Chapter 7
Paper Microfluidics

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1 Introduction

Low cost, paper-based point-of-care diagnostics, often also referred as, paper microfluidics and paper lab-on-a-chip is a new, emerging class of microfluidics devices. This chapter summarizes recent advances in the field of paper microfluidics, describes the underlying physical concepts, design and fabrication guidelines, and presents various examples of application of this technology. In this chapter, we mainly focused on the works published in the period 2010–2016. We apologize in advance if any original works and reviews were not mentioned or overlooked due to the large amount of material that has been published in this topic.

Multiple reviews have been published: reviews giving overview over all aspects of paper devices [1–5], reviews which are mainly focused on paper-based biosensors with EC detection [6], functionality of paper devices [7], detection [7, 8], chemical measurements using microfluidic paper-based analytical device (μPAD) [9], bio-sensing techniques and, specifically, use of nanoparticles [10], working principles and reaction mechanisms [11], history of development [12], thorough review of properties of paper material [13], paper devices for biomarkers and bacterial detection [14], infectious disease [15], paper devices in resource-limited settings for diagnostics and education [16].

Traditionally paper microfluidics has been seen as a low cost diagnostic tool for the developing world. There are still big hopes associated with this technology, mainly that it can help people in low and middle-income countries in areas such as public health, environmental monitoring, agriculture, water and food safety and many others [17]. At the moment, according to the World Bank data, more than 5 billion people are living in the areas that can be characterized as low and middle-

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income. These people might live in low-resource, hard-to-reach areas with a very limited access to primary healthcare and nearby medical institutions. Access to affordable diagnostic tools can be a very positive change in these communities. Another aspect of the same problem: people might lack basic medical education in these areas. If diagnostic tools are easy to use and interpret, many people would be able to apply them to real life problems it e.g. a community health officer at a remote rural village with limited medical education and not only highly educated practitioners could do the initial diagnostic screenings. With time, when reaching technological maturity, this type of technology could be very useful for rapid diagnostics of e.g. non-communicable diseases (e.g. sickle cell, or daily monitoring of blood glucose levels in diabetic patients), but also for communicable (infectious) disease diagnosis (e.g. malaria or ebola). Monitoring quality of drinking water is another application area that still needs attention worldwide, as some regional discrepancies as well as differences between rural and urban regions persist. According to the World Health Organization, 8 out of 10 people living in rural areas do not have access to high quality, clean drinking water sources. Integration of low cost solutions for water quality monitoring would have positive economic effect and improve well-being of people in those regions. Managing the above-mentioned challenges will be more important over the years as the world population will continue to grow. These ideas are few of the core motivating factors behind the development of paper-based microfluidic diagnostic devices.

As the research community on this technology rapidly developed, the field also started receiving a commercial attention as well as interest from non-profit organizations. This is seen following the numerous patents filed in this area in recent times with a few examples referred to here [18–20]. DFA (Diagnostics for All), for example, is a non-profit organization aiming to deliver low-cost, easy-to-use, point-of-care diagnostic devices designed specifically for the developing world [21], and is one of the successful examples of bringing this technology into use. If paper-based devices will be able to overcome such shortcomings of the lateral flow based devices as lack of quantification, specificity and sensitivity, they may potentially revolutionize the field of low cost diagnostics.

The paper microfluidics device works by wicking liquid sample between separate compartments containing assay reagents. The device may consist solely of patterned chromatography paper or may combine several other materials (e.g. polymers, conductive materials, functional nanoparticles, etc.) used in point-of-care. Typically, a paper device has hydrophobic patterns, and hydrophilic areas which are used as chambers and channels, and performing various kinds of fluidic operations. If, for example, a colorimetric assay has been adapted, the result can be read by a phone, or photographed and sent for further analysis to a centralized lab, where a specialized medical practitioner will be making an informed judgement. Time duration of assays on paper is mainly limited by the time needed to wick paper channels of given dimensions and design and by the inherit properties of the assay itself, and typically is within 30 min, which is well sufficient for many analytical applications. Some applications are particularly suitable for paper diagnostics. These are situations where e.g. qualitative results

are of primary interests, the analytes are fairly easy to obtain, some device-to-device variations due to fabrication processes are acceptable and assays can be easily transferred for use on paper substrates [22].

Paper-based devices are so attractive due to these factors:

- Low cost,
- Operating on low volumes,
- Abundant supply,
- Easy to construct devices,
- Suitable for multiplexing, thus multiple samples can be analyzed simultaneously,
- Reagent storage in dry form,
- Facile interpretation of the results (produced signal can be read by eye),
- Do not require additional instrumentation for liquid transport (without pump),
- Can be designed not to require additional instrumentation to read test results [23],
- Possible to combine with portable readout systems [24, 25], smart phones [26–28]
- Highly-skilled staff is not required,
- Light-weight material,
- Does not produce bio-hazardous waste,
- Devices can be mass-produced,
- Portable format of devices, and many others.

Factors that are often mentioned in a positive context can be, however, seen as drawbacks when applied in different settings. In fact, this depends on the specifications to device and the application area.

The following drawbacks are often mentioned:

- Limited sensitivity of many assays,
- Lack of quantitation in existing assay formats,
- Limited number of detection methods can be applied, i.e. colorimetric, electrochemical,
- Often not self-sustained, i.e. to be able to obtain quantitative results paper-based devices need to be integrated with external read-out system, which increases the costs and complexity [22].

