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Paper Microfluidics in Microbial World

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Abstract: ***Microfluidic built systems delivers a potent platform for microbiological assays. The review gives an overview of research activities stirring in the field of paper based microfluidics. It focuses on different techniques used to fabricate paper based miniaturized devices. Finally, applications of paper based microfluidic systems have been discussed.***

Keywords: Microfluidic, Paper based microfluidics, Fabricate, Miniaturized

1. Introduction

Microfluidics is a blooming field of science where fluids in microliters are manipulated in 5 to 500 micron channels. It is an interdisciplinary arena which covers wide area of science and technology. It is used in developing miniaturized devices which has application in biomolecules detection, making diagnostic devices, environmental analysis, microelectronics, clinical diagnostics, drug development and detection, etc. Unique characteristics of microfluidic devices is its small dimension, requirement of small volume of samples and reagent, ease immobilization of reagents on substrate itself, rapid, robust in detection and its sensitivity. A large number of laboratory based techniques can be performed in small fingertip sized chip. Thus microfluidic based devices are cost effective and easily portable to remote countries where sophisticated laboratory and availability of highly skilled technician is the major challenge.

The first microfluidic device was miniaturized gas chromatography developed by Terry and et al in 1970's at University of Stanford (37). However, Whitesides and his coworkers were robust in designing microfluidics devices with various applications. Concept of microfluidics depends upon hydrophobic and hydrophilic components. Stamped channels on a hydrophilic substance are demarcated by hydrophobic polymers. The distinction between suitable hydrophilic-hydrophobic substances results in good capillary action of fluids in micro channels so created. Varieties of hydrophobic photoresist polymers and hydrophilic substrates are available. Also various methods to emboss channels are available, in order to get consistency in pattern of channels. However, Soft lithography is the commonly used technique. It is based on embossing channels on hydrophilic substrates like silicon wafers, silicon dioxide, gold, glass, poly dimethylsiloxane (PDMS), acrylic materials, etc. However, No substrate is perfect. Every substrate has its advantages and disadvantages. Choice of substrate depends on application of work. But all these substrate are costly and they add to the cost in the final manufacturing of the devices. Recently cellulose based paper has been used as substrate to develop low cost microfluidic devices. Paper as a substrate material for fabricating micro analytical devices has many advantages. It is extremely cheap and ubiquitous, thus reducing the cost of manufacturing. It is biocompatible, easing the immobilization of organic compounds, enzymes, hormones, antibodies, etc. It is biodegradable and can be disposed off easily by incineration. Being hydrophilic, it has the ability to wick fluid via capillary action. This allows instrument free manipulation of liquid in small channels. Hydrophobic barrier can be created using suitable material like wax, tape, etc. and test reagents and solutions can be stored on paper. Paper is thin light weight and flexible, and easy for transportation. All these advantages makes paper a suitable substrate for developing point of care devices, paper based biosensor chips, etc. for onsite detection of target molecule and screening of different compounds is new trending application of paper based micro analytical devices. Such devices can be operated by lay men and researchers in resource limited environment. In this review different methods to fabricate paper and its relevant applications have been detailed.

2. Paper as a Substrate to Fabricate Microfluidic Device

Choice of fabricating substrate is important to make micro devices since the manufacturing cost is dependent on it. Many substrates have been reported so far like silicon, gold, PDMS, glass, thread and paper. However paper is very cheap among all. The main advantage of paper based microfluidics devices is to provide a low cost portable platform for assays which otherwise requires huge laboratory based sophisticated instrumentation. Paper has gathered attention for the development of cheap device in scientific community. There are varieties of papers available like Whatmann filter paper, nitrocellulose membrane, cellulose acetate paper, etc. however Whatmann filter papers are widely used as a crude platform.

3. Techniques used to Fabricate on Paper

Microfabrication techniques were developed to prepare semiconductors but now they are also used to make biosensors and microfluidic devices. It allows manufacturing portable, hand handled, light weighed, low cost devices. Fabrication techniques creates hydrophobic barrier around hydrophilic channels thereby restricting the flow of liquid in particular planned manner. Most important microfabrication techniques are photolithography, wax printing and inkjet printing. However, simply cutting and stacking paper is also used to create low cost paper based biosensors.

