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Shantanu Bhattacharya
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Paper Microfluidics

Theory and Applications

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Advanced Functional Materials and Sensors

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Preface

Energy demand has been rising remarkably due to increasing population and urbanization. Global economy and society are significantly dependent on the energy availability because it touches every facet of human life and activities. Transportation and power generation are two major examples. Without the transportation by millions of personalized and mass transport vehicles and availability of 24×7 power, human civilization would not have reached contemporary living standards.

The International Society for Energy, Environment and Sustainability (ISEES) was founded at Indian Institute of Technology Kanpur (IIT Kanpur), India, in January 2014 with an aim to spread knowledge/awareness and catalyze research activities in the fields of energy, environment, sustainability, and combustion. The society's goal is to contribute to the development of clean, affordable, and secure energy resources and a sustainable environment for the society and to spread knowledge in the above-mentioned areas and create awareness about the environmental challenges, which the world is facing today. The unique way adopted by the society was to break the conventional silos of specializations (engineering, science, environment, agriculture, biotechnology, materials, fuels, etc.) to tackle the problems related to energy, environment, and sustainability in a holistic manner. This is quite evident by the participation of experts from all fields to resolve these issues. The ISEES is involved in various activities such as conducting workshops, seminars, and conferences in the domains of its interests. The society also recognizes the outstanding works done by the young scientists and engineers for their contributions in these fields by conferring those awards under various categories.

Third International Conference on "Sustainable Energy and Environmental Challenges" (III-SEEC) was organized under the auspices of ISEES from December 18 to 21, 2018, at Indian Institute of Technology Roorkee. This conference provided a platform for discussions between eminent scientists and engineers from various countries including India, USA, Norway, Finland, Sweden, Malaysia, Austria, Hong Kong, Bangladesh, and Australia. In this conference, eminent speakers from all over the world presented their views related to different aspects of energy, combustion, emissions, and alternative energy resource for

sustainable development and cleaner environment. The conference presented five high-voltage plenary talks from globally renowned experts on topical themes, namely "The Evolution of Laser Ignition Over more than Four Decades," by Prof. Ernst Wintner, Technical University of Vienna, Austria; "Transition to Low Carbon Energy Mix for India," by Dr. Bharat Bhargava, ONGC Energy Center; "Energy Future of India," by Dr. Vijay Kumar Saraswat, Hon. Member (S&T), NITI Aayog, Government of India; "Air Quality Monitoring and Assessment in India," by Dr. Gurfan Beig, SAFAR; and "Managing Large Technical Institutions and Assessment Criterion for Talent Recruitment and Retention," by Prof. Ajit Chaturvedi, Director, IIT Roorkee.

The conference included 24 technical sessions on topics related to energy and environmental sustainability including 5 plenary talks, 27 keynote talks, and 15 invited talks from prominent scientists, in addition to 84 contributed talks and 50 poster presentations by students and researchers. The technical sessions in the conference included advances in IC engines, solar energy, environmental biotechnology, combustion, environmental sustainability, coal and biomass combustion/gasification, air and water pollution, biomass to fuels/chemicals, combustion/gas turbines/fluid flow/sprays, energy and environmental sustainability, atomization and sprays, sustainable transportation and environmental issues, new concepts in energy conservation, waste to wealth. One of the highlights of the conference was the rapid fire poster sessions in (i) engine/fuels/emissions, (ii) renewable and sustainable energy, and (iii) biotechnology, where 50 students participated with great enthusiasm and won many prizes in a fiercely competitive environment. More than 200 participants and speakers attended this four days' conference, which also hosted by Dr. Vijay Kumar Saraswat, Hon. Member (S&T), NITI Aayog, Government of India, as the chief guest for the book release ceremony, where 14 ISEES books published by Springer, Singapore, under a special dedicated series "energy, environment, and sustainability" were released. This was the second time in a row that such significant and high-quality outcome has been achieved by any society in India. The conference concluded with a panel discussion on "Challenges, Opportunities, and Directions for National Energy Security," where the panelists were Prof. Ernst Wintner, Technical University of Vienna; Prof. Vinod Garg, Central University of Punjab, Bhatinda; Prof. Avinash K. Agarwal, IIT Kanpur; and Dr. Michael Sauer, University of Natural Resources, Austria. The panel discussion was moderated by Prof. Ashok Pandey, Chairman, ISEES. This conference laid out the roadmap for technology development, opportunities, and challenges in energy, environment, and sustainability domains. All these topics are very relevant for the country and the world in present context. We acknowledge the support received from various funding agencies and organizations for the successful conduct of the Third ISEES conference III-SEEC, where these books germinated. We would therefore like to acknowledge NIT Srinagar, Uttarakhand (TEQIP) (special thanks to Prof. S. Soni, Director, NIT, UK); SERB, Government of India (special thanks to Dr. Rajeev Sharma, Secretary); UP Bioenergy Development Board, Lucknow (special thanks to Sh. P. S. Ojha), CSIR; and our publishing partner Springer (special thanks to Swati Meherishi).

The editors would like to express their sincere gratitude to large number of authors from all over the world for submitting their high-quality work in a timely manner and revising it appropriately at a short notice. We would like to express our special thanks to Ms. Pulak Bhushan who reviewed various chapters of this monograph and provided their valuable suggestions to improve the manuscripts.

Recent advances in the field of paper microfluidics in innumerable research domains have stimulated considerable efforts to the development of rapid, cost-effective, and simplified point-of-care diagnostic systems. The book is divided into three parts, viz. theoretical background of paper microfluidics, fabrication techniques for paper-based devices, and broad applications. Each chapter of the book is self-explanatory and focuses on a specific topic and its relation to paper microfluidics. Each chapter starts with a brief description of the topic's physical background, essential definitions, and a short story of the recent progress in the relevant field. The book also covers the future outlook, remaining challenges, and emerging opportunities. This book shall be a tremendous up-to-date resource for researchers working in this area.

Kanpur, India — Shantanu Bhattacharya
Singapore — Sanjay Kumar
Kanpur, India — Avinash K. Agarwal

Contents

About the Editors

Prof. Shantanu Bhattacharya (PhD) (ORCID ID: 0000-0002-7902-2119) is a Dr. Gurumukh T. and Veena M. Mehta Chair and Professor of Mechanical Engineering and the Head of Design Program at the Indian Institute of Technology Kanpur. Prior to this, he completed his MS in Mechanical Engineering from the Texas Tech University, Lubbock, Texas and a PhD in Bioengineering from the University of Missouri at Columbia, United States of America. He completed his postdoctoral training at the Birck Nanotechnology Center at Purdue University. Dr. Bhattacharya's main research interests are Design and development of micro-nanosensors and actuation platforms, nano-energetic materials, micro and nanofabrication technologies, water remediation using visible light photocatalysis and product design and development. He has many awards and accolades to his credit which includes the Institution of Engineers Young engineer award, the Institute of Smart structures and systems young scientist award, the national design research best mechanical engineering award, fellowship from the high energetic materials institute at Australia, fellowship of the Institution of Engineers of India etc. Dr. Bhattacharya has guided many PhD and masters' students and has many peer-reviewed international journal publications, patents, books, and conference proceedings.

Dr. Sanjay Kumar (ORCID ID: 0000-0001-8101-097X) is currently a Research Fellow at the National University of Singapore, Singapore. He received his PhD in design and fabrication of functionally engineered materials from the Department of Mechanical Engineering, Indian Institute of Technology Kanpur-208016 India. His research focuses on the development of design and development of acoustic metamaterials, acoustic wave control, paper-based analytical devices for point-of-care diagnostic applications, synthesis of nanoscale materials with controllable size and shape, design of multifunctional materials through self-assembly of nanoparticles, additive manufacturing processes, theoretical modeling and optimization, finite-element based numerical simulation, etc. He has published eight journal papers in peer reviewed international journals, six book chapters, and two US patent (filed). Along with Ms. Pulak Bhushan and Prof. Shantanu Bhattacharya, he received the Gandhian Young Technological Innovation (GYTI 2017) Award at Rashtrapati Bhavan, New Delhi, India for development of a dengue NS1 detection kit. He was also awarded the "IFMBE Young Investigator Award" at the 2nd International Conference for Innovation in Biomedical Engineering and Life Sciences (ICIBEL2017) held in conjunction with the 10th Asia Pacific Conference on Medical and Biological Engineering (APCMBE2017) Malaysia.

Prof. Avinash K. Agarwal joined IIT Kanpur in 2001 and prior to that, he worked at the Engine Research Center, University of Wisconsin, Madison, USA as a Post-Doctoral Fellow (1999–2001). His areas of interest are IC engines, combustion, alternative fuels, conventional fuels, lubricating oil tribology, optical diagnostics, laser ignition, HCCI, emissions and particulate control, and large bore engines. Prof. Agarwal has published more than 270 peer reviewed international journal and conference papers, 32 edited books, 55 books chapters and has 8000+ Scopus and 11100+ Google scholar citations. He is associate editor of ASME Journal of Energy Resources Technology. He has edited "Handbook of Combustion" (5 Volumes;

3168 pages), published by Wiley VCH, Germany. Prof. Agarwal is a Fellow of SAE (2012), Fellow of ASME (2013), Fellow of INAE (2015), Fellow of ISEES (2016), Fellow of RSC (2018), and Fellow of NASI (2018). He is recipient of several prestigious awards such as Clarivate Analytics India Citation Award-2017 in Engineering and Technology, NASI-Reliance Industries Platinum Jubilee Award-2012; INAE Silver Jubilee Young Engineer Award-2012; Dr. C. V. Raman Young Teachers Award: 2011; SAE International's Ralph R. Teetor Educational Award-2008; INSA Young Scientist Award-2007; UICT Young Scientist Award-2007; INAE Young Engineer Award-2005. Prof. Agarwal is the recipient of Prestigious Shanti Swarup Bhatnagar Award-2016 in Engineering Sciences.

Chapter 1
A Historical Perspective on Paper Microfluidic Based Point-of-Care Diagnostics

Sanjay Kumar, Pulak Bhushan, Avinash K. Agarwal and Shantanu Bhattacharya

Abstract Paper-based microfluidic systems have emerged as one of the most favorable technologies used in many potential applications such as point-of-care diagnostics, flexible electronics, energy storage, etc. From the past several decades, paper-based technology has readily accepted in the academic research lab and industries as well. The paper-based devices have changed the life of humankind. The distinguishing characteristics of paper substrate like low cost, biodegradability, biocompatibility, and ease of fabrication helped their adaptability in biosensing applications. This chapter gives a concise overview of the historical perspective of paper-based devices, classification of paper types, and their recent applications.

1.1 Introduction

In the past several decades, people have been migrating towards the most developed cities defying all cross-border concerns, making the world a 'global village'. The significant increase in the interaction between people from across the globe, has led to severe consequences for global health. These worldwide demo-graphical changes have steered the spreading of communicable diseases from one place to another through infected people (McMichael 2000; Pang and Guindon 2004; Tatem et al. 2006). For example, the severe acute respiratory syndrome (SARS), a respiratory viral disease was first recognized in 2003 in Guangdong Province, China and was

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spread out in over thirty countries within a year (World Health Organization 2003). Zika virus, a vector-borne flavivirus was first isolated in a monkey in 1947 in Uganda and reported in a human in 1953 in Nigeria and since then several outbreaks have been encountered in territories like America, Africa, Asia and Pacific (Hayes 2009; Petersen et al. 2016). Other such examples are the outbreak of human infection with avian influenza A (H7N9) virus in March 2013 in China, the epidemic of Ebola virus disease (EVD) in 2013 in West Africa and the dengue outbreak in Africa (Team 2016).

Conventional approaches used for diagnosis of infectious diseases is hindered by long turnaround times, skilled labor requirements, and sophisticated equipment requirements, imposing a financial liability on the healthcare system. According to a 2015 study by the World Health Organization (WHO), 1.8% of the population pays more than one quarter of their total expenditure on healthcare. Moreover, most of these infectious diseases originate in an underdeveloped/developing region where inadequate medical facilities render their timely diagnosis a difficult task. Due to the lack of proper diagnostic and medication facilities, these diseases claim thousands of human life annually (Lee et al. 2010). In 2003, the Bill and Melinda Gates Foundation in partnership with the National Institutes of Health identified the key challenges of diagnostics as, the lack of appropriate laboratory equipment and shortage of skilled personnel. They emphasized on the development of a simple, accurate, reliable and cost-effective strategy for rapid and point-of-care detection that would facilitate healthcare in resource-limited areas. In the context of early diagnostics of infectious diseases, WHO has released a set of guidelines termed ASSURED (i.e. affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free and deliverable to users) for developing point-of-care diagnostic devices (Peeling et al. 2006).

Point-of-care (POC) diagnostic devices enable the collection of a patient's health information at convenient locations generally outside of the main laboratory. They are low cost miniaturized systems integrated with complex functionalities facilitating timely diagnosis, health monitoring, clinical management, and dis-ease surveillance in both developing and developed countries (Wang et al. 2016). These devices have played a vital role in addressing global health needs by enabling early disease detection, preventing outbreaks and significantly affecting the medical outcome of disease treatment. According to the Coherent Market In-sights Analysis, the global POC diagnostic market was valued at US$1764.6 million in 2016 and is expected to witness a robust compound annual growth rate of 14.5% over the forecast period (2017–2025).

1.2 Paper Microfluidics: Historical Perspective

There are varieties of point-of-care technologies such as flow-through, agglutination assays, solid phase, and paper-based microfluidic device, etc. available in the market. The first three processes have been perceived as one of the most powerful

applications owing to their small size, portability, and low volume requirement for samples. Nevertheless, these systems require sophisticated equipment for fluidic handling and other attributes of diagnostic assays such as sample preparation and analyte detection, greatly limiting their usage in remote settings. Among these, paper-based microfluidics is the most widely used technique be-cause of its natural ability to passively transport fluid through capillary action or wicking, eliminating the need for external bulky pump or other equipment. Paper-based platforms possess several other distinguished characteristics such as rapid, inexpensive, ease of use, portability, affordability, low sample volume requirement, faster response, little interference, biodegradability, mass production capability, and external equipment-independent, which fulfills the WHO guidelines for POC (Yager et al. 2006).

From its first invention around 105 A.D. in ancient China (Temple 1998), paper has been used in many application areas. In the early 19th centuries Gay-Lussac developed a litmus paper (Crosland 2004). After that in 1850s to 1950s, researchers have developed paper-based sensors for various applications such as for radial paper chromatography (Weil 1953), urine test (Rocco 2005), detection of dyes and pigments (Müller and Clegg 1949), etc. However, paper-based sensors gained universal recognition after Martin and Synge were awarded Nobel Prize (chemistry) for their invention of paper chromatography in 1952. By the mid-1960s, commercialization of paper-based sensors had started such as, first dipstick assay for glucose detection in human blood, introduced by Ames company in 1964 (Rocco 2005), one-step lateral flow assay for the point-of-care pregnancy test kit launched by Unipath in 1988 (Chard 1992; Davies et al. 2007). Between mid-90s and 2000s, there was a progressive development of paper-based devices for detection of analytes such as diabetes, cholesterol, diagnostics of pathogens, and infectious diseases, etc. There was another major breakthrough in this field when two dimensional microfluidic paper-based analytical device (2D-μ PAD) was introduced by Whiteside's research group in 2007 (Martinez et al. 2007). Three distinguished branches with hydrophobic barriers made of SU-8 photoresist were patterned on chromatography paper using photolithography process and used for glucose and albumin detection. Since the development of 2D-μ PAD, extensive research has been carried out in device design, performance improvement and their utilization in various point-of-care applications in the past decades (Cate et al. 2014; Kumar et al. 2016a, b, 2017a, b, 2018; Morbioli et al. 2017; Li et al. 2017; You et al. 2017; Yeh et al. 2017; Whitesides 2018).

