be determined (Wu and Fang 2003). Single molecule detection is achievable via SERS and this concept has been used to design protein and nucleic acid biosensors (Bizzarri and Cannistraro 2007). A highly SERS-active substrate is the most important factor in producing efficient SERS applications. Previously, aqueous metal colloids were employed in most SERS techniques, but this limits the application of SERS, since specimens analyzed must be water-soluble. Filter paper coated with AuNPs offers a much simpler method and the compounds examined do not have to be water-soluble. Ngo et al. investigated the effect of AuNP-treated paper as a generic platform for SERS detection and quantification of analytes (Ngo et al. 2012, 2013a,b). The effects of AuNP surface coverage, aggregation size, and distribution on paper on the efficiency of SERS were quantified (Ngo et al. 2012, 2013a). Methods of producing AuNP paper for SERS application were optimized. Not all analytes are SERS-sensitive; a better understanding of the SERS efficiency as a function of the analyte molecular structure is needed.

**Test robustness**

The proper life time of paper diagnostics is a major issue. This requires both the microfluidics and the bioanalytical detection system to be stable for at least 6 months, or better, 1 year or more, to ensure proper time for commercialization and distribution. A critical requirement is for the adsorbed biomolecules to be stable and to remain bioactive over paper. Enzymes, antibodies, and antigens are of special interest for biorecognition in diagnostics. To be practical, paper diagnostics must be stored dry until use.

Khan and Garnier measured the stability and the kinetics of enzyme immobilized on paper with and without polymers used as retention aids (Khan and Garnier 2013). The stability of ALP and horseradish

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**Figure 12**: Different length scales of the constituents of gold nanoparticle (AuNP)-treated paper for surface enhanced Raman scattering (SERS) application (Ngo et al. 2011).
peroxidase adsorbed on paper was found to be two to three orders of magnitude higher than in solution, but also two to three orders of magnitude slower (Khan et al. 2010c). Khan et al. (2010c,d) also modeled the thermal deactivation of ALP on paper to predict enzyme activity. Heat deactivates and denatures enzymes by modifying their conformation, due to increased thermal movement and decreased solvent stabilization (Khan et al. 2010c,d). The idea that immobilization of an enzyme on paper prevents aggregation and retards the conformation disorder, by stabilizing the secondary and tertiary structures of the enzyme, was studied. The effects of aging time and temperature on the relative activity of ALP enzymatic papers, with and without polymer treatment, are illustrated in Figure 13. The enzymatic activity quickly dropped within the initial hours of thermal treatment and then gradually decreased at a slower rate. The enzyme deactivation was faster at higher temperatures. The deactivation rates of ALP enzymatic papers treated with polymers were much faster. Zhang and Rochefort (2010, 2011) and Rochefort et al. (2008) investigated enzyme microencapsulation to protect biomolecules. Enzymes microencapsulated in cross-linked PEI and coated on paper using standard technology displayed a marked improvement in stability without losing selectivity (Zhang and Rochefort 2010, 2011, Rochefort et al. 2008). A better understanding on the aging of biomolecules, such as antibodies and antigens adsorbed or immobilized on paper, is required.

**Test design and application**

An important issue is the validity of the test and understanding how design affects the detection threshold and its effect on diagnostics and treatment. A consequence of increasing sensitivity can be the increase in the occurrence of false positive tests. This can be achieved by optimizing the antibody conditions to react with an antigen. A lack of selectivity can also create false positives tests. The opposite is a false negative test, which can result either from too low sensitivity, i.e., level of detection, or from the lack of recognition of the analytical biomolecule. The lack of recognition can be due to poor binding specificity, but also from weak or partial antibody/antigen. Examples are the weak D and partial D antigen on the RBC recognition in blood typing. For blood typing tests, false positive and false negative results can lead to a fatal hemolytic transfusion reaction. A false negative can result in the transfusion of a donor with blood A, B, or D to a recipient of blood O-; conversely, a false positive can cause a O- recipient to be incorrectly identified as A, B, or D, resulting in the same crisis upon improper transfusion. For certain

![Figure 13](image_url)