3.1. Photolithography

It is an expensive method but creates channels of high resolution. The process consists of sequential steps in which

a desired pattern is generated on the surface of a substrate through exposure of regions of light sensitive material, a photoresist, to ultraviolet light. In the first step, a substrate like silicon wafers, glass, etc. is coated with a layer of photoresist polymer. A photomask having a pattern of channels with an opaque material is aligned on the applied photoresist. The entire assembly is then irradiated with ultraviolet light, thus exposing the regions of photoresists not covered by the opaque regions of the photo mask. Two types of photoresists are available: Positive photoresist and Negative photoresist. When positive photoresist is exposed to ultra violet light there is change in the chemical structure of the resist and it becomes more soluble to the developer solvent like acetone, iso propyl alcohol and is washed away in subsequent steps. The exposed resist is thus washed away. Negative photoresist behaves in opposite manner to positive one. Exposure to the ultra violet light causes the negative resist to become polymerized, and more difficult to dissolve. Therefore, the negative resist remains on the surface wherever it is exposed, and the developer solution removes only the unexposed portions. The figure below shows the pattern differences generated from the use of positive and negative resist.

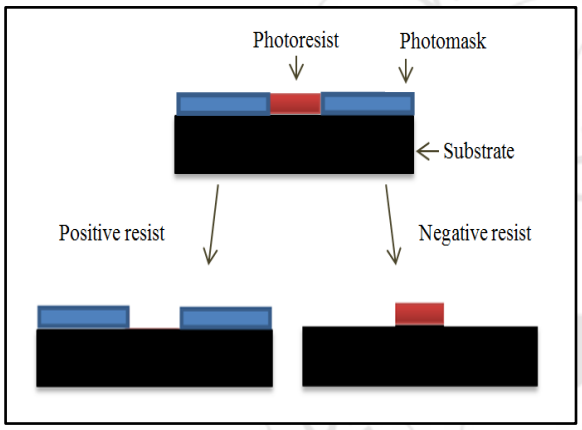

Figure 1: Difference between positive and negative resist

Generally negative photoresists are used to manufacture microfluidic devices. Poly methyl methacrylate, SU-8, etc. are some of the negative photoresists. Thus by photolithography a template is prepared on which an elastomer like poly dimethylsiloxane (PDMS) is poured. After hardening, PDMS is peeled off. This peeled layer is stuck to another substrate like glass slide and the assembly is sealed by keeping it inside oxygen plasma cleaner. Further inlet and outlet channels are pierce through syringe and the chip is ready for further use. PDMS has been the most widely used material in the research and development of microfluidics. PDMS is an optically transparent elastomer. Systems in PDMS are typically fabricated using techniques in soft lithography. Soft lithography involves replication of channels by using photoresist. The process can be carried out in ambient laboratory settings. Duplication can be repeated several times. Soft lithography therefore enables rapid, simple, and inexpensive fabrication processes. Shayne and *et al* in 2016 have used SU-8 to fabricate paper based device. Paper substrate was impregnated with SU-8 photoresist. SU-8 was evenly distributed using a spin coater. Photomask, a transparency having template designed using auto CAD was aligned on these using a mask aligner. The assembly was exposed to ultra violet radiation for 18 seconds. Unexposed photoresist was washed thrice using isopropanol. Microanalysis device was dried with high pressure air and stored in a sealed plastic bag until the time of use (33). Martinez and *et al* in 2010 have impregnated SU-8 photoresist on cellulose paper and allowed its polymerization by exposing it to ultra violet light through transparency having template on it. Unexposed photoresist was washed using acetone. These created patterned channels on cellulose paper (25). Park and *et al* in 2015 also used SU-8 to fabricate on paper and sensory evaluation of different tastes of wine was carried out using chemical dyes (30). Cuicui Ma and *et al* in 2012 used octadecyltrichloro-silane (OTS) solution to create hydrophobic barrier. Hydrophilic filter paper was uniformly coated with a layer of OTS. The coated layer was then treated with UV/O_3 through a patterned template quartz mask, generating hydrophobic barriers. Width of barrier was 130 µm and that of channel was 80µm (22). Jianq and *et al* in 2016 have also used OTS to create hydrophobic barriers on cellulose paper. But hydrophilic channels were created by corona generator. In this method, electrode tip of the corona generator scans the channel region to create hydrophilic channels. The device was used for colorimetric detection of nitrite from saliva samples. Fast lithography activation of sheets commonly called FLASH is a rapid method for prototyping microfluidic devices on paper. FLASH is based on photolithography but requires only ultra violet lamp and a hot plate; no clean room facilities are required. In absence of ultra violet lamp and hotplate FLASH technique can be performed even in sunlight. The method provides channels in paper with dimension as 200 mm in width and 70 mm in height. Photomask for patterning paper was printed using an inkjet printer or photocopier or drawn by hand using a waterproof black pen(13). Table no.1 shows different analytes detected on Whatman filter paper no.1 fabricated using PDMS and table no.2 shows different analytes detected on Whatman filter paper no.1 fabricated using SU-8.