1.3 Outline

Paper microfluidics is one of the rapidly growing technology and shown a tremendous caliber in field of healthcare, biochemistry, environmental monitoring, analytical chemistry, etc. Owing to their distinct advantages such as rapid, easy-to-use, compatible with biological, organic and inorganic entities, chemical

inertness, robustness, economical, environment-friendly and easy disposal, paper microfluidic-based devices have become a prominent alternative for existing technologies POC technologies. In the past few decades, paper-based microfluidics has gained much attention in broad application areas such as point-of-care diagnostics, environmental monitoring, energy storage, fuel cell, flexible electronics, etc.

This monograph presents the different paper microfluidic-based technologies covering various applications such as point-of-care diagnostics, environmental pollution monitoring, public health monitoring, etc. Specific topics covered in the monograph include:

- Introduction to paper microfluidics
- Fluid transport mechanisms in paper-based microfluidic devices
- Fabrication techniques for paper-based microfluidic devices
- Flow control in paper-based microfluidic devices
- Sensing mechanisms in paper-based devices
- Advances in paper based point of care diagnostics for blood/plasma separation
- Evolution of paper microfluidics as an alternate diagnostic platform
- Paper-based microfluidic systems for detection of infectious diseases
- Paper-based microfluidic devices for detection of DNA
- Nucleic acid amplification on paper substrates
- Paper: A versatile material for the fabrication of low-cost wearable devices
- Paper-based devices for wearable diagnostic applications
- Paper-based devices for food quality control
- Environmental monitoring using paper-based devices
- Paper-based devices for energy storage applications
- Future of paper microfluidic systems.

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Chapter 2
Fluid Transport Mechanisms in Paper-Based Microfluidic Devices

Sanjay Kumar, Pulak Bhushan and Shantanu Bhattacharya

Abstract Paper microfluidics is one of the rapidly growing technology and shown a tremendous caliber in field of healthcare, biochemistry, environmental monitoring, analytical chemistry, etc. Owing to their distinct advantages such as rapid, easy-to-use, compatible with biological, organic and inorganic entities, chemical inertness, robustness, economical, environment-friendly and easy disposal, paper microfluidic-based devices have become a prominent alternative for existing technologies POC technologies. The challenge remains, however, in the designing of an efficient paper-based device for specific applications. A great deal of work has been done in this field to address the challenges in its two key enabling parameters namely 'materials properties' and 'fluid-transport mechanism' to achieve the functional paper device in an efficient and predictive way. Keeping in mind the previously published articles, the focus here is primarily on showcasing the fluid transport mechanisms through paper-based microfluidic devices. This chapter intends to provide an understanding towards theoretical modelling from a device perspective. The commonly used paper substrates and critical factors involved with any fluid transport phenomena is also covered.

Keywords Paper microfluidic · Fluid transport · Capillary flow · Lucas-Washburn equation · Paper types

S. Kumar and P. Bhushan have equal contribution in this chapter.

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2.1 Introduction

Paper is a thin, lightweight ($\sim$10 mg cm^{-2}) and flexible material, available in a wide range of thicknesses (Martinez et al. 2009). It is produced by pressing together multiple cellulose fibers with a porous structure (Altundemir et al. 2017). Although a variety of paper substrates is available in the market, among them, mainly two types of paper substrates are used in point-of-care diagnostics—pure cellulose fiber-based materials (filter paper, chromatography paper, etc.) and nitric acid treated cellulose-based materials (nitrocellulose membrane). Cellulose fiber is a linear chain macromolecule composed of β-D-glucopyranose units linked by glycosidic bonds (O'sullivan 1997). It is made from various raw sources such as wood, flax, cotton, jute, bamboo, grass, bagasse (Yetisen et al. 2013), etc. Cellulose is inherently fibrous, hydrophilic, tough, biodegradable and water-insoluble substance. In filter papers, fibers are randomly overlapped over each other (Fig. 2.1a–c) while in nitrocellulose membrane, fibers are granular in shape (Fig. 2.1d). Filter papers such as Whatman filter paper (grade 1–4) are generally used in qualitative analytical techniques for analyte detection. Owing to its wicking ability, filter paper is the most widely used substrate in the fabrication of paper-based devices (Martinez et al. 2009; Liana et al. 2012).

Nitrocellulose, is a microporous structure which is manufactured by partial nitration of refined cellulose (nitration ratio <2.3 substitutions per ring) (Mansfield 2005). Nitration alters the properties of cellulose such as from hydrophilic to hydrophobic and makes it highly adsorbable to proteins. Nitrocellulose membrane is widely used in lateral flow assay based biosensors (Fenton et al. 2008). Nitrocellulose membrane is brittle and weak. The lower tensile strength of nitrocellulose membranes (<2 lb/in) make them very difficult to handle. Therefore, adhesive polyester film backing is generally used to strengthen the nitrocellulose membrane (Mansfield 2009). Table 2.1 shows a summary of different types of paper substrate, their characteristics, and broad applications.

The selection of paper substrate in biosensing depends on various characteristics such as capillary flow time (the time required for a liquid sample to flow through the pores in the lateral direction), thickness of the paper, pore size, porosity (% of air present in porous structure) and surface quality. The capillary flow time is inversely related to the capillary flow rate and expressed as s cm^{-1}. Practically the capillary flow rate is difficult to measure accurately because the fluid flow rate through the nitrocellulose membrane decays exponentially as the fluid front transports along it. The estimation of capillary flow time helps in deciding the position of test line and control line on the membrane.

Paper thickness is another critical parameter for paper-based microfluidic devices for several reasons. The amount of sample required for a successful run of the assay depends on the pore volume. A thick membrane would need higher sample volume for the saturation of pores. But if the membrane thickness is too thin, then the membrane will be weak and might get damaged during handling. Also, when the known volume of capture reagent (Ab/Ag, in case of LFA) is either printed or

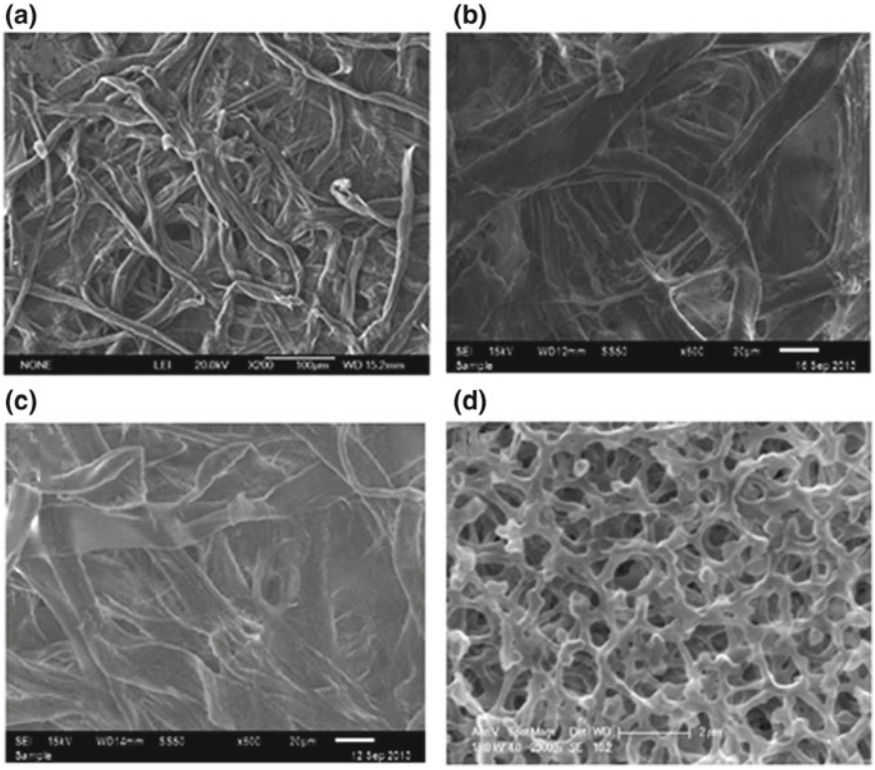

Fig. 2.1 SEM images of Whatman filter paper **a** Grade 1 at 200× magnification. Reprinted with the permission from Liu et al. (2015) ©Elsevier Ltd., 2014. **b** Grade 3 at 600× magnification (fiber thickness: 14.8 ± 1.4 μm) and **c** Grade 4 at 600× magnification (fiber thickness of 20.4 ± 3.6 μm). Adapted with permission from Evans et al. (2014) ©The Royal Society of Chemistry, 2014. **d** SEM images of nitrocellulose membrane with 0.2 μm pore size. Reprinted with the permission from Png et al. (2015) ©The Optical Society, 2015

pipetted onto the thin membrane, it will spread out resulting in poor color visibility. Commercially, the nitrocellulose membrane is available in the range of 100–150 μm thickness (Mansfield 2009).

Pore size is directly related to the particle retention capabilities of the paper. Pore size can be determined experimentally using the bubble point method (Yu et al. 2010). In this method, after soaking the porous structure of the membrane into liquid, air pressure is applied to transport the fluid into the pores of the membrane. The minimum pressure at which first air bubble is formed corresponds to the largest diameter of the pore in the filtration direction of the membrane, known as bubble point. At this point, the nominal pore size can be calculated from the following equation: $d = \frac{4\gamma \cos\theta}{P}$, where d is the average pore diameter, γ is the liquid surface tension, P is the minimum air pressure required to reopen the pore and θ is the

Table 2.1 Different types of paper substrates and their characteristics

Paper types	Characteristics	Applications
Whatman Filter paper grade 1	Fibrous structure, weight: 88 g m^{-2}, particle retention: >11 μm, thickness: 180 μm, porosity: 10.5 s	Detection of glucose (Kumar et al. 2017), lactate, uric Acid (Kumar et al. 2016)
Whatman Filter paper grade 2	Fibrous structure, weight: 103 g m^{-2}, particle retention: >8 μm, thickness: 190 μm	Monitoring of contaminants in the atmosphere, soil testing
Whatman Filter paper grade 3	Highly absorbent, Fibrous structure, weight: 187 g m^{-2} particle retention: >6 μm, thickness: 390 μm, porosity: 26 s	Suitable for suction filtration, used in Büchner funnels
Whatman Filter paper grade 4	Fibrous structure, weight: 96 g m^{-2}, particle retention: >25 μm, thickness: 205 μm, porosity: 3.7 s	Detection of nitrite ion (NO_2-) (Li et al. 2010)
Nitrocellulose membrane	Granular structure, reasonably uniform pore size, particle retention: >0.02 μm	Lateral flow assay, e.g. immobilization of antibody/antigen (Yen et al. 2015), DNA, enzymes, etc.
Cellulose glossy paper	Made of cellulose fiber blended with an inorganic filler, non-degradable and relatively smoother surface	Detection of ethanol (Arena et al. 2010)
Bioactive paper	Obtained by the modification of paper matrix with biomolecules	Detection of Pathogens (Pelton 2009)

contact angle between the liquid and pore wall. In actual condition, membranes contain a range of pore sizes known as pore size distribution (PSD) of the membrane. Using the PSD, the capillary flow rate can be estimated as a function of the pore size (Yetisen et al. 2013).

Porosity, a non-dimensional quantity is an inherent characteristic of the porous paper substrate. It is a measure of the extent to which its surface allows the penetration of a gas or liquid. It represents the volume fraction of open space in the paper substrate (Singh et al. 2017). Porosity can be obtained experimentally by measuring the volume of liquid absorbed by the paper substrate (Parolo et al. 2013). In this method, a paper strip of specific dimensions is dipped into a known amount of fluid such as phosphate-buffered saline (PBS). Then, the change in volume of liquid (pore volume) is measured after the strip is taken out. Finally, porosity can be calculated empirically by calculating the ratio of pore volume and the total volume of the paper.

The permeability of a porous substrate is a physical parameter that characterizes the degree of resistance to fluid flow (Rudman and Patterson 2001). The air permeability is partially dependent on the porosity. For fiber porous materials (Fig. 2.1a), it can be estimated using the following empirical relation (Van der Westhuizen and Du Plessis 1996):

$$k = r_f^2 \frac{\pi\varphi(1-\sqrt{1-\varphi})^2}{24(1-\varphi)^{1.5}} \tag{2.1}$$

where r_f is the average fiber radius. For random fibrous media, the permeability can also be calculated using the following permeability-porosity correlation (Nabovati et al. 2009).

$$k = C_1 r_f^2 \left(\sqrt{\frac{1-\varphi_c}{1-\varphi}} - 1 \right)^{C_2} \tag{2.2}$$

where φ_c is the critical value of porosity, and C_1 and C_2 are the geometrical factors of the network. Also, for the granular isotropic porous materials such as nitrocellulose membrane (Fig. 2.1d), permeability can be predicted through the Kozeny-Carman equation (Bear 2013; Choi et al. 2016).

$$k = \frac{d^2\varphi^3}{180(1-\varphi)^2} \tag{2.3}$$

2.2 Fluid Transport

Paper microfluidics deals with the fluid flow without any requirement of external force. The capillary action is the driving force for the passive movement of fluid through the paper substrate. The interaction between the paper and contacting surface of the fluid is generally governed by two opposite forces, i.e., cohesive force and adhesive force. When fluid is brought in contact with the paper, there is an intermolecular interaction between liquid molecules at the liquid–air interface (cohesion) and also between the solid-liquid interfaces (adhesion). The adhesive force is responsible for spreading of the liquid on to the porous substrate while the cohesive force such as surface tension is responsible for the reduction in the area of the liquid-air interface. So, the fluid flow occurs only when the effect of adhesion surpasses that of cohesion. The wicking depends on various physical and geometrical properties of porous media such as paper materials, the structure of the paper, pore size, permeability, paper size and shape and also on the physical properties of the liquid. In general fluid transport can be categorized in two groups namely, wet-out process and the fully wetted flow. In the first kind of flow, the fluid front is wicking along the dry porous media and can be modelled using the classical Lucas-Washburn equation. In the second case, the fluid transport occurs along the wetted porous media and is governed by the Darcy's law.

2.2.1 Classical Lucas-Washburn Equation (Capillary Flow)

Let us consider a rectangular porous paper strip of length L, uniform width w and thickness h. r_a is the average pore radius of the paper. When a small amount of liquid is dropped onto the paper substrate, three types of interfacial layer is formed due to surface tension, i.e., solid-liquid interface, liquid-vapor interface, and vapor-solid interface. The relationships between these interfacial surface tensions in an equilibrium condition (Fig. 2.2a) may be described as $\gamma_{SL} + \gamma_{LV} \cos\theta = \gamma_{SV}$, where γ_{SL},γ_{LV} and γ_{SV} are the interfacial surface tension (energy per unit area) between solid-liquid, liquid-vapor and vapor-solid interface respectively, and θ is the angle between the tangent of the liquid-vapor interface and the surface at the point of contact, known as contact angle. The contact angle is a measure of the shape of a liquid droplet on a solid surface. Generally, there are two types of liquid droplets, i.e., 'wetting' droplet $(\theta < 90°)$ and 'nonwetting' droplet $(\theta > 90°)$ and depending on its value, the surface can be either hydrophilic (liquid can wet the surface) or hydrophobic (liquid repellent surface). The chemical composition of the solid surface and liquid are the critical parameters for the contact angle.