**Figure 13** Effect of time and temperature on the relative activity of enzymatic papers. Alkaline phosphatase (ALP) stabilized on paper using: (A) ALP paper without polymer; (B) ALP cationic polyacrylamide (CPAM); (C) polycrylic acid (PAA); and (D) polyethylene oxide (PEO) papers. (Khan et al. 2010c,d)
applications, such as blood typing of primary and secondary groups, total accuracy and the absence of any false positive/negative results are imperative. For other applications, in which a paper test is used as the first detection line, reproducibility/sensitivity can be traded for simplicity and low cost, allowing wider and more frequent public use. Unfortunately, very few studies have analyzed the issue of false positive/negative results in diagnostics and their potential effects.

Simple detection on/off binary tests identify the presence or absence of a critical analyte. The main advantage of these tests is their simplicity and ease of use and interpretation. The best example is the pregnancy test; the patient is either pregnant or not. Of interest is the ability to control the critical analyte concentration at which a “positive” is communicated. For example, cholesterol and prostate specific antigen are becoming health issues only over a certain concentration. A binary test only reporting concentrations above these critical thresholds would be very useful for home testing.

Significant research effort has been dedicated for multiple paper test use, as it had been identified as a major weakness. But is that so? The higher the test complexity, the higher the price and the lower the robustness, ease of use, and interpretation. At the limit, these tests become non-competitive relative to the traditional tests and methods they were designed to replace in the first instance. Research progress has demonstrated that paper tests can be designed to provide alternatives to most diagnostics needed. However, paper diagnostics will make sense and be successful only should they provide a significantly cost saving and simplicity over their targeted replacement. Research prowess and high level publication is a facet of paper diagnostics; public acceptance and commercialization is another. These should not be confused.

Conclusion

The development of paper diagnostics analytical devices (PADs) for medical applications has exploded in recent years. This is because paper is cheap, widely available, easily engineered, disposable, sterilizable, hydrophilic, and easy to functionalize and process into diagnostic devices. Paper is a very attractive substrate to develop a generic low-cost diagnostic platform. Paper can serve four functions in a diagnostic: 1) transport and measurement of samples and analytes, 2) reaction support, 3) separation of reactants from products, and 4) communication of results. A plethora of manuscripts present new concepts of rapid/instantaneous medical analytical tests and analyzing the performance of PADs. Many ingenious and sometimes complex 1D, 2D, and even 3D flow prototype diagnostics have been developed. Printing has emerged as the manufacturing technique of choice for PADs, first by printing microfluidic systems on paper and then the reagents and biomolecules. From literature, paper diagnostics have become ubiquitous for medical applications; but surprisingly, very few commercial applications exist.

This article has attempted to provide a roadmap, by reviewing the different types of paper tests, their principles, and medical applications and has analyzed their performances and limitations. Paper tests for medical applications must meet two requirements: very low cost and ease of use. The challenge is to ensure the selectivity and sensitivity of the diagnostics, while maintaining the simplicity and affordability that paper diagnostics attracts. The need to create more complex PADs to suit multi-step processes has often hindered the ability for many designs to be applicable in the field and commercialized. Additionally, each biomedical application differs in its own right, making it impracticable to design a single PAD to suit multiple diseases and disorders. Development is needed to reconcile the potential of very low cost, meaningful, and robust paper tests for specific medical analyses with increased sensitivity, flexibility, and reliability of the concept.

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Gil Garnier has been Director of the Australian Pulp and Paper Institute (APPI) and Professor in the Department of Chemical Engineering at Monash University, Australia, since 2005. He recently founded the Bioresource Processing Research Institute of Australia (BioPRIA). Previously, Dr. Garnier was a Senior Research Scientist and team leader at Kimberly-Clark for 5 years. For 8 years, he held the dual position of Research Engineer at the Pulp and Paper Research Institute of Canada (Paprican) and Adjunct Professor in the Chemical Engineering Department of McGill University. His expertise includes the application of (bio)polymers and (bio)colloids to surface engineering and papermaking. His current research is on bioactive paper, nanocellulose applications, and biorefining.