Table 1: Analytes detected on Whatman filter paper no.1 fabricated using PDMS.

Target Analyte	*Reference*
Glucose, Bovine serum albumin	Crooks 2011 (20)
Glucose	Park 2015 (30)

Table 2: Analytes detected on Whatman filter paper no.1 fabricated using SU-8.

Substrate	*Target Analyte*	*Reference*
Cellulose paper	Wine ingredients	Park 2014 (38)
Whatman filter paper no 1	Glucose, proteins	Martinez 2008 (26)

3.2 Wax printing

Wax, a hydrophobic substrate is used to create barrier around hydrophilic channels. Wax is an easily available low cost alternative to fabricate paper based microfluidic devices. It can be directly imposed on paper through wax printer. The process is easy to fabricate and can be finished within 5 to 10 minutes without using clean room facilities (21).Dungchai and *et al* in 2010 have also used wax printing to create hydrophobic barriers on paper. But they rubbed solid wax through a screen onto filter papers. The printed wax by this method is then melted by using hot plate oven which results in formation of hydrophobic barrier. The minimum width of

hydrophilic channel was 650 mm and that of hydrophobic was 1300 mm. On comparing this technique with photolithography it was found that wax printed channel are easy to fabricate and can be used as alternative to photolithography techniques (7). Xu and *et al* in 2016 have fabricated paper with wax pen. Electrophoretic separation of carmine and sunset yellow was achieved within few minutes by applying potential on the channel using a simple and inexpensive power supply(41).Henry and *et al* in 2014 have used parafilm to create hydrophobic barriers on paper. This method was advantageous to the other fabrication method because it had higher solvent compatibility. This device was used to carry out series of enzymatic reactions(10). Table no.3 shows different analytes detected on Whatman filter paper no 1. fabricated using wax.

Table 3: Analytes detected on Whatman filter paper no 1. fabricated using wax

Target Analyte	*Reference*
Horse radish peroxidases, Bovine serum albumin, Glucose	R. Lu, W. Shi 2009 (21)
Glucose, protein	Martinez 2010 (25)
Alanine aminotransferase, Aspartate aminotransferase	Vella 2012 (39)
Drug induce Liver+ injury marker	Pollock 2013 (31)
Organopesticide	Somashekar 2013 (35)
Alanine aminotransferase	Jain 2015,Swanson 2015 (36)(12)
Beta hydroxybutarate	C. wang 2016 (40)

3.3 Inkjet printing

It is a process in which individual ink droplet between 10 µm to 100 µm diameters are placed at software configurable location on a substrate. Inkjet printing is a recent manufacturing technique which is easy to learn and has a high fabrication speed. It offers a great planar resolution of 20 µm and also enables flexible designs due to its inherent capacity to deposit into thin film. In its very basic principle an inkjet printer performs the contactless dispensing of pico liter sized droplets of liquids onto a user defined position of a substrate. It is used for the deposition of nucleic acid in fabrication of DNA chips (19). Iron metal template with a specific pattern was placed on a piece of chromatography paper. A permanent marker was then used to directly plot the pattern of the template on the paper surface. The resultant patterned paper was subsequently left at room temperature, to evaporate the solvent in ink marks. The plotting time mainly depended on the complexity of the device's pattern. Number and color coded devices for multianalyte analysis were fabricated by marking the different test regions of the corresponding patterned paper with different numbers and colors, respectively. Contamination resistant devices were additionally created by sandwiching the patterned paper with two pieces of transparent adhesive tape. It is difficult to handle small volume of fluidics in small channels and requires external force to pump the solution from the channel. Prakash and *et al* in 2015 have devised a punch card programmable microfluidics device for easy manipulation of fluids. They used rotating disc which squeezes the channel from behind thereby pumping the fluid forward (17). Principles of origami paper folding was used to fabricate 3d paper based device by Liu and *et al* in 2011. The device was made by folding by hand (20). Nuchtavorn and *et al* in 2016 have used low cost fabrication method using desktop digital craft plotter and technical drawing pens. They created barriers on paper using permanent marker ink. The device was used to colorimetrically estimate flavonoids and phenolic compounds (43).