The capillary pressure which is equal to pressure difference at liquid-solid (paper) interface in the wetted region is governed by the Laplace pressure (Washburn 1921),

$$\mathrm{P_c} = \frac{2\gamma\cos\theta}{\mathrm{r_a}} \tag{2.4}$$

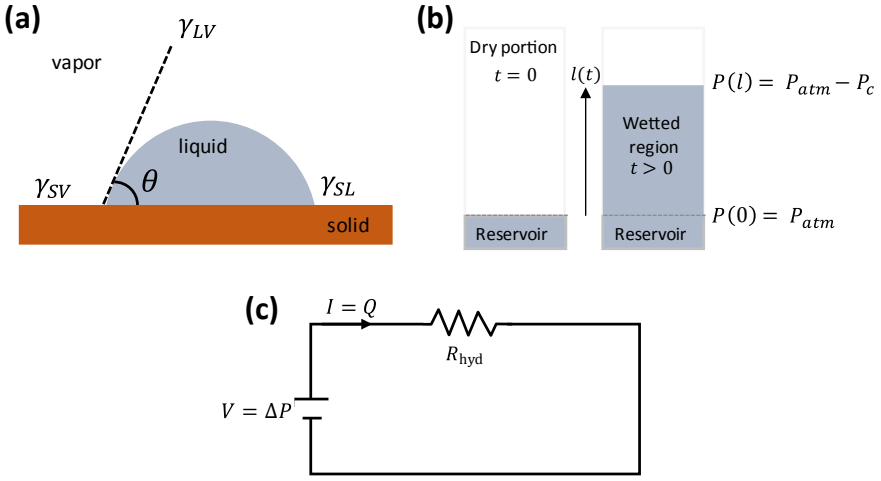

Fig. 2.2 **a** Surface tension force balance at a three-phase contact line between a liquid droplet, its vapor, and a non-deformable solid surface. **b** Schematic of fluid flow in a paper strip (porous media) through capillary action. **c** Electrical circuit analogy (Ohm's law) for fluid transport in a paper-based fluidic circuit

where γ is the interfacial surface tension of the liquid, and r_a is the average pore radius. For the dynamic systems, the contact angle may not have a static value and depends on the meniscus velocity (the Capillary Number Ca). (Fries and Quéré 2010) The dynamic contact angle can be determined by an empirical relationship proposed by Jiang et al. (1979) (based on data by Hoffman 1975),

$$\frac{\cos\theta_d - \cos\theta}{\cos\theta + 1} = -\tanh\left(4.94\mathrm{Ca}^{0.702}\right) \tag{2.5}$$

At the onset of capillary flow, the average liquid velocity is very small and assumed to be zero and therefore, the contact angle remains constant. Now, by neglecting the gravity effect and the inertial terms and combining Eq. (2.4) and the Hagen-Poiseuille's equation for fully developed flow with capillary action caused by surface tension, the penetration/wetted length of one-dimensional single phase fluid transport in paper substrate can be described by the classical Lucas-Washburn equation as (Mendez et al. 2009),

$$l(t) = 2\sqrt{\frac{k\gamma\cos\theta}{\varphi\mu r_a}}\sqrt{t} \tag{2.6}$$

where $l(t)$ is the length of wetted region of the paper after time t, k is the permeability of the paper which is a measure of how easily fluid can flow through a given paper substrate and depends upon pore size and pore geometry, μ is the fluid viscosity and t is the liquid penetration time. Equation (2.6) is derived under some assumptions such as single-phase homogenous one-dimensional fluid flow in a constant temperature and ambient pressure, perfectly wetting fluid, a uniform cross-section of the porous surface, no frictional or inertia losses due to the displacement of air by liquid, no viscosity induced pressure losses, etc. Since γ, θ, μ and r_a are all constant for a given fluid wicking through a given paper substrate, so, the wicking length is proportional to square root of time ($l(t) \propto \sqrt{t}$), i.e., the fluid-front velocity decreases with time because of the flow resistance offered by the surface of porous media (Fu et al. 2011).

The Lucas-Washburn equation is a fundamental equation for modelling the fluid flow in porous media. By considering the effect of tortuosity on the capillary flow, the Lucas-Washburn equation can be expressed in following form,

$$l(t) = \sqrt{\frac{r_a\gamma\cos\theta}{2\mu\tau^2}}\sqrt{t} \tag{2.7}$$

where tortuosity $\tau = (L_e/L)^2$, L_e effective path length between two intermittent points in the liquid and L straight path length. The value of τ is always greater than one.

2.2.2 Darcy's Law for Fluid Flow

The fluid flow through the fully wetted paper substrate is described by the Darcy's law. It was first established experimentally by Henry Darcy in 1856. By investigating the flow of water through sand, it was found that the viscous pressure loss can be described by

$$\boldsymbol{u} = -\frac{k}{\mu}\nabla P \tag{2.8}$$

where $\boldsymbol{u}$ is the average fluid velocity vector, k is the medium permeability, μ fluid viscosity, and P is the pressure. On further expanding, the above equation, the imbibition rate of the fluid ($\widehat{u}$) through the paper substrate can be determined by Darcy's law, as per the following equation,

$$\widehat{u} = \frac{k_i \Delta P}{\mu l(t)} \tag{2.9}$$

where $k_i = \frac{k}{\varphi}$ is the interstitial permeability of the paper strip, $\varphi = 1 - \frac{m}{\rho_c h}$ is the porosity of the medium(Hong and Kim 2015), m is the basis weight, ρ_c and h are the density and thickness of the porous substrate, and ΔP is the pressure difference over the wetted region known as Laplace pressure. Darcy's law describes the flow rate Q for a fluid through a porous medium (Fig. 2.2b) under a pressure differential ΔP. The relationship can be derived from the Navier–Stokes equation (Fu et al. 2011),

$$\mathrm{Q} = -\frac{kwh}{\mu L}\Delta P \Rightarrow \Delta P = -\frac{\mu L}{kwh}Q \tag{2.10}$$

In this expression, $\Delta P = P(0) - P(l)$, where $P(0)$ is the pressure at x = 0, and $P(l)$ is the average capillary pressure at the fluid front, $w \times h$ is the cross-sectional area of the paper strip. The flow domain for the considered model system also includes a hydrodynamic load term, which involves a generic flow resistance $R_{hyd} = \frac{\mu L}{kwh}$ at satisfies,

$$Q = -\frac{\Delta P}{R_{hyd}} \tag{2.11}$$

Equation (2.11) is an analogous to the Ohm's law of an electric circuit,$\Delta V = RI$, where I is the electric current, R is the electrical resistance and ΔV is the potential drop (Fig. 2.2c). In hydrodynamic systems the volumetric flow rate, Q is the volume per unit time, while in electric system current is the charge per unit time. Also, ΔP (energy per volume) is analogous to potential drop (energy per charge).

Table 2.2 List of the relevant dimensionless numbers for paper-based microfluidics

Numbers	Equations	Forces	Characteristics
Capillary number (Ca)	$\frac{\mu v}{\gamma}$	$\frac{\text{viscous}}{\text{surface tension}}$	Describes the capillary flow
Weber number (We)	$\frac{\rho v^2}{\gamma/r_a}$	$\frac{\text{dynamic pressure}}{\text{capillary pressure}}$	Predicts the disruption of an interface under the action of inertial forces
Reynolds number (Re)	$\frac{\rho v d}{\mu}$	$\frac{\text{advective mass transport}}{\text{momentum transport}}$	Describes the flow regime. Predicts whether the system will be dominated by viscosity or momentum
Schmidt number (Sc)	$\frac{\mu}{\rho D}$	$\frac{\text{momentum transport}}{\text{diffusive mass transport}}$	Describes the mass-momentum transfer
Péclet number (Pe_L)	$\text{Re} \times \text{Sc} = \frac{v}{D/d}$	$\frac{\text{advective mass transport}}{\text{diffusive mass transport}}$	Describes the diffusion and appears in mixing phenomena

The Darcy law is only valid for small Reynolds numbers where convective terms can be neglected and viscous terms dominate. Due to the varying pore geometries of the paper, no specific critical pore Reynolds number can be provided for the validity of the Darcy law, as they vary between 0.1 and 75 (Dullien 2012). Table 2.2 lists the some important dimensionless numbers related to paper-based microfluidics.

2.2.3 *Fluid Transport in the Porous Media of Varying Cross Section/Arbitrary Shape*

Apart from the straight channels, various researchers have proposed alternative models to govern the fluid transport through the paper strips with different shapes with specific dynamics such as circular (Conrath et al. 2010), trapezoidal (Mendez et al. 2009), sector-shaped (Wang et al. 2013), multi-section medium (Shou et al. 2014), and other arbitrary shapes (Elizalde et al. 2015). For n number of connected rectangular paper strips with a piecewise varying cross-section (Fig. 2.3a), the volumetric flow rate can be evaluated using the equation,

$$Q = -\frac{k\Delta P}{\mu \sum_{i=1}^{n} L_i/w_i h_i} = \frac{\Delta P}{\sum_i^n R_{hyd_i}} \tag{2.12}$$

The total time required to transport the fluid through the entire length (L) of the paper strip is given by

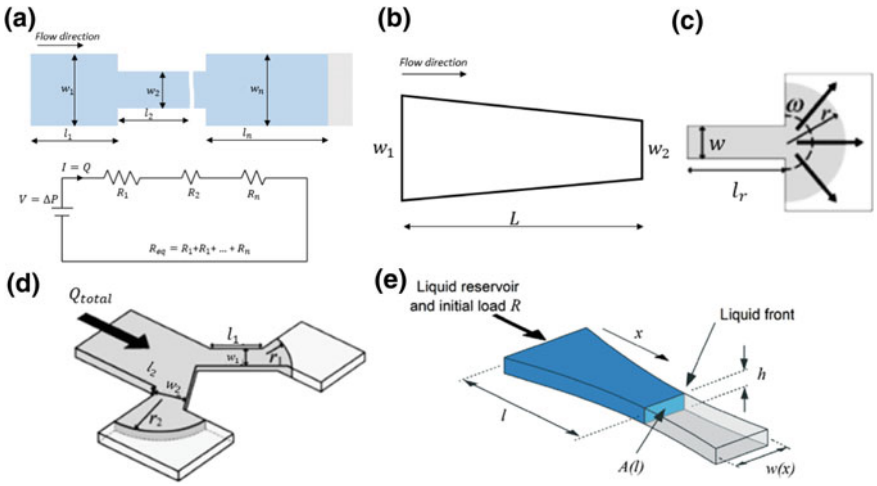

Fig. 2.3 Schematics of paper strips with various shapes. **a** Piecewise varying width, **b** Trapezoidal, **c** Fan-shape, **d** Y-shape, and **e** Paper strip with non-uniform cross-section. Reproduced from Elizalde et al. (2015) with permission from the Royal Society of Chemistry

$$t' = \frac{\mu}{k\Delta P}\left(\sum_{i=1}^{n} w_i L_i\right)\left(\sum_{j=1}^{n} \frac{L_j}{w_j}\right) \tag{2.13}$$

The fluid flow time in paper substrate of trapezoidal shape (Fig. 2.3b) can be estimated using the following equation (Shou et al. 2014),

$$t = \frac{L^2 w_1^2 C}{4(w_1 - w_2)^2}\left\{\left(\frac{w_2^2}{w_1^2}\right)\left[\ln\left(\frac{w_2^2}{w_1^2}\right) - 1\right] + 1\right\} \tag{2.14}$$

where C is a constant term equal to $\mu/\left(J(s)\gamma\cos\theta\sqrt{k/\varphi}\right)$, where $J(s)$ is a Leverett J-function of water saturation. The volumetric flow rate for a straight paper channel connected with a fan-shaped expansion zone (Fig. 2.3c) can be calculated by (Mendez et al. 2009),

$$Q = \frac{kh\Delta P}{\mu}\left[\frac{l_r}{w} + \frac{1}{\omega}\ln\left(\frac{2r}{w}\right)\right]^{-1} \tag{2.15}$$

where l_r and w are length and width of the straight portion of the paper, ω is the central angle of the fan and r is the radial distance of imbibition in the expansion zone. Equation (2.15) can be further modified by taking into account the hydrophobic outer boundaries into paper strip (Hong et al. 2016).

$$Q = \frac{khA\Delta P}{\mu}\left[\frac{l_r}{\alpha_r w} + \frac{1}{\alpha_s \omega}\ln\left(\frac{2r}{w}\right)\right]^{-1} \tag{2.16}$$

where α_r and α_s reduction coefficients corresponding to the straight channel and expansion zone, respectively. The reduction coefficient (α) is defined as the ratio of imbibition rate through a paper channel with and without the wax boundaries. The value of α_s is be assumed to be 1 for a large channel width (>3 mm). Also, since $\alpha_r < 1$, so Eq. (2.16) suggests the volumetric flow rate is reduced in a paper channel with wax boundaries. The first term within the bracket in right-hand side of Eq. (2.16) is associated with flow resistance in the straight channel (R_s) while the second term is associated with the flow resistance in expansion zone (R_e). Since the value of r in flow resistance term for expansion zone varies with wetted length and time t, so the flow rate would be unsteady this term is dominant. However, if $R_s \gg R_e$ then fluid transport through the channel can be assumed as a steady flow.

The volumetric flow rate for flow bifurcation system as shown in Fig. 2.3d can be written as $Q = Q_1 + Q_2$. It can also be described in terms of hydrodynamic flow resistance.

$$\frac{Q_2}{Q_1} = \frac{R_1}{R_2} = \frac{\frac{l_1}{\alpha_1 w_1} + \frac{1}{\omega}\ln\left(\frac{2r_1}{w_1}\right)}{\frac{l_2}{\alpha_2 w_2} + \frac{1}{\omega}\ln\left(\frac{2r_2}{w_2}\right)} \tag{2.17}$$

For a specified fluid velocity or mass transport rate the cross-sectional shape of the paper strip can also be estimated using the empirical model developed by Elizalde et al. (2015). Figure 2.3e shows the schematic of the flow domain used for modeling. The time required for imbibition of fluid in a paper strip of varying cross-section $A(x)$ and length (l) is described as:

$$\frac{kR}{\mu}\int_0^l A(l')dl' + \int_0^l \left[A(l')\int_0^{l'}\frac{dx}{A(x)}\right]dl' = Dt \tag{2.18}$$

where R is the flow resistance, and D is the diffusive coefficient, a constant term in this model which is equal to $k\Delta P/\mu$. Applying the Leibniz rule, Eq. (2.18) can be converted into following expression to obtain an explicit solution for the unknown cross-sectional profile $A(x)$ of the paper strip to attain a desired flow rate $Q(x)$:

$$A(x) = -\frac{Q(x)^2}{D}\left[\frac{dQ(x)}{dx}\right]^{-1} \tag{2.19}$$

By putting the $Q(x) = v(x)A(x)$ the equation was further expanded into:

$$A(x) = \frac{v_0 A_0}{v(x)} \exp\left[-\frac{1}{D}\int_0^x v(x')dx'\right] \tag{2.20}$$

where $v(x)$ is the velocity function, v_0 and A_0 are the velocity and cross-sectional area at x = 0 respectively. These equations can be used to determine the unknown cross-sectional shape required for a specified fluid velocity $v(x)$ and mass flow rate $Q(x)$.