Significant progress has been made in increasing functionality of paper-based fluidic devices. At the moment, many fluidic operations are possible including fluidic timers, valves, sophisticated detection units and even power sources (batteries)—therefore, potentially it is possible to design complex assays in single paper-based device. If, some of the promising design concepts could be mass-produced in paper at a price comparable to commercially available rapid tests, they may be commercialized in the near future.

2 Application Areas

The idea of low-cost microfluidics responding to global public health needs has been around before paper microfluidics started developing as an individual field, and, undoubtedly, there was and still is an actual need for monitoring devices used in resource-poor settings [29]. The world Health Organization (WHO) provides the guidelines ("ASSURED" concept) to how devices should be better suitable for being used in developing countries. Paper devices meet such requirements as being affordable, sensitive (sufficient sensitivity for some applications), specific, user-friendly, rapid and robust, equipment-free, portable and target-delivered (to those who in need of the technology) [1]. Essentially, paper devices can be applied to analysis of various bodily fluids and their synthetic substitutes (whole blood [30], human serum [31, 32], artificial serum, synthetic urine, urine, saliva). Detection of a variety of biomarkers has been demonstrated i.e. uric acid and glucose [24, 31], cholesterol [33], simultaneous detection of glucose, lactate and uric acid in urine, ketones [34], salivary nitrite [34], proteins [34], lactate [35], triglyceride [36], nitrates [34].

An overview of the history of the development of this field can be found elsewhere [1]. An important step in growing awareness of this technology was introduction of paper-based ELISA and the first demonstration of the colorimetric glucose assay on paper, which is still used by many researchers as a model application system [27, 37].

While a killer-application, that is only possible on paper, is still missing, a wide range of interesting applications has been reported:

- Medical diagnostics

    Paper fluidics can be successfully implemented in medical diagnostics. Especially it is useful for systematic, routine diagnosis, analysis of patients' samples in places distant from hospitals, analysis of asymptomatic diseases, and evaluation of disease progression [1]. Examples include early cancer detection [38] using multiple biomarkers, among those are r-fetoprotein (AFP), carcinoma antigen 125 (CA125), carcinoma antigen 199 (CA199) and carcinoembryonic antigen (CEA) [32], isolation of extracellular vesicles [39], blood typing [30, 40], drug monitoring (example of induced liver failure [41]), diagnostics of non-communicable diseases (including cardiovascular disease and cancer) [42].

- Veterinary diagnostics

    This application area mainly addresses infectious and viral diseases passed from animals to humans. A wide array of communicable diseases can be transmitted from livestock or wildlife to humans in various ways. Some of these disease can potentially become pandemics [1].

- Food safety and control, agricultural field

    Determination of toxic agents with main examples including salmonellosis and campylobacteriosis infections (via eggs, poultry, and unpasteurised milk), enterohaemorrhagic Escherichia coli (O157:H7 serotype) and cholera

(via water, rice, vegetables, seafood) [1], water analysis by electronic tongue device [43], detection of pesticides [44, 45], foods analysis e.g. wine and beer [46].

- Environmental monitoring

  Today, very few developing countries have adapted testing routines for water supplies in rural regions. Contamination due to industrial and agricultural activities, as well as environmental pollution monitoring is still challenging and needs to be tackled. Examples of monitoring of these factors include detection of heavy metals [47], low cost monitoring of environmental pollutants in air [48], measuring the metals (Fe, Cu, Ni, and Cr) content in welding fumes [49, 50], biosensor for organic pollutants in water (e.g. L-DOPA and catechol [51]), bacterial detection (Salmonella and Escherichia Coli) [28].

- Energy storage and generation

  In fact, paper as a substrate has entered other fields, besides traditional point-of-care diagnostics. Batteries and other types of energy storage devices can be constructed based on paper [52], fuel cell harvesting electricity from bacterial metabolism [53], devices generating power when liquid sample is applied [54].

- Pharmaceuticals

  Detection of pharmaceuticals has been demonstrated on paper [55].

- Forensics

  Detection of explosives is a new area of application [56].

3 Physical Principles

The ability to control the flow rates of liquids in paper channels is necessary for successful operation of the device. This section gives an overview of basic physical principles behind flow in paper. For more details, one can refer to the informative articles on this topic [57, 58].

3.1 Flow Through Paper

Darcy's law describing flow through porous media is the basis for estimation of liquid flow through a paper channel. For the constant width of channels fluid flow can be expressed as

$$Q = -\frac{kWH}{\eta L}\Delta P$$

Where Q is the volumetric flow rate, k—permeability of the paper, η—viscosity, WH—area of the channel perpendicular to flow, ΔP—pressure difference over the length of channel L.

When the width of channel is varied:

$$Q = -\frac{\Delta P}{\left(\frac{\forall}{k}\sum_{i=1}^{N}\frac{L_i}{W_iH_i}\right)}$$

W_iH_i is the area perpendicular to flow, L_i is the length in the direction of flow, and ΔP is the pressure difference across the length of the channel.

Using parallels between fluidic and electrical resistance, i.e. liquid flow Qi is an equivalent for current, ΔPi pressure drop along the channel is equivalent to voltage drop, and $\mu Li/(kWiHi)$ is an equivalent to fluidic resistance of each individual channels, one can estimate total flux. If several fluidic elements are connected in series or in parallel, the total flux through this network will follow analogy of Ohm's law, i.e. sum of individual fluxes when connected in series, and reciprocals when connected in parallel.