3.4 Other techniques

Recently adhesive tape was used as hydrophobic barrier to restrict flow of liquid inside the channels. 3D microfluidic paper based device was made by stacking layers of patterned papers and water impermeable double sided adhesive tape. Assembly was made that channels the flow of liquid within and between the layers of paper. Further this was used to detect protein and glucose from urine samples. Stacked paper based device was easy to fabricate and more conventional to prepare than the one made by using photolithographic techniques (24). Punch card based programmable microfluidic devices made by Prakash and *et al* in 2015 also uses tape to demark hydrophobic barriers. The devices are foldable, easier for operation and donot require any additional equipment (16). Similar 3D and bendable device was made by Martin and *et al* in 2017. They adhered patterned paper onto transparent tape. A simple open channel microfluidic device was made (23). Liu hongand *et al* in 2011have used origami principle to fabricate paper based device using adhesive tape. The device was used for colorimetric estimation of glucose and bovine serum albumin (20).

4. Applications in Immunoassays

Current analytical technique fails to meet recent demands of patient in rapid testing of antigens. Microfluidics based point of care devices are turning boon in this regards.

i. Malarial antigen detection:
African countries are especially fruitful because of availability of device which detects malarial antigen in cost effective manner. These antigens are generally detected by ELISA test, PCR analysis, etc(18). However these techniques are time consuming. By applying microfluidics principal Sumingchen and *et al* in 2016 have detected *Plasmodium falciparum*histidine rich protein 2 antigen. They also adopted mass spectrometry principle on paper. A paper based mass spectrometry immunoassay was developed that employs ionic probes to detect antigens. They immobilized cellulose paper with anti-Pf HRP2 antibodies by reacting the amino group of antibodies with aldehyde functionalized on paper. Further serum solution containing Pf HRP2 antigen was added on to the paper. This was followed by adding a solution having antibody conjugated to the cleavable probe. Fragments of ions were quantified in a mass detector with LOD 500 pM. Their method was with 98.1% sensitivity and 96.2% specificity(5). Moody and *et al* in 2002 had compared three commercially available dipstick devices which targets malarial antigen HRP2 as shown in table no.4 (27)

Table 4: Commercially available dipsticks to detect malarial antigen

Name of the Commercial Kit	*Sensitivity*	*Specificity*
Parasight F	77 to 98 %	83 to 98%
ICTPf	96 %	99%
PATH Falciparum Malaria IC test	96%	99%

ii. HIV Antigen detection

Chao Cheng and *et al* in 2010 have developed paper based ELISA method using paper microfluidics technique. This device was used as a bioanalytical platform in poor countries to detect HIV virus rapidly and in cost effective manner. They fabricated 96 microzone on paper using hydrophobic polymer and used this miniature paper to successfully detect gp 41 enveloped antigen from HIV I virus (6). Table no.5 shows different ELISA techniques performed on Whatman filter paper.

Table 5: Different ELISA techniques performed on Whatman filter paper

Target Antigen	*Reference*
IL 6	Assadollahi 2009 (3)
gp 41	Cheng 2010 (6)
Prostate specific Ag	Nie 2012 (28)
Rabbit Ig G	Oyola2015 (29)
Pf HRP2	Suming Chen 2016 (5)

iii. Hepatitis C virus detection

Nitrocellulose membrane was immobilized with human Ig G against HCV and was able to detect HCV from pathological samples. The detection was done by using chemiluminescene principle and on site detection was done. The assay was able to detect the virus within 15 minutes as compared to other laboratory techniques which generally requires 24 hours(42).

4.2. Applications in enzymology

i. Transaminases

Functioning of liver is detected by checking the level of transaminases from blood samples. In order to detect its level in resources limited areas aspartate aminotransferase(AST) and alanine aminotransferase(ALT) measurements are done on devices which works on microfluidics principles. Pollock and co-workers in 2012 have developed low cost paper based multiplexed transaminase test. Their device is of small dimension where a small drop of blood sample is required. Onsite detection and quantification of enzymes were reported within 10 minutes by comparing color with standard graded strip. LOD was 53 U/l for ALT and 84 U/l for AST (12, 31, 32). Vellaand *et al* in 2012 quantitated AST, ALP, ALT,GGT, LDH on paper fabricated with wax barrier. Results were analyzed in 15 minutes by using digital camera and comparing the intensity by using image analysis programme. LOD values were 44U/l for AST and ALT, 15 U/l for ALP, 7 g/l for GGT and LDH (39). Swanson and *et al*in 2015 used portable reader which works on light transmittance to quantitate AAT from fingerprick blood sample. Results were automated within 10 minutes on site without using image analysis software. LOD was 6 U/l (36).

ii. Horse radish peroxidases

A simple portable paper microfluidics based analytical device was made by Lori and *et al* in 2016 (33, 34). The device was coupled with colorimetric detection principle for sensitive and rapid detection of horse radish peroxidases. Cellulose based Whatman filter paper no 41 was impregnated with 10 mM tetra methylbenzidine, a redox dye. Blue coloration was observed in presence of HRP. The device was sensitive to detect 6 ng/ml HRP in 10 minutes. This concept was further used by Alonso and *et al* in 2002 to detect rifampicin. HRP based biosensor was developed by them to detect rifampicin amperomatrically from biological samples (2).