2.2.4 Radial Fluid Transport in Porous Media

When a drop of liquid penetrates into the surface of the paper substrate for the first time, it wicks radially outward. Unlike, the cylindrical capillary, the liquid-vapor interfacial area continuously increases with time in radial flow. The radial penetration of liquid in thin porous media was theoretically studied for the first time by Marmur (1988). Since then, many researchers have theoretically and experimentally investigated the radial capillary transport mechanism in the porous substrate (Conrath et al. 2010; Xiao et al. 2012; Wang et al. 2013; Kumar et al. 2016). Conrath et al. (2010) developed a theoretical model to estimate the time required to reach the liquid by radial wicking in a paper strip of desired radius R (Fig. 2.4a) which is given as:

$$t(r) = \frac{r_a \mu \varphi R^2}{8\gamma k h \cos\theta}\left[\ln\left(\frac{r(t)}{r_0}\right)^2 + \left(\frac{r_0}{r(t)}\right)^2 - 1\right] \tag{2.21}$$

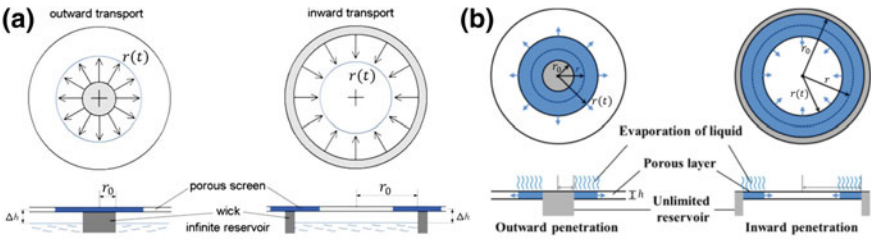

Fig. 2.4 **a** Schematic illustration for outward (left side) and inward (right side) capillary fluid transport. Reprinted from Conrath et al. (2010) with permission from ©Springer Science + Business Media B.V. 2009. **b** Radial capillary penetration incorporating evaporation. Reprinted with permission from Liu et al. (2016) ©2016 American Chemical Society

where $r(t)$ and r_0 are the radii of the wetted fluid front at time t and the liquid reservoir, respectively. This relation can be further utilized to predict the volumetric flow rate of circular wicking front in a paper substrate,

$$Q = \frac{2\pi k\gamma h \cos\theta}{\mu \ln\left(\frac{r(t)}{r_0}\right)^2} \tag{2.22}$$

If the ambient condition like, evaporation effect is taken into account, then the radius up to which the fluid penetrates radially in thin layer porous medium (Fig. 2.4b) for the given evaporation rate can be determined as (Liu et al. 2016),

$$\left(\frac{r_c}{r_0}\right)^2 \left[1 - 2\ln\left(\frac{r_c}{r_0}\right)\right] = 1 - \frac{\dot{m}_e^c}{\dot{m}_e} \tag{2.23}$$

where R_c is the critical radius, $\dot{m}_e$ and $\dot{m}_e^c$ are actual and critical evaporation rate of liquid respectively. The critical evaporation rate which depends on the liquid properties, physical and geometrical parameters of the porous medium, can be estimated using the following equation,

$$\dot{m}_e^c = \frac{4\sigma\cos\theta_s\rho}{\mu}\frac{2k}{r_{eff}}\frac{h}{r_0^2} \tag{2.24}$$

where r_{eff} is the effective pore radius of the porous medium.

2.2.5 Diffusion-Based Fluid Transport

In case of mass transport in the paper substrate, the behavior of fluids can't be fully predicted by the Navier-Stokes equation as it does not consider diffusion. The motion of a chemical or biochemical species in the wetted paper matrix are described with a set of governing equations.

Fick's first law of diffusion:

$$J_i = -D\frac{\partial C_i}{\partial x} \tag{2.25}$$

Fick's second law of diffusion:

$$\frac{\partial C_i}{\partial t} = D\nabla^2 C_i \tag{2.26}$$

Here, the quantity J_i is the flux of solute i, C_i is the concentration of solute i in mass per unit volume. The quantity D is the diffusion coefficient of the solute in the carrier fluid, which can be estimated by the Stokes-Einstein relation. $D = \frac{kT}{6\pi\eta r_h}$, where k is a Boltzmann constant, T is the temperature, η fluid viscosity, and r_h is the hydrodynamic radius of the solute. In the equation, the term in the numerator represents the kinetic energy of the molecule, while the denominator represents the friction force acting on the molecule. Fick's second law governs the change in concentration field with time due to the diffusion. For the capillary pressure driven flow, the Fick's second law can be formulated as

$$\frac{\partial C_i}{\partial t} + \boldsymbol{u}\nabla C_i = D\nabla^2 C_i \tag{2.27}$$

This is known as a convection-diffusion equation. The diffusive flow field can be modeled using the above equation with specified boundary conditions. The mass transfer in the moving fluid through the porous media are generally governed by the molecular diffusion and by the fluid velocity field. The Péclet number (Pe_L) plays a vital role in mass transport phenomena. If $Pe_L < 100$, the mass transport is controlled by the molecular diffusion and if $Pe_L \geq 100$, mass transport is controlled by the average fluid velocity (Piquemal 1993).

2.2.6 *Lateral Flow Immunoassay (LFIA)*

Lateral flow assay (LFA) based POC devices is the fastest growing technology for qualitative and quantitative analysis. According to the Markets and Markets report, the lateral flow assay had covered 28.2% of the global market for clinical diagnostics of infectious diseases in 2016. The LFIA based market is expected to reach $8.24bn by 2022 from an estimated $5.55bn in 2017, at a compound annual growth rate of 8.2%. (Lateral Flow Assay Market worth 8.24 Billion USD by 2022 August 2017) From the past several years, the lateral flow-based immunoassays have garnered much attention in diagnostics of infectious diseases such as malaria, dengue (Wang et al. 2014; Zhang et al. 2015; Kumar et al. 2018), Ebola (Yen et al. 2015), HIV, Zika (Bosch et al. 2017), etc.

Components and Principles

The LFIA biosensing platform mainly consists of four components namely, sample pad, conjugate pad, nitrocellulose membrane, and an absorbent pad, which are all held together on a plastic backing card using a pressure-sensitive adhesive with a specified overlap onto one another (Fig. 2.5a). The component overlaps allow the unrestricted capillary flow of the samples from the sample pad to the absorbent pad.

Sample pad's primarily role is to transport the target analyte to other components of LFA with high efficiency and providing the homogeneous distribution of the sample onto the conjugate pad. It also controls the fluid flow rate. It is generally

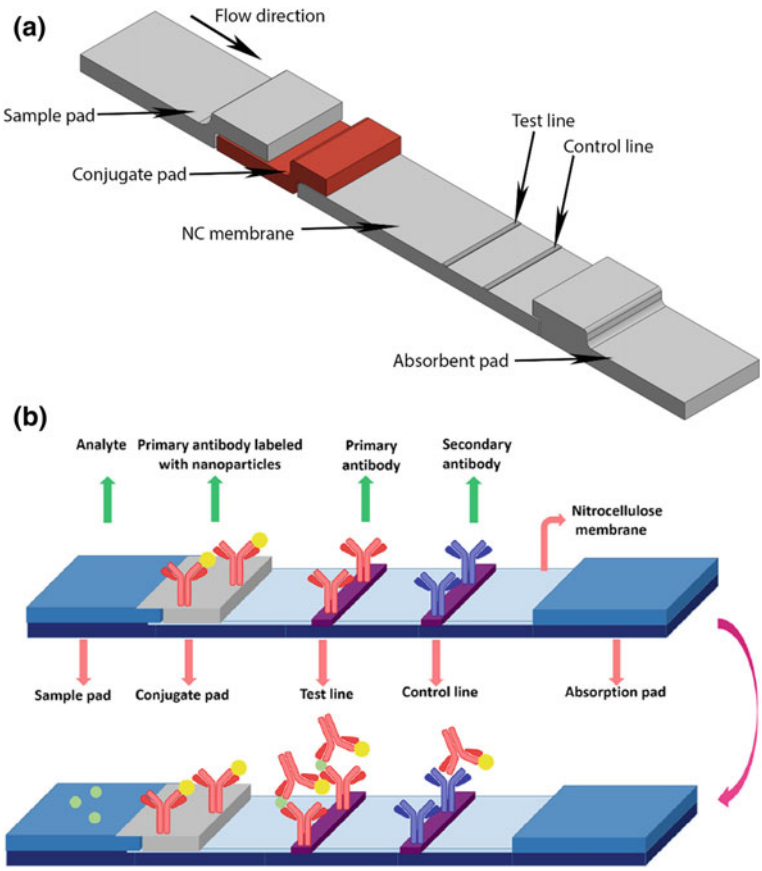

Fig. 2.5 **a** Typical configuration of the lateral flow test strip. **b** Schematic of sandwich format of LFIA. Reprinted from Bahadır and Sezgintürk (2016) with permission from Elsevier B.V.

made of cellulose, rayon, glass fiber, woven meshes, Whatman filter paper grade 1, etc. The choice of material for the sample pad depends on the sample types which is to be handled.

The role of conjugate pad is to allow the immobilization of nanoparticles-Ab conjugates, holds the conjugates for a long time in dried form and releasing the labeled conjugate efficiently upon contact with the incoming aqueous sample from sample pad. So, the hydrophilic porous materials with high flow rates such as non-woven filters, glass fiber, cellulose, rayon, polyesters, polypropylene, etc. are used in the fabrication of conjugate pad.

The membrane is an essential and critical component of the lateral flow test strip. The primary purpose of the membrane is to allowing its surface for binding of proteins at the test line and control line. Nonspecific adsorption of proteins at the test and control line may results in reduced sensitivity, so an ideal membrane should

have the high protein-binding capacity. Nitrocellulose membrane is most widely used material for the membrane fabrication. It binds protein by electrostatic interaction between strong dipole of the nitrate ester with the strong dipole of the peptide bonds of the protein. Various types of NC membranes are available in the market with specific capillary flow time.

The absorbent pad is the engine of a lateral flow test. It helps in maintaining the capillary flow rate through the nitrocellulose membrane and prevents the backflow of excess reagents by soaking. It also allows the use of larger target sample volumes resulting in a higher sensitivity of the device. The materials used for the fabrication of absorbent pad is highly absorptive. Cellulose filters are mostly used for the absorbent pad.

Although the lateral flow assay is the one-step process, the proper selection of materials for each component, its processing and assembly are very crucial. The optimization of experimental conditions is needed to develop a LFA test strip with excellent performance and high sensitivity (Bahadır and Sezgintürk 2016).

In a typical LFIA, the target analyte such as human blood, plasma, serum, urine, saliva, etc. is loaded onto the sample pad after which it migrates to the conjugate pad which contains pre-immobilized (dried) nanoparticles-antibody (Ab) conjugates. When the sample liquid reaches the conjugate pad (CP), the antigens (Ag) present in the sample bind with the conjugated NP–Ab to form NP–Ab-Ag complexes. These complexes continue to flow towards the test line by capillary action through a nitrocellulose membrane. The test line contains immobilized capture antibody specific to Ag on it. If an antigen is present in the sample, then the antibodies on the test line conjugate with the complex making a sandwich like structure (NPs-Ab-Ag-Ab). A distinct color change can then be observed at the test line due to localized surface Plasmon resonance (LSPR) of aggregated nanoparticles. Whereas, if antigens are not present in the sample, no sandwich structure is formed resulting in no color change at the test line. The control line is loaded with secondary antibodies specific to the primary antibodies conjugated to the NPs. Here, the bounded/unbounded conjugated NPs–Ab binds with secondary antibodies (anti-IgG) to demonstrate a color change (in both cases when the antigen is present in the sample or not). The change in color at the control line confirms the successful completion of the colorimetric assay. The role of the absorbent pad is to soak up the extra liquid.

Immunochemical Reaction Mechanism

In a typical lateral flow assay, the liquid sample containing specific target analyte migrates through the sample pad and conjugate pad and reaches to the test line in detection pad. During this fluid transport, several mechanisms such as convection, molecular diffusion, immunoreactions between antibodies and antigens on the surface of reporter particles play a significant role. The sensitivity of the LFA is directly proportional to the signal intensity at the test line. The signal intensity depends on several parameters such as the concentration of reporter particles, primary and secondary antibodies, analyte flow rate, and reaction time for the target analyte to form a complex in capture site. To improve the performance of LFAs,

these terms must be considered in theoretical modeling. From the past decade, several research groups have attempted to model the surface binding mechanism involving the molecular diffusion, advective transport (movement of reactants by the bulk motion of the liquid), and chemical reactions (Hansen et al. 2012; Aguirre et al. 2014). Normally, following immunoreactions, takes place in a standard lateral flow assay. When the sample migrates through the conjugated pad, the specific target analyte (A) binds with the conjugate reporter nanoparticles-Ab/Ag (P) to form reporter nanoparticles-analyte complexes (PA).

$$[A] + [P] \rightleftarrows [PA] \tag{2.28}$$

These complexes move ahead towards the test line by capillary action through the detection pad (typically made of the nitrocellulose membrane). Test line contains immobilized capture Ab/Ag-specific to the analyte on it. At the test line, the free analyte (A_f) and conjugated nanoparticles-analyte complex (PA) interact with capture Ab/Ag (R) to form complexes RA ($[A_f] + [R] \rightleftarrows [RA]$) and RPA ($[PA] + [R] \rightleftarrows [RPA]$) respectively. Additionally, unbounded reporter particles (P_i) may bind to complex RA to form the complex RPA ($[P_i] + [RA] \rightleftarrows [RPA]$). The control probe (C) (i.e., secondary Ab specific to the primary Ab) is immobilized in control line. Here, the concentration of above complexes are functions of space x and time t (i.e., $[C](x,t)$) and can be described by the convection–diffusion–immunoreaction equations as follows (Qian and Bau 2003):

$$\frac{\partial [A]}{\partial t} = D_A \frac{\partial^2 [A]}{\partial x^2} - u \frac{\partial [A]}{\partial x} - (F_{PA} + F_{RA}) \tag{2.29}$$

$$\frac{\partial [PA]}{\partial t} = D_P \frac{\partial^2 [PA]}{\partial x^2} - u \frac{\partial [PA]}{\partial x} - \left(F_{PA} + F^1_{RPA}\right) \tag{2.30}$$

$$\frac{\partial [P]}{\partial t} = D_P \frac{\partial^2 [P]}{\partial x^2} - u \frac{\partial [P]}{\partial x} - \left(F_{PA} + F^2_{RPA}\right) \tag{2.31}$$

$$\frac{\partial [P]}{\partial t} = F_{PA} \text{ and } \frac{\partial [RPA]}{\partial t} = F_{RPA} \tag{2.32}$$

where D_A and D_P are the molecular diffusion coefficients of the analyte and the reporter particles, u is the average fluid velocity. F_{PA}, F_{RA} and F_{RPA} are the rate of formation of the particle-analyte complex and capture Ab-analyte complex respectively. F^1_{RPA} and F^2_{RPA} are the rate of formation of capture Ab-analyte-detection Ab complex due to the interactions between ($[PA] + [R] \leftrightarrow [RPA]$) and ($[P] + [RA] \leftrightarrow [RPA]$) respectively. The diffusion coefficient (D) for proteins and nanoparticles can be estimated by the Stokes-Einstein equation (Filipe et al. 2010),