3.2 Spreading of Wax and Width of Patterned Channel

Spreading of the molten wax and width of final channels can be predicted using the Washburn equation:

$$L = \sqrt{\frac{\gamma Dt}{4\eta}}$$

Where L—distance covered by the wax front, η—viscosity (function of time and temperature to which device was exposed during bake), γ—effective surface tension, D—average pore diameter, t—time. The same equation can be used to predict transport of the fluid front in the channel.

The final inner width of the channel formed by wax can be defined as

$W_C = W_P - 2L$, where

W_C—inner width of the hydrophobic channel, W_P—inner width of the printed channel, L—the additional distance that the wax spreads perpendicular to the length of the channel. L is a function of time, heat and the structural properties of paper.

3.3 Transport Time

Transport time through a multi-segment geometry can be calculated using the modified Darcy's law equation:

$$t = \frac{VR_{eq}}{\Delta P}$$

Where V is the volume of the geometry, ΔP is pressure difference and R_{eq} expressed as

$$R_{eq} = \frac{\mu}{k} \sum_{i=1}^{N} \frac{L_i}{W_i H_i}$$

If we assume that permeability, viscosity, and pressure difference are constant, differences in the transport times in two channels would be only due to geometrical differences

$$t = \frac{\mu}{k\Delta P} L^2$$

This means that all channels of the same length would have the same transport time regardless to other geometrical dimensions.

Of course, in reality, channels often have several sections characterized by different length and width. For a channel that has two sections, both characterized by constant length and width (and same heights), the equation for transport can be modified to

$$t = \frac{\mu}{k\Delta P} (L_1 W_1 + L_2 W_2) \left(\frac{L_1}{W_1} + \frac{L_2}{W_2} \right)$$

3.4 Signal Visibility

The visibility of the signal is a function of the thickness and the opacity of paper:

$$\% \, Visible \, signal = \frac{Visible \, depth}{Thickness}$$

4 Main Formats of Paper Devices

Many design concepts in paper microfluidics are inspired by bio-sensing principles and structure of the lateral rapid tests and dipsticks [59]. Examples of the commercial point-of-care tests are shown in Fig. 7.1. If focus in lateral flow assays and dipsticks was their robustness and ease of interpretation (often yes/no answer), paper microfluidics explored various ways to enhance the functionality, increase design complexity while still trying to keep the costs down. Current status on fluidic operations in paper can be found elsewhere [60]. Design concepts of paper fluidic devices are constantly under development. Currently several groups succeeded in integration of sample preparation with simultaneous detection of a biomarker of interest using the same paper device [61].

Transport of liquid through paper is equivalent to transport of liquid through the porous media. It occurs as soon as paper is brought in contact with liquid reagents and therefore has to be confined and directed. To gain some understanding on

Fig. 7.1 Commercial
point-of-care tests. (**a**)
DetermineTM HIV 1/2
Ag/Ab Combo, (**b**)
DetermineTM TB LAM Ag
test, (**c**) QuickVue Influenza
A + B test, (**d**) Clearview1
Malaria P.f. Test, (**e**)
DirectigenTM EZ Flu
A + B, (**f**) ICON HP (**g**)
ImmunoCard STAT!1
E. coli O157 Plus, (**h**) A
multiplex lateral-flow
assay. RAIDTM 5 for the
determination of biological
threat agents. Adapted
from [1] with permission
of Royal Society of
Chemistry

correlation between the shape of channels and liquid transport, one can also refer to
[58]. Most of the channels have rectangular shape, but there is also experimental
work with channels with varied width [58]. Typical width of the hydrophilic
channel is 0.5–4 mm, hydrophobic barrier can be about 1 mm.

All paper devices can be classified by these three main formats:

- 2D format for paper networks

  Two-dimensional paper networks were the first microfluidic designs. A few
typical examples are shown in Fig. 7.2. These are mostly channels fully made
out of paper, which can either be cut or patterned with a liquid-repelling material
to a required shape. The channels may vary in width, length, may contain several
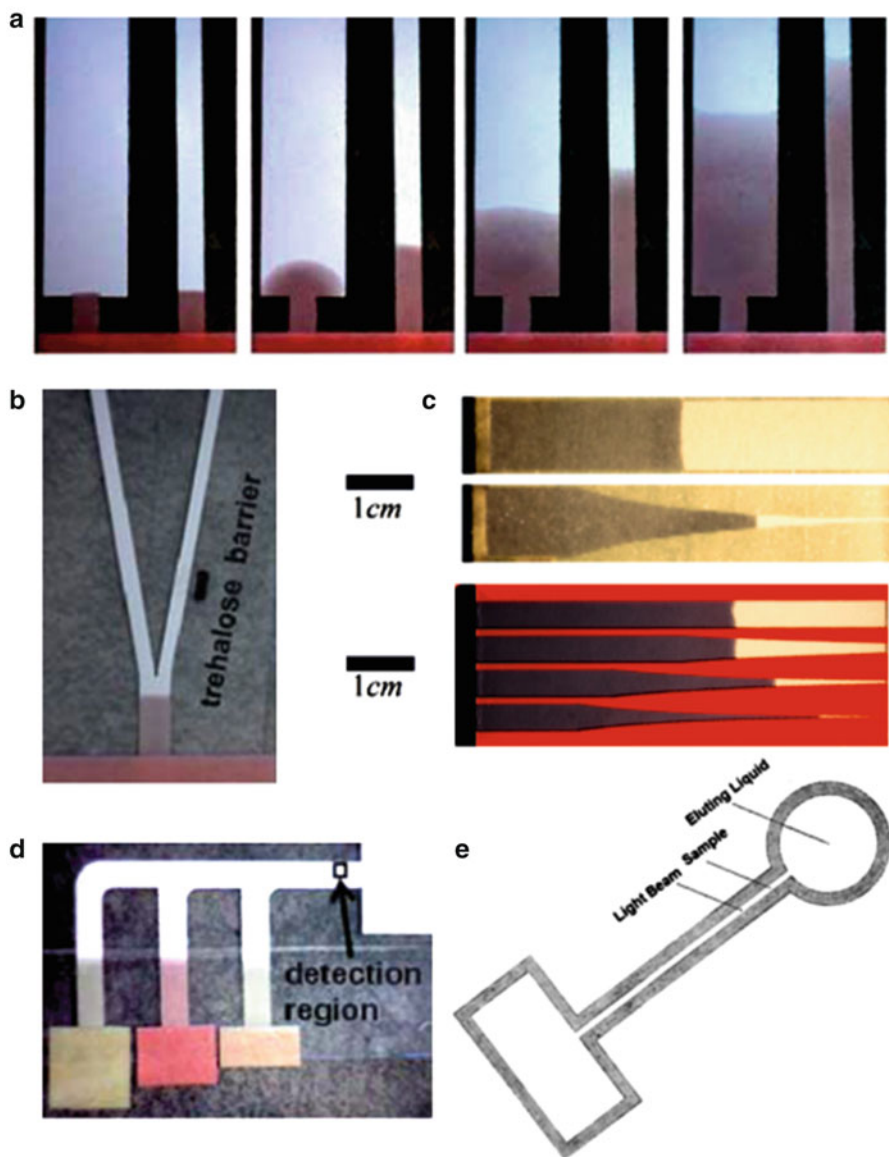
Fig. 7.2 2D paper channels of various designs: (**a**) channels of different width (liquid introduced via the same inlet of the same width) shows faster transport in the strip of smaller width. Images were taken at 2, 10, 50, and 210 s after introduction of fluid to the inlet (Adapted with permission [65] Royal Society of Chemistry); (**b**) Y-shaped channel with dissolvable barrier can be used to create a delay in the transport time of a fluid along the channel (Adapted with permission [65] Royal Society of Chemistry), (**c**) Slit-shaped channels and correponding differences in liquid transport (Adapted with permission [58] Royal Society of Chemistry); (**d**) A simple 2D paper network. The arrival time of multiple reagents at the "detection region" of the paper strip is staggered by placing 3 inlets along the common segment of the device. The geometry of the inlets results in the sequential arrival of fluid from each of the three inlets. (Adapted with permission [65] Royal Society of Chemistry); (**e**) One of the first paper microfluidic designs with inlet, transport channel and reaction zone. (Adapted from [1] with permission of Royal Society of Chemistry)

segments varying in size for storage of liquid and dry reagents. Modifying channel length and width allows controlling time and spatial distribution of reagents and samples.

To prevent contamination between individual fluidic compartments hydrophobic barriers, absorbing pads or physical separation of reacting zones can be applied. Flow in these channels follows the relationships discussed in the previous sections. Some of the early publications discuss various scenarios of liquid transport in 2D networks such as: Y-shaped and T-shaped devices, structures for hydrodynamic focusing, size-based separation, mixing and dilution [57, 62–65], hydrophobic barriers for time-controlled transport of liquid reagents [66]. The majority of the newly published works are using this design principle probably due to its simplicity.

- 3D format for paper networks,

  Three-dimensionality was an important step in developing device complexity. In these devices, liquid transport occurs in both vertical and lateral directions [32, 67–71]. 3D devices, shown in Figs. 7.3 and 7.4, have two main advantages (1) better suitable for multiplexing, a higher number of tests can be simultaneously integrated; (2) more complex assays can be integrated as three-dimensionality (liquid flow can now be controlled in both lateral x-y and vertical z directions) allows for more complex fluidic operations and more suitable for multi-step assays[72, 73]. Since liquid flow can be transported vertically, the distribution times between various reaction zones can be significantly reduced, so the required sample volume.

- Centrifugal, i.e. paper-disc format,

  Flow in 2D and 3D devices are governed by capillary force. Centrifugal paper-based systems are operating on principles of interplay of centrifugal and capillary forces [36, 74, 75]. This adds more possibilities to time and spatial time control as reagents can be recirculated within the same channel multiple times and the flow rate through the paper can be well controlled. Compared to this, in 2D and 3D networks, the flow through channel is typically constant and reverse flow is not possible. Two examples of centrifugal systems are shown in Fig. 7.5.

- Various hybrid formats, that cannot be strictly assigned to either of the above mentioned categories.