4.3. Applications in molecular detection

Martinez and *et al* in 2010 fabricated paper with wax and made a prototype which would simultaneously detect glucose and protein from urine sample. The device works on simple colorimetric principle where change in color indicates presence of analyte and color intensity was used to quantitate the analyte(25). Dungchai and *et al* in 2010 have used paper based microfluidics device to simultaneously quantify glucose, lactate and uric acid from single sample. Their approach was successfully applied to quantify 0.5mM glucose, 1- 25 mM lactate and 0.1 - 7 mM uric acid from clinical samples (7). Toluene based hydrophobic barrier were created using inkjet printing technology on paper where qualitative analysis was done of urine sample to detect total proteins, glucose and pH. Fabricated paper based device was sensitive to detect 0.46 µM protein and 0.28 mM glucose (1). Longfei and *et al* in 2014 fabricated cellulose paper with hydrophobic substrate silane and used the template to determine glucose from human serum samples (4). Nie and *et al* in 2009 have fabricated three electrodes made of carbon ink or Ag/AgCl ink on paper. Using chronoamperometry technique 0.22 mM glucose quantification was carried out (28).

Beta hydroxybutyrate is a biomarker for detecting diabetic ketoacidosis. Chien and *et al* in 2016 have developed a paper based pop up electrochemical device to detect the same from blood sample of patients. Pop up device was made by folding a single sheet of paper into a 3 D shape. By folding and unfolding shape of device is changed and fluids are manipulated. LOD of their device was 0.3 mM as compared to commercial test strip of LOD 0.12 mM(40). On the same line Liu and *et al* in 2011 have fabricated 3D paper microfluidics device based on origami paper folding principle to detect BSA and glucose (20). Godino and *et al* in 2014 quantified triglyceride from blood samples on siphon paper. Siphon paper strip was loaded with 10 µl of whole blood sample at one end and the strip was centrifuged with special automated centrifuge. Separated triglycerides were detected by using enzymatic reaction (8). Yang and *et al* in 2016 used lipase enzyme to quantify triglycerides from food sample. In this method lipase enzyme was immobilized on nafion membrane. This membrane was placed above graphite epoxy transducer and potentiometric detection of triglyceride was done (43).

4.3 Applications in environmental pollutant detection

A bioactive paper was made to detect heavy metals (table no.6) from lake water samples. Whatman filter paper no 1 was fabricated using sol gel based bioink (beta galactosidase) which qualitatively detects the heavy metals. Chromogenic

substrate loses its original color in presence of these heavy metals which were quantitated by image analysis software (11).

Table 6: LOD of heavy metals detected from lake samples using paper based microfluidics

Heavy Metal	*LOD*
Hg II	0.001ppm
Ag I	0.002ppm
Cu II	0.02 ppm
Cd II	0.02ppm
Pb II	0.14 ppm
Cr VI	0.15 ppm
Ni II	0.23ppm

A paper based colorimetric sensor was developed to detect iodine from environmental samples. Sensor was prepared by soaking paper in starch and oxidant solution. On drying the paper it was ready to be used as sensor. Violet blue coloration was scanned by adobe photoshop(15).Semi quantitative detection of organic drug and antibiotic oxytetracycline used in aquaculture was done by simple paper based microfluidics assay. Cellulose paper was modified with reagents used to detect the antibiotic. On application of small volume of sample there results a color change that was quantitated by camera and paint software (9).Table no.7 shows different pollutant detected on Whatman filter paper no 1.

Table 7: Shows different pollutant detected on Whatman filter paper no 1

Pollutants	*Reference*
Organopesticides	Kavruk , Somashekar 2013 (14, 35)
Perfluorinated compounds, Surfactant, transition metals	Henry 2014 (10)
Surfactant	Brennan 2014 (11)

5. Conclusion

In this review, we have described different techniques used to fabricate paper based microfluidic devices and application of it in various fields is been discussed. Paper based microfluidic devices are proving to be a boon to people living in remote interior parts of world. They have received wide interest in being robust and eco-friendly in nature. External pump free delivery of samples and reagents, thereby manufacturing low cost device is the major attribute of it. The device has applications in healthcare monitoring, environmental analysis, etc. The future of microfluidics offers various opportunities in molecular biology, biochemistry, virology, etc.

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