$$D = \frac{\kappa_B T}{6\pi\mu R} \tag{2.33}$$

Here, κ_B is the Boltzmann constant, T is fluid temperature, μ is the fluid viscosity, and R is the hydraulic radius of the analyte or signal particle. The color signal intensity in the test line is proportional to the concentrations of reporter nanoparticles-Ab/Ag $[P]$, conjugates $[PA]$, and sandwich complex $[RPA]$ which is given as (Liu et al. 2017),

$$S = [P] + [PA] + [RPA] \tag{2.34}$$

For a given concentration of analyte, the color intensity in the test line depends on the amount of reporter nanoparticles-analyte complex reaches at the test line (Kumar et al. 2018). The optimum concentration of complex (PA) can be estimated using the following expression (Ragavendar and Anmol 2012),

$$S[PA]_{opt} = 0.9\left\{A_0 + P_0 + k_{d1}/k_{a1} - \left((A_0 + P_0 + k_{d1}/k_{a1})^2 - 4A_0P_0\right)^{0.5}\right\} \tag{2.35}$$

where A_0 and P_0 are the initial concentration of analyte in sample and concentration of conjugated Ab. k_{a1} and k_{d1} are the equilibrium reaction constants. These constants can be estimated by following expressions (Berg and Purcell 1977; Zwanzig and Szabo 1991),

$$k_{a1,particle} = \frac{4\pi aDNk_a}{Nk_a + 4\pi aD} \tag{2.36}$$

$$k_{d1,particle} = k_d\left(1 - \frac{Nk_a}{4\pi aD + Nk_a}\right) \tag{2.37}$$

where a is the nanoparticle radius, N is the number of receptors on the surface of the nanoparticle, D is the diffusion coefficient for the ligand. k_a and k_d are the association rate for complex formation and the dissociation rate constant, respectively. The convection–diffusion–immunoreaction Eqs. (2.29–2.32) and (2.35) as mentioned above, can be used in the prediction of optimized test line location where the signal intensity is maximum. Several recent publications reported the numerical models for lateral flow assay. Berli and Kler (2016) developed a quantitate LFA model for predicting the relative concentration of antigen-antibody complex at the test line. Liu et al. (2017) developed a one-dimensional mathematical model to study the effect of report particle characteristics like the binding sites on the overall performance of LFAs. They successfully validated their model with the experimental results. Schaumburg et al. (2018) reported a three-dimensional multi-membrane model for lateral flow assays. Liu et al. (2018) developed a

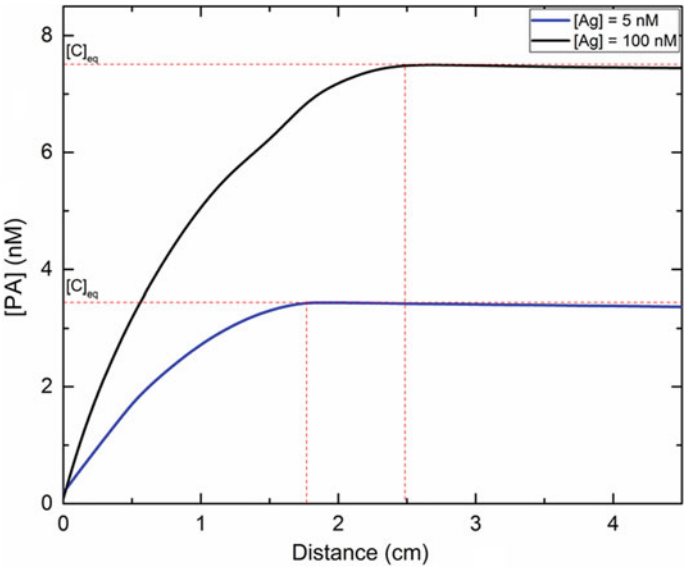

Fig. 2.6 Variation of the concentration of conjugated complex as a function of fluid transport distance along the nitrocellulose membrane for capillary flow time = 200 s/4 cm. Reprinted with permission from Kumar et al. (2018), ©Author(s)

semi-quantitative convection-diffusion-reaction model to investigate the effect of inlet reporter particle concentration, initial capture probe concentrations and association rate constant on overall performance of the lateral flow device. The association and dissociation rate constants were calculated by considering the kinetics of renaturation of nucleic acids. They were able to theoretically estimate the detection limit of the device and validated by direct comparison with experimental data.

In recent times, researchers have utilized the convection–diffusion–immunoreaction models to design the lateral flow assay for point-of-care diagnostic applications. Kumar et al. (2018) utilized the convection–diffusion–immunoreaction model to optimize the dimensions of the lateral flow device. The location of the test line was theoretically estimated and successfully validated by experimentation. The test line distance was optimized such that sufficient reaction time is given to the conjugated complex (PA) to achieve equilibrium concentration since, after that, no change in [PA] occurs (Fig. 2.6). Based on the theoretical study, they presented a unique lateral flow immunoassay device that used a tapered NC membrane and gold nanoparticles-graphene oxide (Au-rGO) nanocomposite as a detection label for highly sensitive detection of dengue NS1.

2.3 Summary

Paper-based microfluidic systems are one of the most widely used technology in the field of point-of-care diagnostics. The transport of fluid containing molecules, particles, analytes, etc. is a critical process in several paper-based microfluidic devices. The theoretical understanding of fluid transport phenomena helps in designing of a paper-based device. The capillary-based wicking phenomena of paper substrates theoretically eliminate the requirement of external equipment for fluid transport. This chapter provides a theoretical background of fluid transport in paper substrates, paper types, critical parameters involved in fluid flow, etc. This chapters also gives a brief overview of different kinds of governing equations used in designing of paper-based devices.

The present brief overview may aid in providing physically and synthetically intuitive guidance for the design of paper-based microfluidic devices such as dip-stick assays, lateral flow assays, etc. with a high sensitivity and wide working range.

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Chapter 3
Fabrication Techniques for Paper-Based Microfluidic Devices

Mohammed Rashiku and Shantanu Bhattacharya

Abstract Paper-based microfluidic devices (μPADs) offer numerous advantages like low cost, wide range of applications, ease of fabrication/operation, high wicking properties, biocompatibility, non-toxicity, and biodegradability over other analytical devices. We present various fabrication techniques for μPADs reported to date. The fabrication methods reviewed in this chapter can be briefly divided into two types: two-dimensional (2D) and three-dimensional (3D) methods. This chapter discusses the various fabrication methods that are well established over the years towards as well as novel and emerging methods for rapid and cost effective fabrication of paper based microfluidic. The various techniques that has been used for fabricating paper based microfluidic devices(μPADs), including photolithography, embossing, flexographic printing, plasma treatment, laser treatment, PDMS screen printing, wax screen printing, ink-jet printing, and two-dimensional shaping (using X–Y knife plotters) are discussed. A comparison between the fabrication techniques with relative advantages and disadvantages is also provided to make the selection of a method of fabrication easier for a particular application.

Keywords Paper-based microfluidics · μPADs · Photolithography · PDMS screen printing · Screen printing · Flexographic printing · Inkjet printing · Alkyl ketene dimer (AKD)

3.1 Introduction

Paper-based microfluidics is an upcoming domain wherein the focus is on creation of devices which are affordable, easily fabricated, simple, disposable and portable. The paper-based microfluidic devices may be single–analyte, multi-analyte, qualitative,

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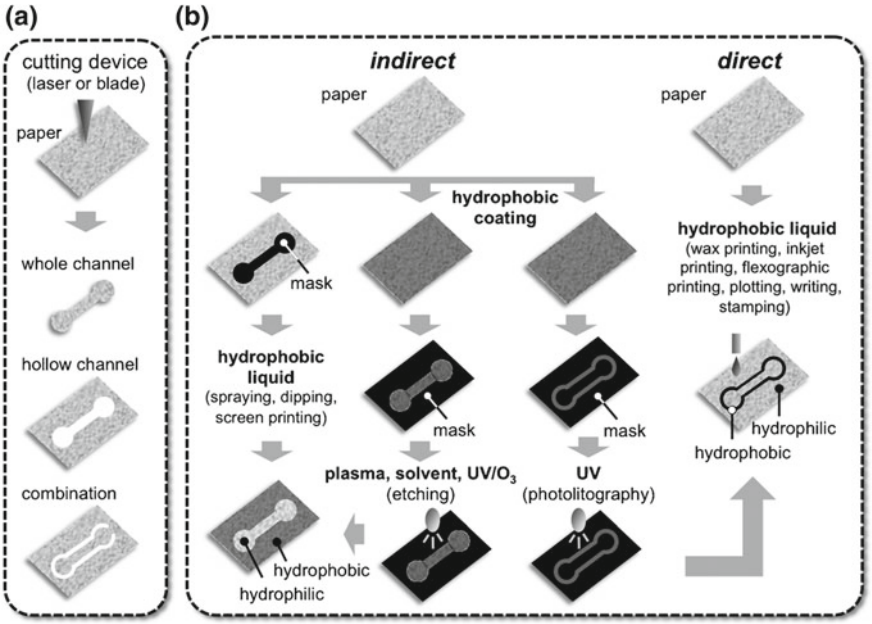

Fig. 3.1 Fabrication methods to form microfluidic channels **a** mechanical cutting and **b** treatment with hydrophobic materials. Reprinted with permission from Yamada et al. (2015). Copyright (2015) John Wiley & Sons, Inc.

semi-quantitative or quantitative (Xia et al. 2016). The growth in interest of paper based devices can be accredited to the favorable properties of paper. Paper is light weight, widely available, easily disposable, cheap and biocompatible (Kumar et al. 2016a). Paper eliminates the need for an external pump since liquid transfer in paper takes place by capillary action (Songok and Toivakka 2017). The fabrication methods to form microfluidic channels by creating patterns of hydrophobic regions on paper substrates can be broadly divided into two: (1) mechanical cutting and (2) treatment with hydrophobic materials (Kumar et al. 2016b). The fabrication methods involving treatment with hydrophobic materials can be further divided into two; direct and indirect. In direct method, the hydrophobic liquid is directly transferred to select regions on the hydrophilic paper substrate whereas in indirect method, a mask is used at various stages to selectively apply hydrophobic material. Direct methods include wax printing, inkjet printing, flexographic printing, laser cutting, etc. Indirect methods include plasma etching, photolithography, laser treatment, etc. These classifications are shown in Fig. 3.1.

A μPAD consists of paper, with hydrophilic channels separated by hydrophobic barriers created with different polymers. A digital camera or a mobile phone camera can be used to capture images. The obtained images may be sent to centralized laboratories for analysis (Sher et al. 2017). The fabrication of μPADs involves the separation of paper into hydrophilic and hydrophobic areas. The hydrophilic areas wick the sample and allow its transport through the paper to the test region where

the reagents are present. The hydrophobic regions direct the movement of the sample solely along the hydrophilic regions (Yamada et al. 2015). There are three strategies to create hydrophilic-hydrophobic contrast. The first strategy is to print hydrophobic materials onto paper substrate so as to create hydrophilic-hydrophobic contrast. Examples of this strategy include wax printing, inkjet printing [reagents such as alkenyl ketene dimer (AKD) and poly(styrene)], etc. The second strategy is transfer of hydrophobic materials into the paper through a metal mask with a definite pattern. The metal mask allows selective deposition of hydrophobic materials like wax, and permanent ink into the paper substrate. The third strategy is to selectively form the hydrophobic substrate hydrophilic or vice versa. This can be done by various methods like photolithography, laser treatment, and plasma treatment (Cai et al. 2014). Paper-based microfluidics can be employed in POC (point-of-care) diagnostics, environmental testing and food quality testing. μPADs have advantages like low sample and reagent consumption and fast analysis compared to conventional analytical methods (Cai et al. 2014).

3.2 Fabrication Methods

μPADs can be fabricated by using 2D or 3D methods. In 2D μPADs, the transport of fluids take place horizontally alone whereas in 3D μPADs, the transport of fluids take place both horizontally and vertically across various layers stacked on top of one another.

3.2.1 2D Fabrication Methods

2D fabrication methods create hydrophilic-hydrophobic contrast in a hydrophilic paper. These methods convert selected regions of paper (hydrophilic) to hydrophobic. The various methods used for fabrication of 2D μPADs are: (1) wax screen printing, (2) embossing, (3) flexographic printing, (4) inkjet printing, (5) laser treatment, (6) photolithography, (7) FLASH, (8) plasma treatment, (9) PDMS screen printing, and (10) Two-dimensional shaping (using X–Y knife plotters).

Wax screen printing

Wax screen printing (Dungchai et al. 2011) is a method in which patterns are imprinted onto the printing surface by forcing ink across a mesh. The screen can be made of silk wherein the design is produced with the areas where no transfer should take place blocked by using a coating of an impermeable film. Printing materials may include dyes and liquid inks.

In this method, the first step is to create a mask on a transparency film which can be subsequently used to create the screens. The mask is created by using CorelDraw or any other similar software and then printing it on a transparency film. A laser

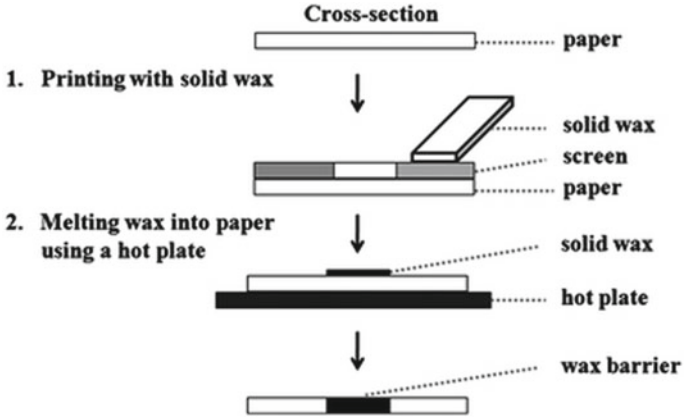

Fig. 3.2 Fabrication steps for wax screen-printing. Reprinted with permission from Dungchai et al. (2011). Copyright (2007) Royal Society of Chemistry

printer is used for printing the mask. Black areas on the transparency film correspond to hydrophobic regions, and colorless areas correspond to hydrophilic features. The screens are created using the transparency film. Wax is transported to the paper through the screen. To form hydrophobic barriers, the printed wax is melted on a hot plate. The paper absorbs the wax forming the hydrophobic barriers. The patterned paper is withdrawn from the hot plate and is cooled to room temperature. The screen can be reused by heating it for 60 s on a hot plate by placing it on a tissue paper to absorb the wax residue. In this method, melting temperature and time needs to be properly adjusted so as to minimize wax spreading. The method has various advantages like no need for sophisticated instruments, low cost, inexpensive and is highly reproducible which makes it suitable for production on a large scale. Figure 3.2 illustrates the steps in the fabrication of μPADs through wax screen-printing.

Embossing

The principal component in embossing (Bandarage et al. 2014) is omniphobic paper. Omniphobic paper (repels almost every liquid) is a paper whose surface is modified by treating with highly fluorinated alkylsilane (Salinization). salinization of paper opens up a wide range of applications in microfluidics. By combining the silanized paper (also called RF paper) with materials like polymer tapes or metal films, it is possible to produce many devices which are lightweight and have low cost. RF paper is highly hydrophobic, is lightweight, is inexpensive and is thermally and electrically insulating, which makes it a better choice for many applications compared to conventional materials.