  Combination of paper devices with other materials and the advances in other areas of microfluidic research gave rise to some interesting hybrid device concepts. Various efforts for hybrid integration of electrodes into paper has been demonstrated: electrical circuit used for electrical readout can be attached externally [53], functionalized paper can be placed on top of screen-printed electrode [76], or electrodes can be incorporated as a part of the fluidic network directly in paper [43, 77]. Since patterning of electrodes on paper or hybrid integration with external devices are relatively established process, paper microfluidics can be combined with other areas such as e.g. digital microfluidics, which allows development of some complex assays [78]. These areas can potentially profit strongly from each other as low power and flexibility of operational control of digital microfluidics can be well combined with suitability
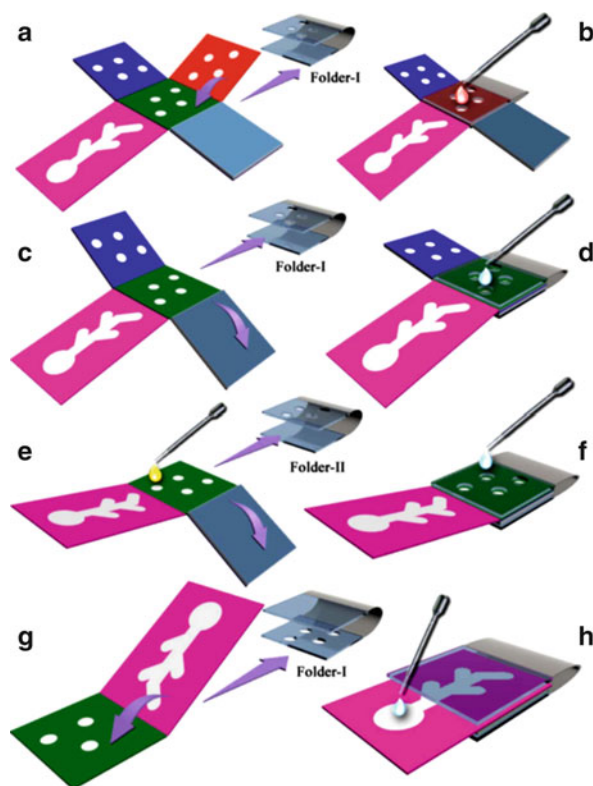
Fig. 7.3 Example of an assay based on 3D origami immunodevice. (**a**) The filter tab was folded above the test pad; (**b**) the blood sample was added into each paper microzone on the folded filter tab with the aid of Folder-I; (**c**) the filter tab was removed and one waste tab was folded below the test pad; (**d**) the washing buffer was added into each immunozone on the test pad to wash the immunozones with the aid of the used Folder-I; (**e**) the used waste tab was removed and a solution of AgNPs-luminol/Ab2 was added into each immunozone on the test pad; (**f**) the remaining waste tab was folded below the reversed test pad and the device was washed again with the aid of Folder-II; (**g**) the used waste tab was removed and the reagent tab was folded above the test pad; (**h**) the hydrogen peroxide solution was added into the reagent cell to trigger the CL reactions with the aid of a new Folder-I. Reproduced from [72] with permission of from Royal Society of Chemistry

for rapid prototyping of paper fluidics. Fabrication of electrodes for digital microfluidic devices in a classic clean room would be significantly more expensive. Another example is a combination of paper with hydrogels, where hydrogels are used for liquid storage and upon application of external stimulus release liquid into paper [79]. Sugars were applied to paper for programmable time-delays, that can be used for sequential reagent delivery [80].

Fig. 7.4 Example of a process flow for fabrication of 3D microPADs using a toner. (**a**) Schematic of the four steps of device fabrication. (**b**) The *top* of the device after addition of aqueous dyes to the sample inlets and wicking across the channels for 2 min. (**c**) The *bottom* of the device after addition of aqueous dyes. (**d**) Cross-section of the device showing the layers of paper and toner. The toner is used for bonding of the layers of paper together, it prevents fluids in channels from mixing. Ports in the toner layer allow wicking between layers of paper. Reproduced from [73] with permission of Royal Society of Chemistry
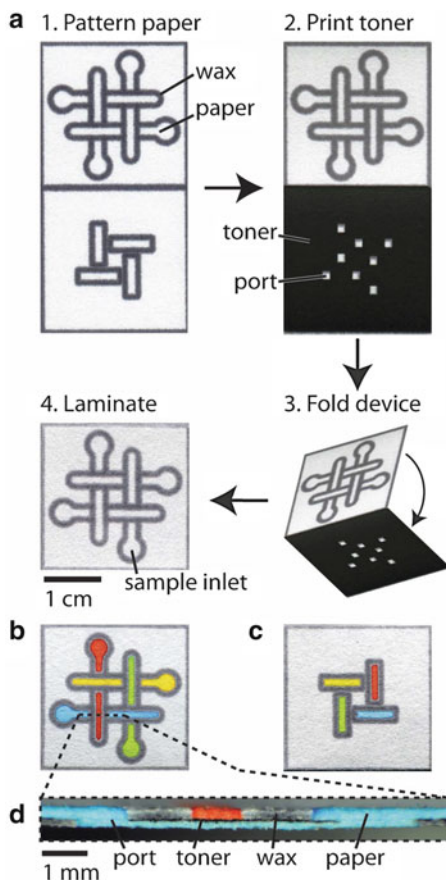
5   Types of Paper and Its Functionalization