Polymeric dies are designed using CAD software and produced through 3D printing (using ABS copolymer). Open channels are embossed on paper (Whatman #1 filter paper) using the polymeric dies. ABS dies are used for embossing since paper can be easily released from dies after embossing. To fabricate the open channel

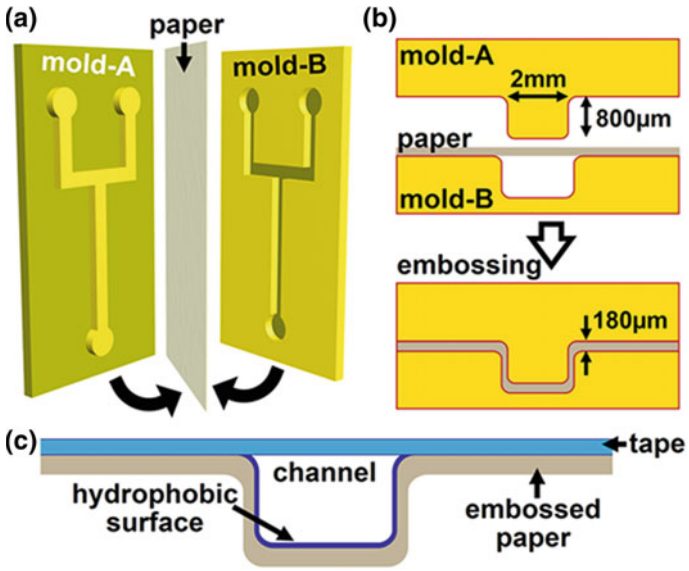

Fig. 3.3 Steps in embossing for fabrication of μPADs. **a** Compressing of sheet of paper between two plastic moulds. **b** Cross-sectional view of the embossing process. **c** Cross-sectional view of the paper channel with adhesive tape covering it on top. Reprinted with permission from Thuo et al. (2014). Copyright (2014) American Chemical Society

microfluidic device, a sheet of paper is sandwiched between two dies having complementary shapes as shown in Fig. 3.3a, b. A pressure of approximately 0.2 kg/cm^2 is applied on the paper through the polymeric dies to compress the paper into a channel. Ethanol (few drops, ~400 μL) is added to the paper to increase its moldability and reduce the glass transition temperature. Embossing is finished in approximately 2 s. Once done, the paper is dried in an oven (set at 60 °C) for 30 s. The channels are now covered with transparent adhesive tape made of PET or EVA or LDPE (see Fig. 3.3c). The inlet and outlet holes are laser cut on the adhesive tape.

3.2.2 Flexographic Printing

In flexographic printing, (Olkkonen et al. 2010) a substrate paper (usually chromatography paper) is attached to the impression roll. Ink is loaded into the ink tank, which is transferred to the anilox roll. Different concentrations of polystyrene solution in an organic solvent (such as toluene or xylene, which are most commonly used) are used as ink (hydrophobic barrier). The anilox roll is enveloped by a large number of cells, which determine the quantity (volume) of ink transported to the printing plate which has a pattern called relief pattern. Any excess ink leftover in the anilox roll is removed by the doctor blade. Once printing begins, the anilox roll rotates to the printing speed. The anilox roll is rotated four times and the plate roll

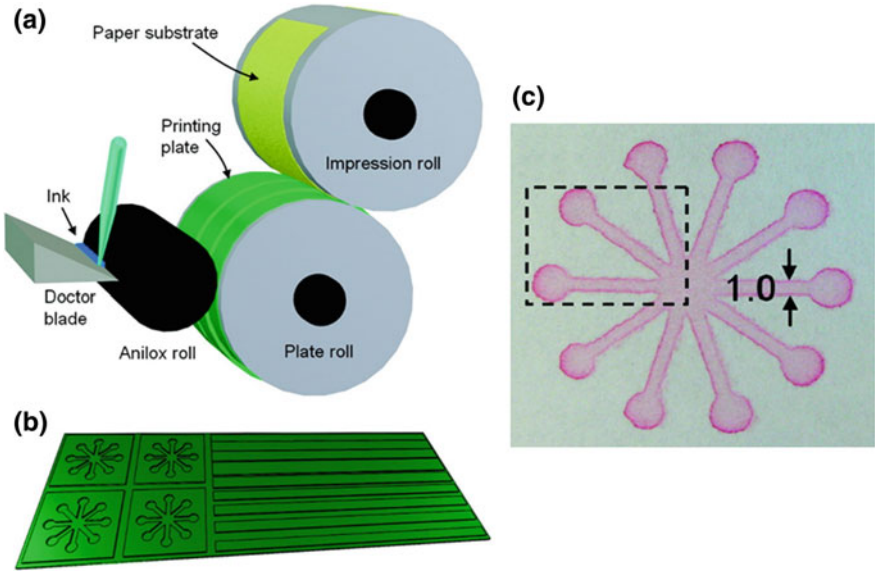

Fig. 3.4 **a** Schematic diagram illustrating flexographic printing. **b** Printing plate with relief patterns. **c** Microfluidic structures created using flexographic printing in chromatography paper. Reprinted with permission from Olkkonen et al. (2010). Copyright (2010) American Chemical Society

and impression roll are rotated once to deliver the ink onto the paper. The printing speed and pressure between rolls may have to be optimized to get adequate penetration of ink into the paper substrate. The pipet is set so that after printing one layer, ink is automatically added into the ink tank. There is a time lag of 5–10 s between printings of two layers. The anilox roll can print few ink layers (typically 10–20) without significant degradation in quality of print. The anilox roll has to be washed after completion of few ink layers, without which the quality of print starts to degrade. The number of layers printed influences the hydrophobic properties of the printed layers. An illustration of flexographic printing process is shown in Fig. 3.4.

Inkjet Printing

The method of creating hydrophobic barriers on paper by inkjet printing (Abe et al. 2008; Li et al. 2010) to produce microfluidic devices can be divided into two. The first method is called patterned hydrophobization while the second method is termed as patterned de-hydrophobization. In patterned hydrophobization, selected regions of filter paper are made hydrophobic by using a modified digital ink jet printer which dispenses an alkenyl ketene dimer–heptane solution to specific areas on the paper to generate a hydrophobic pattern. The filter paper with printed patterns are then cured by heating in an oven at a high temperature ($\sim$100 °C) for a short period ($\sim$5–10 min). An ink jet printed paper produced by patterned hydrophobization is shown in Fig. 3.5.

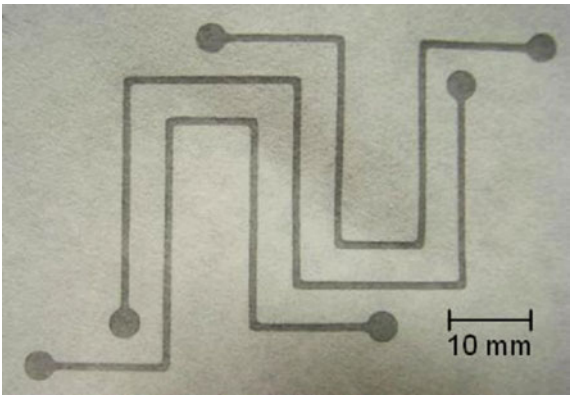

Fig. 3.5 Ink jet printed channels and zones defined by the hydrophilic regions separated from the hydrophobic regions. Reprinted with permission from Li et al. (2010). Copyright (2010) Elsevier

In the case of patterned de-hydrophobization, the whole filter paper is first soaked in a hydrophobic polymer solution and then specific areas are etched to locally dissolve the hydrophobic polymer at select regions. As the first step, the filter paper is immersed in a hydrophobic polymer solution (1 wt% solution of polystyrene in toluene, an organic solvent) and leave it for 2 h. The selection of polystyrene solution in toluene is justified due to its suitability for inkjet etching and high hydrophobicity. Take the filter paper out of the hydrophobic polymer solution and allow it to dry at room temperature for 15 min. Dip coating is used instead of spin coating to ensure three dimensional hydrophobicity. Spin coating only leads to a surface coating. Once the drying is complete, toluene (ink) is applied onto the hydrophobized filter paper using the inkjet printer. Toluene (organic solvent) dissolves polystyrene at selected regions. The excess polymer gets collected outside the liquid droplets. This is due to the "coffee ring effect" that occurs on vaporization of toluene. This is absorbed by the surrounding paper. The toluene droplets are continually applied. This turns the selected hydrophobic regions once again hydrophilic. A dye is doped with the polystyrene solution to view the inkjet printing process. An illustrative drawing of the process is provided in Fig. 3.6. The chemical sensing inks can also be delivered to the sensing area using the same inkjet printer.

Laser treatment

In this method, (Chitnis et al. 2011) a hydrophobic surface coated paper is treated with laser to modify specific regions of the coated paper from hydrophobic to hydrophilic. Wax paper, palette paper or parchment paper may be used as the starting material for the process. A CO_2 laser is utilized to form hydrophilic patterns.

Hydrophobic surface coated paper is spread and placed on the laser platform. A CO_2 laser cutting system is used for surface treatment. The necessary pattern (hydrophilic areas) is created using Coreldraw or any similar software, which is

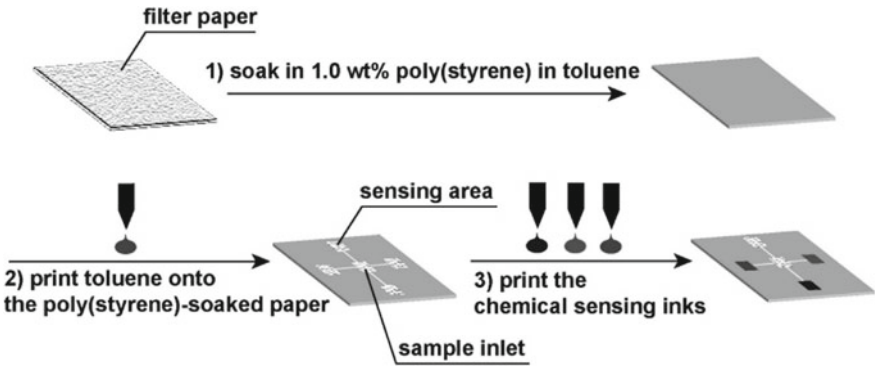

Fig. 3.6 Schematic drawing of the inkjet-printing process for fabricating microfluidic multianalyte chemical sensing paper. Steps 2 (patterning) and 3 (chemical sensing reagent application) are carried out on the same inkjet printer. (Pen symbol shows inkjet printing). Reprinted with permission from Abe et al. (2008). Copyright (2008) American Chemical Society

Fig. 3.7 Schematic diagram showing laser treatment method for fabrication of μPADs

then printed on the paper through raster scanning of laser beam. The operational parameters like the laser power and scanning speed are kept at a certain value so as to avoid cutting of paper. Typically, a low laser power and high scanning speed are required. A simplified diagram showing the steps in laser treatment process is shown in Fig. 3.7.

Photolithography

In this method, (Martinez et al. 2007) chromatography paper is soaked in a photoresist (SU-8 2010) for a small period of time (∼30 s). SU-8 is a negative photoresist. In case of negative photoresist, the parts which are exposed to UV light become cross-linked. The unexposed portions are dissolved during development. The soaked chromatography paper is spun at a high speed (∼2000 rpm) for 30 s, and baked in an oven (set at 95 °C) to eliminate cyclopentanone present in the photoresist. The photoresist is now exposed to UV light (405 nm, 50 mW/cm^2) for 10 s through a photomask. A photomask allows light to pass through in a defined pattern. The photomask can be created on a transparency film using inkjet printing, using laser engraving, using a photocopier, or even drawn by hand. The photomask was aligned properly with the photoresist. Once exposed, the paper is baked again

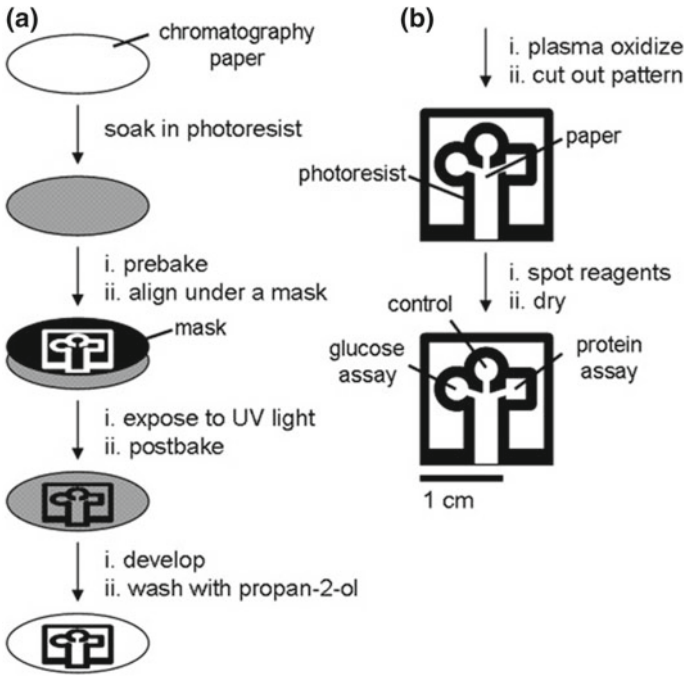

Fig. 3.8 Diagram showing the photolithography process. Reprinted with permission from Martinez et al. (2007). Copyright (2007) John Wiley & Sons, Inc.

at 95 °C for 5 min. This baking causes cross-linking of the portions exposed to UV light. The unexposed portions are removed during development. In the development phase, the paper is soaked in propylene glycol monomethyl ether acetate (PGMEA) for 5 min and is washed with propan-2-ol. The entire surface is treated with oxygen plasma for 10 s to remove any residual resist and to improve the hydrophilicity of the paper. The whole process can be understood more easily from Fig. 3.8.

FLASH (Fast Lithographic Activation of Sheets)

FLASH (Martinez et al. 2008b) is a fabrication method which has its foundations on photolithography. The basic instruments needed for FLASH are a UV lamp and a hotplate. Unlike photolithography, there is no need for clean room in FLASH. FLASH method can be effectively executed even in sunlight if UV lamp and hotplate are unavailable. With this method, up to 200 μm small hydrophilic channels can be easily patterned in paper. Photomasks are prepared as explained before.

In FLASH process, photoresist is poured and spread equally throughout the paper. Once the photoresist is absorbed by paper, it is baked at 130 °C for 5–10 min on a hotplate to vaporize the propylene glycol monomethyl ether acetate (PGMEA) present in the photoresist. The paper is cooled to room temperature.

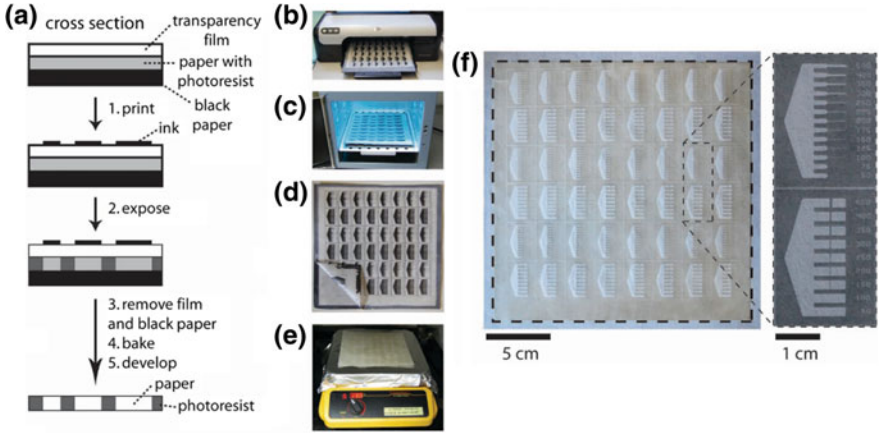

Fig. 3.9 **a** Schematic diagram of FLASH process. **b** Direct printing of microfluidic channel design onto FLASH. **c** Exposure of FLASH paper (UV light). **d** Removal of the paper. **e** Baking of paper on hotplate. **f** Development of the paper in acetone and 70% isopropyl alcohol. Adapted with permission from Martinez et al. (2008b). Copyright (2008) Royal Society of Chemistry

One surface of the paper is covered using a transparency film and the other with black construction paper to minimize the reflection of UV light when exposed. Hold the three components together and seal the transparency film border to the construction paper (the paper is usually smaller by approximately 0.5 cm on the sides compared to transparency and construction paper). To create the distinction between hydrophilic and hydrophobic regions, black patterns are printed on the transparency film. The FLASH paper is now exposed to UV light for a few seconds. Next, the transparency film and construction paper are peeled off. The paper is soaked in acetone for a minute and washed with 1× acetone and 2× isopropyl rubbing alcohol (70% solution in water). The paper is allowed to dry under ambient conditions. The whole process is outlined in Fig. 3.9.

Plasma Treatment

Alkyl ketene dimer (AKD) dissolved in n-heptane solution is used for hydrophobization of filter paper (Li et al. 2008). The filter paper is immersed in the solution and are instantly removed and kept in a fume hood to vaporize heptane. The filter paper is now cured in an oven. This treatment makes the filter paper turn from hydrophilic to hydrophobic. To form hydrophilic patterns, plasma treatment is used. Metal masks are created in stainless steel with the desired patterns of hydrophilic regions cut out mechanically. Next, the treated paper (hydrophobic) is sandwiched between the patterned metal masks and are placed in the vacuum plasma etcher for 15 s at an intensity of 15 W. Proper care is taken to ensure that the masks are aligned properly. A vacuum level of 0.6 mbar is used. The areas on the filter paper which are exposed to plasma treatment become hydrophilic and allows wetting by water and thus can be used for transport of aqueous solutions.

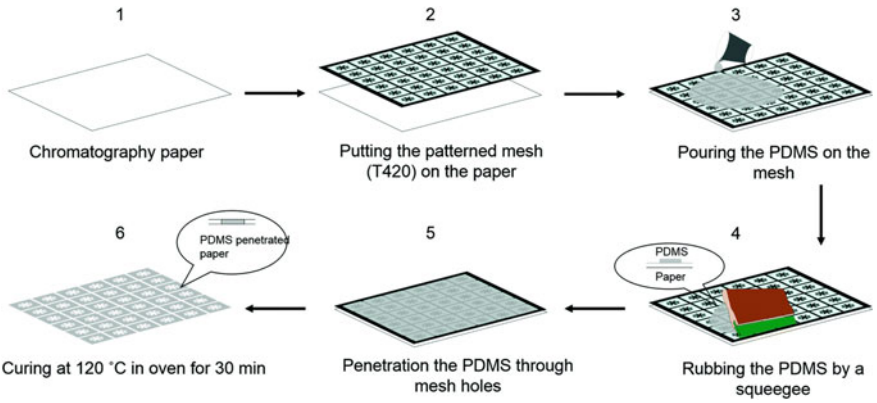

Fig. 3.10 Schematic diagram representing PDMS-screen-printing (1), (2) the screen is placed directly on the surface of chromatography paper; (3), (4) the screen is rubbed with PDMS with a squeegee squilgee; (5) PDMS penetrated into the paper; (6) PDMS-screen-printed paper is cured in an oven at 120 °C for 30 min. Mohammadi et al. (2015)—Published by The Royal Society of Chemistry

PDMS screen printing

PDMS (instead of SU-8 photoresist) is used for creating hydrophobic barriers on chromatography paper because of the flexible nature of PDMS materials (Mohammadi et al. 2015). A printing table is required for the process which allows movement of substrates in x and y directions. A screen stencil with nylon mesh of pore size 35 µm is patterned according to the requirements. The screen stencil is positioned on top of the chromatography paper and PDMS rubbed on to the surface using a squeegee. This drives the PDMS through the mesh into the chromatography paper and slowly passes through into the paper to produce the PDMS pattern. This paper is kept in an oven at 120 °C for 30 min for curing. The patterned paper is taken out from the oven and is cooled to room temperature. The process schematic is shown in Fig. 3.10.

Two-dimensional shaping (using X–Y knife plotters)

An X–Y knife plotter (computer-controlled) is used for shaping different kinds of porous media (Fenton et al. 2009). The knife is rotated freely on a turret which enables cutting of small radius corners and holes. The blade angle and the downward force has to be properly adjusted to enable cutting of most of the porous media (polyester-backed NC membrane, photocopy paper, etc.) in a single pass. Three sequential cuts are required to cut chromatography papers without tearing. The first two cuts can made in chromatography paper penetrates only part way through. After cutting of required patterns, the unwanted material was removed manually. It takes approximately 1 min to set up the instrument. The cutting finishes in 5–15 s. The time taken for cutting depends on the porous media being cut and the shape complexity. Removal of unwanted material may take 10–100 s.

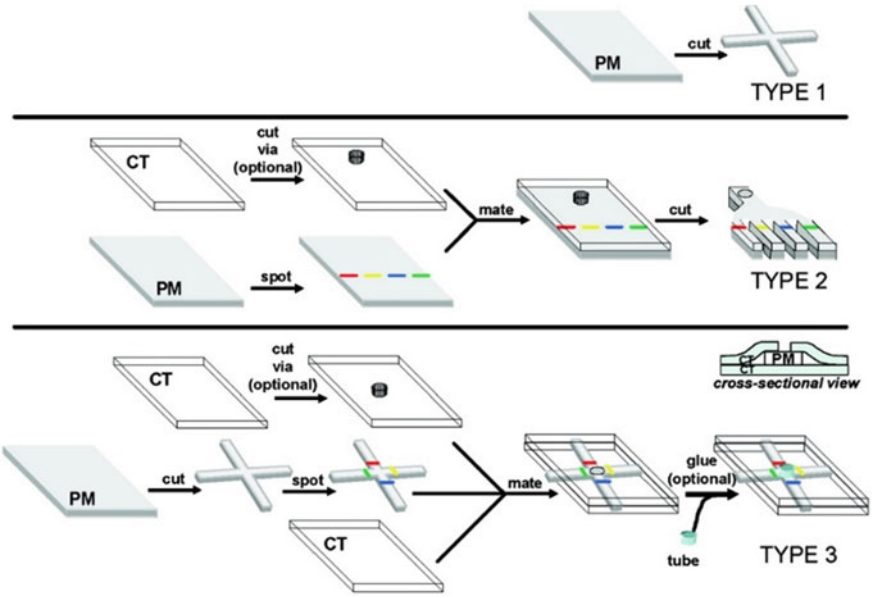

Fig. 3.11 Three methods of paper microfluidics device fabrication using two-dimensional shaping. Reprinted with permission from Fenton et al. (2009). Copyright (2009) American Chemical Society

Figure 3.11 depicts three different types of fabrication of paper based devices which yields three different device types.

Type 1: Porous media is shaped by an X–Y knife plotter. (Direct method—without any intermediate step).
Type 2: Reagents are deposited on the porous media. A cover tape is attached to the porous media. This composite is shaped using the X–Y knife plotter. A cylinder is fixed atop via using glue, which acts as a reservoir.
Type 3: Porous media is shaped using the X–Y knife plotter. The reagents are deposited on the porous media. The sheet of porous media is squeezed between two sheets of cover tapes. A cylinder is fixed atop via using glue, which acts as a reservoir.

Printed Circuit technology

The patterns of the channel are designed using an electronic design software and printed on a transfer paper (Zhang and Zha 2012). The patterns are copied from the paper to a sheet copper (0.3 mm thick—attached to a blank printed circuit board to avoid bending) using thermal transfer printer. This transfers the carbon powder on transfer paper free of patterns to the sheet copper. This sheet copper is etched in ferric trichloride water solution. The regions of sheet copper unprotected by carbon

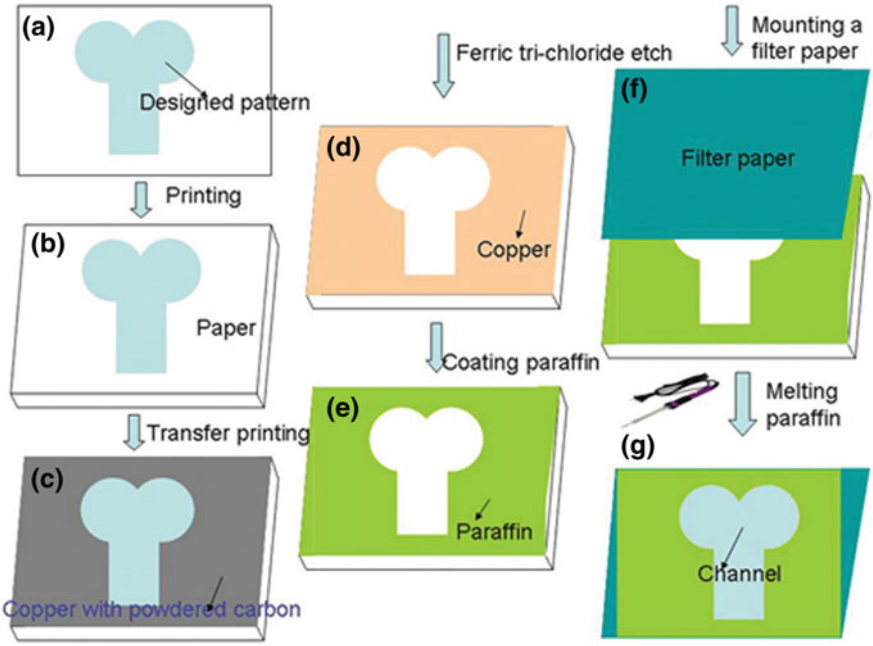

Fig. 3.12 Printed circuit technology for fabricating μPADs. Reprinted from Zhang and Zha (2012). Copyright (2012) AIP Publishing

powder is etched. The etching process is allowed to complete. The sheet copper is detached from the blank printed circuit board and washed and dried. Paraffin is applied on the sheet copper and filter paper mounted on top. The other side of sheet copper is heated using an electric iron. The paraffin gets melted and is absorbed by the filter paper. The areas where paraffin is penetrated into the filter paper becomes hydrophobic and the remaining regions remain hydrophilic. A schematic of the process is shown in Fig. 3.12.

3.2.3 3D Fabrication Methods

Stacking of patterned paper

Here, we discuss a technique (Martinez et al. 2008a) for creating 3D μPADs by assembling recurring layers of paper and double-sided adhesive tape (waterproof). Both paper and adhesive tape are patterned to direct fluid flow within individual layers and between different layers of paper. A hydrophobic polymer is used to pattern the paper into distinguished channels. The adhesive tape isolated the channels in adjacent layers of paper. Holes are cut up from the tape so as to allow vertical flow of fluids.

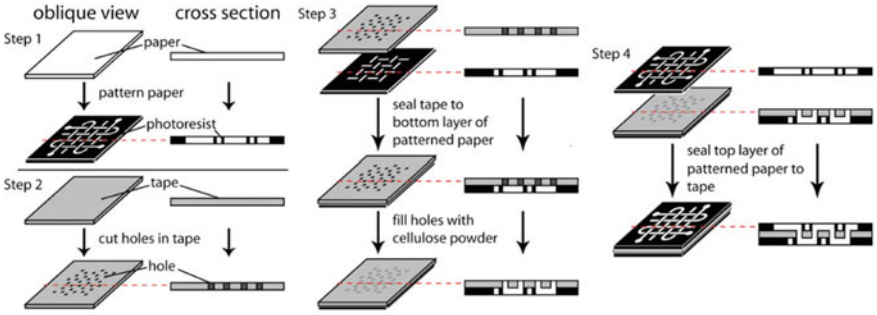

Fig. 3.13 Fabrication of 3D μPAD. Adapted with permission from Martinez et al. (2008a). Copyright (2008) National Academy of Sciences

The layers of paper are soaked in SU-8 2010 photoresist. After drying, the paper is exposed to UV light through a transparency film. This paper is developed using acetone and isopropyl alcohol. Holes are cut into the adhesive tape using a laser cutter or using a hole punch. The layers of paper and tape are assembled by piling them on top of one another. A layer of patterned paper was attached to a layer of adhesive tape (adhesive tape has a protective film on one surface). A paste made of cellulose powder and water is used to fill the holes in the tape. A second layer of paper is oriented with respect to the previous layer and attached to the other side of the tape after removing the protective film. The stacking process is repeated till completion of the device. The cellulose powder-water paste is utilized so that fluids wick between two neighboring layers of paper (Fig. 3.13).

3D wax printing

Wax printing is extended to form multiple layers of channels on a sheet of paper (Li and Liu 2014). Here, the penetration depth (of wax) is controlled by adjusting property of printed wax (density) and heating time. The wax is printed on both sides and allowed to wick only to a controlled depth of penetration.

Two parameters are regulated to keep the wax penetration depth at the required value. (1) Density of printed wax: Once heated, wax of higher density gets absorbed by paper faster than lower density wax along the paper thickness, which resulted in different depth of melted wax. Figure 3.14 A shows different grey scale values corresponding to different wax densities. Lower the grayscale value, higher the density (2) Heating time: Depth of penetration is greater for longer heating time (wax density remaining same). The heating temperature may also be adjusted to manage the penetration depth of the melted wax.