Traditionally, rapid point-of-care tests use nitrocellulose membranes (e.g. membranes available from Millipore). The term "membrane" is exclusively used to describe nitrocellulose in the lateral flow format [1]. Nitrocellulose-based technologies are in close relationship to paper microfluidic devices. Attempts to replace nitrocellulose in the diagnostics industry with other materials (nylon, polyvinylidene fluoride and Fusion 5 from Whatman) were not successful due to high-costs, manufacturing challenges and need for additional optimization of chemistry. In order to replace current manufacturing practices, to which industries are typically reluctant, paper-based devices have to compete in manufacturing price, signal-to-noise ratio, robustness and functionality. The term "paper" refers to cellulosic materials (i.e. filter paper and chromatography paper used in microfluidic devices). 90 % of paper is produced from wood stock, however for diagnostic purposes a paper from cotton is desired (to eliminate interferences from
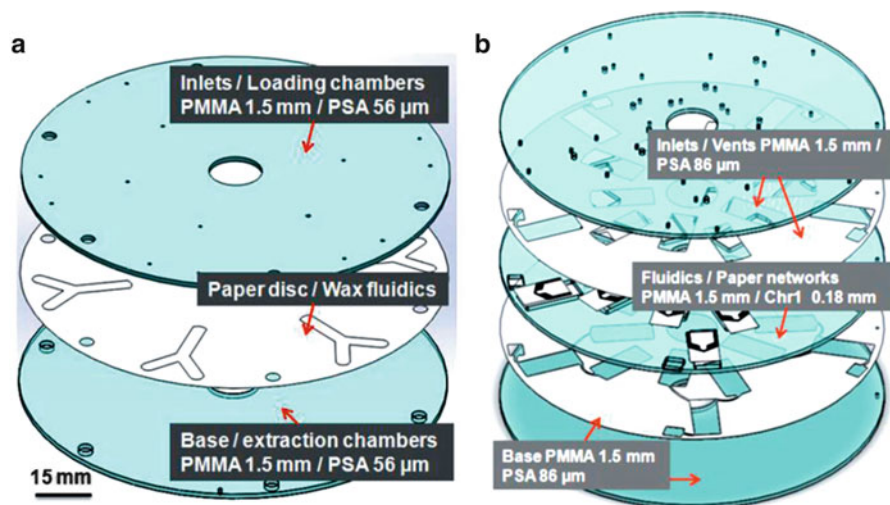
Fig. 7.5 Centrifugal paper-disc devices: (**a**) whole paper disc is used in this device assembly and fluidic structures are patterned in paper by wax, (**b**) paper inserts with wax patterns are cut and integrated into polymeric parts. Adapted from [75] with permission of IEEE

lignin coming from wood). Unlike nitrocellulose membranes exhibiting hydrophobic properties due to its cellulose acetate blends, filter and chromatographic papers are hydrophilic and do not require the deposition of surfactants to improve wetting properties. Additionally, there are several other requirements to paper material: suitability for processing of biological samples in small volumes and within short times (e.g. wicking time between reaction zones), specificity and sensitivity comparable to commercial rapid tests [1].

Another crucial factor is sufficient protein binding to allow for the formation of sharp and intense capture zones while keeping the signal from nonspecific background low.

There is no universal type of paper that will suit all applications. Choice of design is highly dependent on the type and structure of paper. Structural properties, insights into physics and chemistry of paper [81] can be found in dedicated reviews. Utilization of different papers, and different channel width were explored by some researchers earlier [36, 82]. Some experimental work on papers of different grades can be found in [75]. Commercial paper grades differ in flow rate, pore size and porosity, thickness, color, particle retention and other properties. Ideally, in paper microfluidic devices there should be high consistency (within single device as well as batch-to-batch) in surface pore size, thickness, protein binding capacity (irreversible capture of reagents at the detection zones), flow characteristics, magnitude of obtained signal during detection and stability during storage. Binding characteristics of selected paper should be tested during the development stage of device. Protein binding capability will dependent on the paper surface area available for

capture. It should also be verified that binding capabilities are not altered during the manufacturing process or when assay is run [13].

Paper requires chemical activation to immobilize antibodies. Many researchers address that it is suitable for variety of (bio)functionalization procedures [13]. Techniques for functionalization of paper, and main factors influencing functionalization procedures such as structure and surface chemistry are discussed [82, 83]. Early works describing strategies for treatment of paper surface with DNA, both physical adsorption and covalent binding [84], salinization [45], in situ polymerization of molecular imprinted polymer on paper [45], modification of paper with poly (vinyl pyrrolidone) and polyaniline [33]. Functionalization with polymers gives numerous active sites to build up a sensitive detection method [85, 86].

There are, however, some concerns associated with possible integration of paper in diagnostic devices. For example, colloidal gold and latex labels, used in the industry, require more open pore materials such as glass fibre and polyester for optimized stabilization and release from it during the assay run. Surface quality is another key parameter in optimizing the performance of paper-based microfluidics. Paper has relatively rough surface characteristics, which might cause various challenges in reproducibility of quantitative measurements. Recently, a novel class of materials, so-called synthetic paper, with controlled porosity characteristics, has been introduced and may have potential for integration in point-of-care devices [87].

Finally, note that it is possible to combine nitrocellulose, filter, and chromatographic paper in one device where positive sides of each type of these materials can be utilized. All these materials can be cut by laser or PC-controlled knife plotter, and assembled in a single process.

6   Existing Fabrication Technologies

This section reviews technologies that are applied for fabrication of paper-based devices. Because paper is a very flexible material, the following techniques are often applied (Fig. 7.6): inkjet, wax, flexography, or screen-printing all use non-toxic reagents. In the majority of the works a paper device needs to go through two main fabrication stages, i.e. patterning of hydrophobic channels for liquid confinement and assembly [88]. On the laboratory level, some deviations from the described low-cost methods are possible, but due to the limited space we only provide general descriptions.

6.1   Technologies for Patterning of Hydrophobic Barriers

Paper is a flexible material; therefore, various printing techniques are well suited to form a pattern. Physical blocking of pores in paper with hydrophobic material is a
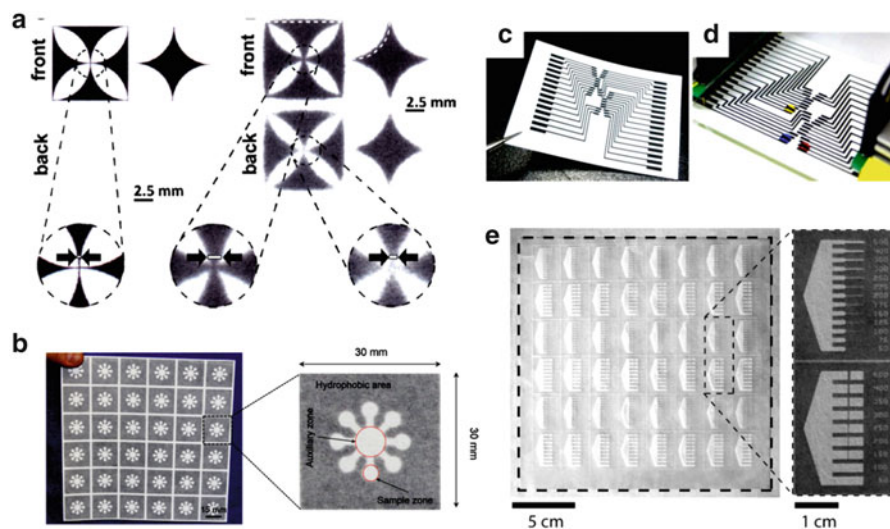
Fig. 7.6 Examples of paper-based structures fabricated by (**a**) wax-printing (Adapted from [92] with permission of Royal Society of Chemistry), (**b**) screen-printing (Adapted from [99] with permission of Royal Society of Chemistry), (**c**) inkjet-printing (Adapted from [104] with permission of John Wiley and Sons) and (**d**) photolithography assisted method (FLASH) (Reproduced from [108] with permission of Royal Society of Chemistry)

widely used method. The rise of this technology goes back to 2007 when a description of the process for creating hydrophobic pattern in paper and using hydrophilic channels for pumpless liquid transport was reported by Whitesides and co-workers [89]. Hydrophobic barriers can be made using wax, SU-8 and other photoresist materials, PDMS, alkenyl ketene dimer [5], polystyrene, ethyl cellulose, silicones, rosin, paraffin, printer varnish, cellulose esters, hydrophobic gels [90] and possibly others. All these materials are impervious to water and allow for implementation of various structures for transport and storage of liquids. Width of channel structures that can be achieved by these methods varies and can go down to 200-300 μm with some optimization.

6.1.1 Wax Printing

Wax printing [91, 92] is the most commonly used technique. It is easy, fast, and low cost, and can be easily applied for small-scale manufacturing. Typically, the channel width is in the range of 1–5 mm. This technology is very straightforward: a design is sent to a printer and printed directly on a sheet of paper, this paper can be sandwiched between a metal foil and placed on a hot-plate at ca. 70 °C for 1 min. For better penetration of wax into paper it can be baked from both sides. Baking temperature, baking time and printing mode are parameters that can be used for optimization in each specific case. Speading of wax in paper after bake can be

predicted (see description in the earlier section). Reproducibility of channel dimensions and printing resolution will depend on quality of the printer and homogeneity of heat applied during the bake. Wax printing has been widely applied to 2D, 3D fluidic devices as well as in centrifugal paper-based systems. Details of the method for fabrication of 3D µPADs by wax printing can be found elsewhere [93]. Permeability of the paper is another parameter describing flow through it. Permeability has been alternated using papers that were impregnated with wax [94]. Accurate control over the penetration depth of melted wax, printed on both sides of a paper substrate allows formation of multilayers of patterned channels in the substrate [95].

6.1.2 Screen-Printing

Screen-printing [96, 97] is a versatile technique where liquid material is transferred onto substrate via a screen (a grid with a stencil attached or formed directly on it) manually or using an automatic tool, which regulates pressure applied on substrate and amount of printed material. After applying the material, the paper substrate is allowed to dry, and maybe subjected to heat or other treatments. What is characteristic for this method is that much thicker layers of printed material can be applied compared to wax printing by a commercial printer. There are also less strict requirements to size, planarity, shape and thickness of the selected paper substrate. Advantages of screen-printing technique [98] are fast fabrication times, low costs, flexibility (different materials can be printed) and capability of mass production. Several instrument-free, single-step screen-printing methods on chromatographic paper were demonstrated, e.g. patterning of PDMS solution with minimum channel width of ca. 600 µm [99], patterning of polystyrene through a patterned screen [100] with minimum channel width of ca. 670 µm (minimum width of hydrophobic barrier was ca. 380 µm). Another variation of this technique utilizing spraying of material through a pre-defined micro stencil instead of squeezing through a screen as in conventional screen-printing, i.e. a mask containing pattern to be transferred [101]. This technique is also widely applied to form electrodes in paper [77].

6.1.3 Inkjet-Printing

Inkjet-printing is also one of the early methods applied for fabrication of paper-based devices [102–104]. Review of inkjet-printed technologies applied on paper can be found elsewhere [105, 106]. In short, this technology is based on transferring material directly into paper via a nozzle (nozzle is activated e.g. piezo-electrically or thermally). Material is jetted in a close proximity from paper surface and follows a required pattern. Printed lines are formed from hydrophobic inks, e.g. PDMS, which was also adapted to roll-to-roll technology [103, 107]. Besides the setup of the inkjet-printer (jetting pressure applied via the nozzle), the chemistry and

wetting properties of the printed material is probably the most crucial optimization parameter to achieve good quality of the printed image.

6.1.4   Flexographic Printing

Flexographic printing is method utilizing a flexible plate containing a pattern which is transferred by applying a liquid ink. It is another variation of the screen-printing technique, which is widely applied in industrial settings. It is suitable for various inks and substrates. One example is flexographic printing of polystyrene applied using the roll-to-roll technology. Several layers need to be applied to achieve hydrophobic channels where width of channels is typically 1 mm [107].

6.1.5   Photolithography-Assisted Methods

Conventional UV lithography can be used to produce pattern on photo-curable polymer that was brought in contact with paper substrate by one or other method. Paper can be impregnated with the photoresist via dipping prior to patterning using a UV lamp through a glass or polymeric mask containing desired patterns. The UV-assisted methods, i.e. FLASH method, was one of the first methods introduced for patterning of paper-based devices [108]. UV lithography can aid very defined and reproducible structures but fabrication costs are high and not every lab has access to photolithography facilities. A variety of hybrid fabrication methods were demonstrated, for example, an interesting method where photolithographic patterning of Parafilm® was subsequently followed by embossing of the film into paper [109].

6.1.6   Plasma Treatment

Definition of hydrophilic and hydrophobic regions can also be achieved by exposure to plasma [110]. Oxygen plasma can also be used to introduce new fluidic functionalities in paper, e.g. multiple-use valves [111].

6.2   Technologies for Assembly of Devices

After paper devices were patterned with hydrophobic materials to form channels and reaction zones, they still need to be assembled. Common methods include cutting, stacking several layers of paper together, shaping of paper by cutting or folding (origami-like technique [71]) with or without use of adhesive tapes [70, 112]. Paper can also be laminated prior to cutting and stacking by methods similar to production of ID cards [113, 114]. Cutting can be done by knife plotter

controlled by PC (down to 0.5–0.8 mm with some optimization of cutting parameters), or CO_2 laser can be used for cutting cellulose (down to 1–1.5 mm). Maintaining contact between hydrophilic features in each layer of a 3D paper-based microfluidic device is the key fabrication challenge for these devices. Dry-stored reagents are deposited onto individual layers before assembling the final 3D device [1].

6.3   Technologies for Fabrication of Electrodes in Paper

Many detection methods applied on paper require reproducible fabrication of paper electrodes. Electrodes, typically conductive inks (carbon, Ag/AgCl), can be formed by various techniques, including those discussed above: screen-printing [96, 97], electrospraying [33], painting or dipping paper in conductive ink, or e.g. solution of carbon nanotubes [115, 116], combination of direct printing of Au and Ag stripes and subsequent electrochemical deposition of AgCl layer, and even by using a regular pencil [44]. Combination of fluidic networks in paper printed over electrodes allows for many applications involving measurement of electrical signals [107] such as e.g. digital microfluidics [117]. Functionality of paper can be extended by modifying its properties or hybridly combining it with conductive materials for various electronics applications [118].

7   Detection Methods

7.1   Colourimetric

Colourimetric method has been widely applied in paper for quantification of concentration of analytes [35, 51]. Colourimetric detection may include or not an enzymatic stage. The method is very common for medical laboratories and, as a result, many assays are based on it. It is also a convenient method for multiplexing [119] and can be combined with other detection methods, e.g. electrochemical [120]. Readout of colourimetric signals can be achieved with a smartphone or dedicated readout system.

7.2   Electrochemical

Detection methods which are using change in electric current or potential as a result of biochemical reaction occurring in a paper device are called electrochemical [38, 44, 121, 122]. Cyclic voltammetry is a very common measurement technique

[97, 103], as well as potentiometric [103, 123]. There are also more exotic variations of these such as streaming potential measurements [124].

7.3 Chemiluminescence and Electrochemiluminescence

Detection of electromagnetic radiation as light emitted during chemical reaction is the basis for chemiluminescence [125, 126]. Electrochemiluminescence method is luminescence generated by electrochemical reactions [47, 127].

7.4 Fluorescence

Fluorescence-based assays also have been integrated into paper [128, 129]. Overall, although fluorescence sensing brings new opportunities to paper-based detection, cost reduction and miniaturization of fluorescence readers are still on-going issues [1].

7.5 Nanoparticles

Many detection practices using nanoparticles are coming from the lateral flow assays. Antibody-conjugated gold nanoparticles and monodisperse latex, coupled with fluorescent dyes, are widely utilized in commercial rapid point-of-care tests. In general, colloidal gold particles are a preferred option due to higher colour intensity compared with coloured latex particles; they also can be dispersed in higher density as they typically ten times smaller than monodisperse latex particles [1, 130].

7.6 Other Methods

There are also a few methods, which are less popular and cannot be strictly assigned to any of the methods described above. Paper-based microfluidic calorimeter has been proposed [131]. This method would only suit for assays producing or consuming sufficient amount of heat to be detected as the method is not highly sensitive. Distance-based detection method where distance travelled by analyte producing colorimetric response is used as an analytical signal [50, 132]. Similarly, time to obtain response in the reference area of the device can be used as readout [23, 114].

Various efforts were also made to achieve visual detection just by naked eye which is a very resource-poor settings inspired idea. Specific designs aiming at

solely visual determination of test result, e.g. counting bars that turned colorful after analyte has reached it were demonstrated [23]. Electrical measurements were applied for monitoring of bacterial movement through the paper, which both has fundamental interest (measurement of speed of movement of bio-entities through paper network is challenging) and potentially interesting applications [133]. Raman spectroscopy [55] was also applied as a detection method, and although this technique might be very feasible in certain applications, it still remains a sophisticated and expensive technique.

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