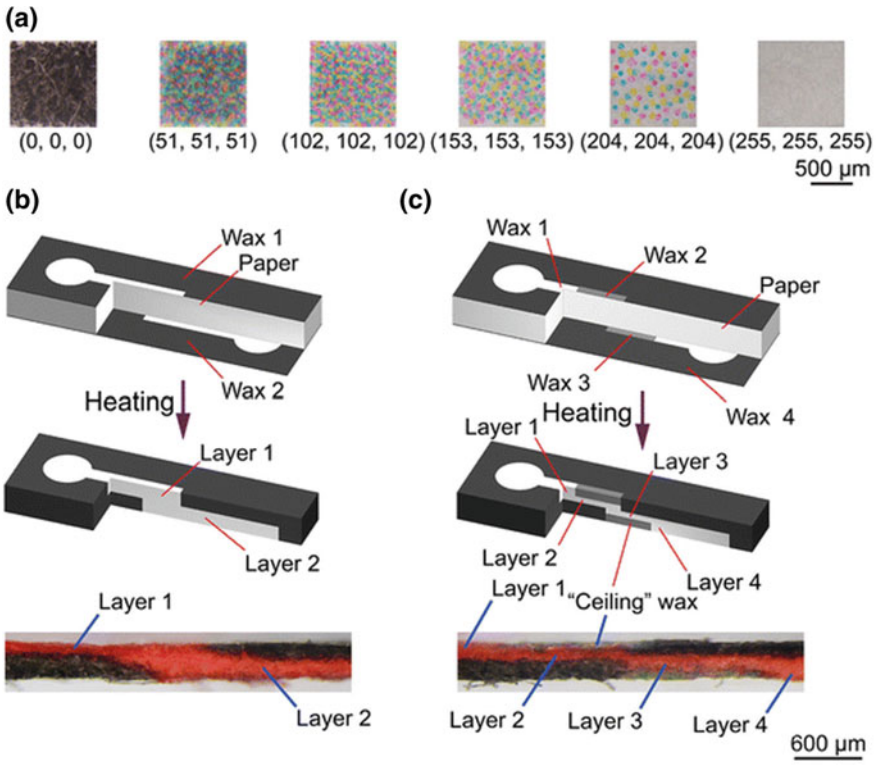

Fig. 3.14 Formation of 3D microfluidic channels using solid wax printing, **a** photos of wax printed at different wax densities (grayscale levels), **b** schematic drawing showing two-layer channels formed by 3D wax printing and **c** schematic drawing showing four-layer channels formed by 3D wax printing. Reprinted with permission from Li and Liu (2014). Copyright (2014) Springer Nature

Whatman® 3MM chromatography paper is selected as the paper substrate due to the greater thickness compared to Whatman No. 1 chromatography paper. Figure 3.14b shows the method of formation of two channel layers. Here, both surfaces of the paper are printed using same density wax. The wax penetrates into the paper and forms two layers of channels. The depth of penetration is controlled by controlling the heating time. The overlap between un-patterned areas interconnected the two channel layers. In Fig. 3.14c, separate areas on either surface of paper are printed different density wax. On heating, four layers are formed of which two are open channels (1 and 4) and two are closed channels (2 and 3).

Table 3.1 Various fabrication methods and their advantages and disadvantages

Fabrication methods	Advantages	Disadvantages
Photolithography	Very narrow channel width ~200 μm; sharp hydrophobic barriers	Sophisticated equipment required; complicated and lengthy process; needs expensive photoresist
Plasma treatment	Very inexpensive hydrophobic material (AKD)	Different masks are needed to create different patterns
Wax printing	Used to produce large devices, simple fabrication process	Needs specially designed wax printers
Ink jet printing	Uses very inexpensive hydrophobic material (AKD); simple and fast fabrication process (in less than 10 min); allows mass production	Low resolution; customized ink jet printers are required
Flexographic printing	Can be used for large-scale production; printing speeds of 60 m/min is possible	Minimum two sequential prints are needed for creating hydrophobic regions, print quality depends on the smoothness of paper surface
Screen printing	Produces devices with simple process	Low resolution; need for different printing screens to create different patterns
Laser treatment	High resolution	Channels do not allow lateral fluid flow; extra coating is required for liquid flow

3.3 Comparison of Various Fabrication Methods

The comparison between various fabrication techniques highlighting the advantages and disadvantages of each method is given in Table 3.1.

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Chapter 4
Flow Control in Paper-Based Microfluidic Devices

Siddhant Jaitpal and Debjani Paul

Abstract Paper-based microfluidics has been extensively used in recent times to perform automated multi-step reactions. One of the requirements to carry out sequential reactions is the ability to control the fluid flow accurately on paper substrates, comparable to what is achieved in conventional microfluidic devices. Flow control still remains a major roadblock in the translation of more complex paperfluidic devices from research labs into the commercial domain. In this chapter, we describe the advantages and limitations of the reported flow control strategies in paperfluidics. Modelling the flow on paper and its implication on the device design are also discussed. Finally, we summarize the challenges for commercialization of these flow-switching devices.

4.1 Introduction

Paper-based analytical devices have a great potential for developing single-use diagnostic tests for use at the point of care (POC). Paper is inexpensive compared to conventional microfluidic substrates, making these devices widely affordable and accessible. It allows passive wicking of fluids by capillary action, eliminating the need for external pumps or power sources. Reagents can easily be patterned on paper by bio-printing. Used devices can be disposed by incineration to avoid contamination. Most of the commercial paperfluidic diagnostic tests employ a lateral flow assay (LFA) format. In this format, a sample containing the target analyte is added to an absorbent pad, where it gets mixed with the detection molecules. It then flows along a nitrocellulose membrane to reach the detection region. The presence of the target analyte is confirmed by a change in color of the detection line, while the excess sample is collected by another absorbent pad. Pregnancy strips, which detect the presence of human chorionic gonadotropin

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(hCG), is an example of a LFA. These assays are restricted to the detection of a single analyte in a single flow channel. Detection of multiple analytes using complex assays, similar to conventional microfluidic devices, cannot be performed in the existing LFA format (Posthuma-Trumpie et al. 2009). The interest in paper as a substrate for fluid flow was rekindled within the microfluidics community when glucose, nitrite and protein were detected in a single multiplexed assay on a paperfluidic chip (Martinez et al. 2007). Since then, several two- and three-dimensional paperfluidic networks to perform complex multi-step assays by sequential and selective delivery of reagents have been demonstrated (Byrnes et al. 2013; Fu and Downs 2017; Yetisen et al. 2013). However, these multiplexed devices have fallen short in their bid for commercialization. This is primarily due to a lack of analytical sensitivity and specificity compared to the devices that test for a single analyte. Integrating sample processing onto a paperfluidic detection device has turned out to be another challenge. Pre-processing of complex samples (e.g. whole blood and sputum) containing multiple analytes is typically performed in separate off-chip modules. Finally, achieving accurate and reproducible flow control to allow for timed reactions still remains a major hurdle in paperfluidics. In this chapter, we focus on the last problem, namely, flow control in paperfluidic devices. We discuss in detail different strategies reported in the literature. We also articulate the challenges that impede the commercial adoption of these lab-grown control technologies and suggest some ways forward.

4.2 Fluid Flow Through Porous Substrates

A parameter critical to the performance of paper-based microfluidic devices is the fluid flow rate, as it affects all time-dependent processes (e.g. the residence time of analytes with capture molecules, enzyme kinetics, etc.). Fluid flow in a porous material depends on a number of parameters including the pore size, the liquid-air surface tension, and the liquid-solid contact angle. The paperfluidics community, thus far, has largely relied on two mathematical models (e.g. Lucas-Washburn and Darcy's equations) to describe the flow through these devices.

4.2.1 Lucas-Washburn Equation

The Lucas-Washburn model was originally proposed to describe flow inside a single capillary. While adapting this equation to describe wet-out flow in a paper substrate, the matrix is considered to be made of a bundle of identical capillary tubes. The position of the wetting front is related to the elapsed time by Eq. (4.1).

$$L^2 = \frac{\gamma r_{pore}}{2\mu} t \tag{4.1}$$

In this equation, L is the distance traversed by the fluid front, r_{pore} is the average radius of the pore, γ is the effective surface tension of the fluid–air interface, and μ is the dynamic viscosity of the fluid. This equation assumes one-dimensional saturating flow in a homogenous porous membrane, and cannot be used to accurately predict the flow rate in real devices. It cannot be used to describe the fluid flow in heterogeneous substrates or for partial-saturation flow regimes. Even the simplest of the lateral flow strips have at least two different porous membranes (e.g. nitrocellulose membrane and a cellulose wicking pad).

4.2.2 Darcy's Equation for Fluid Flow

Darcy's Law can be used to describe the flow rate in a paperfluidic network containing multiple channels and multiple porous substrates. It gives the volumetric flow rate in a porous media, as shown in Eq. (4.2).

$$Q = \frac{\kappa A \Delta P}{\mu L} \tag{4.2}$$

The flow rate (Q) is a function of the membrane permeability (κ), the cross-sectional area (A) perpendicular to the flow, the pressure difference (ΔP) across a channel, the viscosity of the fluid (μ), and the length of wetted membrane (L).

While Darcy's law can be used to model the flow through multiple porous domains connected in a network, it requires solving a moving boundary problem which is computationally complex. Mendez et al. (2009) successfully provided a numerical solution to the problem by finite element analysis. They observed that a lack of knowledge of certain parameters, such as the permeability and the capillary pressure, hinders practical application of their model. This method has not been widely adapted by the paperfluidics community due to its complexity and high computational cost. As such, Darcy's law has been mostly used to model flow in fully wet domains.

4.2.3 Richard's Equation for Partially Saturated Flows

It has been recently been argued that none of these two flow models can effectively capture the complexities of flow through wetting porous materials due to the assumption of the existence of a fully saturated wetting front. On the other hand, Richards's equation is widely used in the modeling of fluid flow in porous hydrogeological systems (e.g. soil). It assumes the existence of a partially saturating

fluid front. In this model, only a fraction of the total pores is filled with the fluid. As a result, the extent of saturation varies with material-dependent changes in the capillary pressure and permeability. When considering the flow in paper-based devices of varying geometries and material properties, we need to also consider the varying fluid properties.

Richard's equation, given by Eq. (4.3), models the change in saturation of a porous media due to gradients in the pressure head.

$$\frac{\partial \theta}{\partial \mathrm{t}} = \frac{\partial}{\partial \mathrm{z}} \left[\mathrm{K}(\theta) \frac{\partial \mathrm{H}(\theta)}{\partial \mathrm{z}} \right] \tag{4.3}$$

Here, θ is the volumetric water content, t is the elapsed time, K is the hydraulic conductivity, H is the pressure head, and z is the position of the fluid front. Both the hydraulic conductivity and pressure can be determined experimentally by constructing the water retention curves for specific substrate materials (Buser et al. 2019). With an increase in saturation, the porous media allows for better fluid conduction and generates imbibition profiles like those predicted by the Darcy's Law. Recently the Toley group outlined a method for determining the effect of partial saturation on fluid flow in porous membranes by experimentally determining the capillary pressure and permeability as a function of saturation. These relationships were used to solve the Richards equation and quantify the flow rates in arbitrary two-dimensional geometries. The imbibition rates and the extent of partial saturation predicted by the Richards equation accurately matched up with the experimental observations (Rath et al. 2018).

4.3 Controlling the Fluid Flow in Paperfluidic Devices

A number of flow control strategies for paperfluidics have been reported in the literature. Most of these techniques aim to control the capillary flow by modifying the pore size of the substrates or the fluid properties (e.g. surface tension and viscosity). Other methods consist of flow control tools (physical or chemical) incorporated within open channel devices to manipulate the overall fluid transport. Yet another class of methods rely on mechanical actuation (i.e. valves), either through manual intervention by the user or automatically via a device element responding to an external stimulus. We divide the flow control tools into two main categories: (i) modulating the fluid velocity within the confines of a channel by physically manipulating the fluid-substrate interactions, and (ii) using valves to turn on/off the fluid flow.

4.3.1 Techniques to Achieve Flow Control Without Valves

4.3.1.1 Changing the Channel Dimensions

The simplest way to alter the flow rate in a paper substrate is to alter the channel dimensions. Fu and others (Fu et al. 2010a) showed that simple changes in width and length of the channel can modulate fluid flow rate for wet-out flow conditions. Mendez and Fenton (Mendez et al. 2009) observed that changes in dimensions of the wicking area of thin porous membranes can induce quasi-stationary flow during imbibition. Their hypothesis was that an increase in the cross-sectional area and unwetted pore volume increases the capillary force at the wicking area, which stabilizes the fluid flow through the channel. Fu et al. also demonstrated that for fully-wet conditions, the flow can be simply modeled as a function of the fluidic resistance after which volumetric flow rates can be easily predicted using Darcy's Law (Fu et al. 2011; Masoodi and Pillai 2010).

4.3.1.2 Creation of Alternate Flow Paths

Changes in the speed of the advancing fluid front can also be achieved by creating alternate flow paths to the primary direction of flow as shown in Fig. 4.1. Sandwiching a porous membrane between two flexible films speeded up the fluid flow by a factor of 10 when compared with uncovered devices (Jahanshahi-Anbuhi et al. 2012). Presence of the flexible films led to the formation of alternate flow paths, thereby increasing the flow rate. The strength of this method was demonstrated by selective increase in the fluid flow velocities for wash steps and for transport between reaction chambers, leading to a cumulative decrease in the total assay time. This method could allow a user to integrate in their paperfluidic device multiple reaction steps requiring different incubation times and wash steps. A potential downside to this method is the use of several different materials and the manual assembly steps needed to manufacture the slow and the fast channels. Giokas et al. (2014) created additional flow paths by manually etching narrow trenches in the porous substrate (Fig. 4.1a). The principle of operation of their device was that trenches parallel to the flow direction accelerated the fluid flow, while trenches perpendicular to direction of flow decelerated it. The length and number of etched trenches were used to control the flow speed. This method is compatible with fabrication processes using knife plotters or laser cutting. The disadvantage with this method is the lack of robustness with respect to reproducibility. This problem was evidenced by obtaining similar flow rates upon perturbation of the design parameters. We anticipate an increase in the complexity of the device design when this technique is applied to multiplex, multi-analyte detection schemes.

Working along these lines, the Crooks group crafted hollow channels (Renault et al. 2013, 2014) within paper by removing sections of the paper substrate that

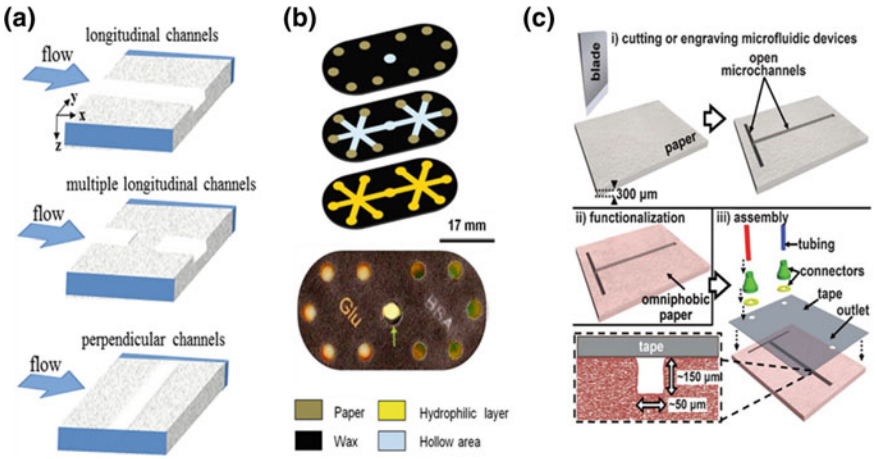

Fig. 4.1 Strategies to change the speed of fluid flow in a porous material by creating additional pathways. **a** Razor-crafted channels parallel to the flow increase fluid speed, while channels perpendicular to flow slow down the fluid. Adapted with permission from the American Chemical Society (Giokas et al. 2014). **b** Hollow paper channels crafted by removing cellulose from the channels. Flow rates increase by a factor of seven. Adapted with permission from the American Chemical Society (Renault et al. 2013). **c** Fabrication of omniphobic paper-based analytical devices by craft-cutting and silanization. Adapted with permission from the Royal Society of Chemistry (Glavan et al. 2013)

normally comprise the hydrophilic channel (Fig. 4.1b). This device was bonded to a hydrophilic layer at the bottom. This bonding step was crucial in achieving high flow speeds under a low pressure without the use of an external pump. They also demonstrated slowing down of the flow by increasing the hydrophobicity of the wax barrier or through the introduction of varying lengths of cellulose in the flow path. Furthermore, they constructed three-dimensional devices by printing wax on the inside of the base hydrophilic layer to form hemispherical or fully enclosed channels (by simple cutting, folding and melting of wax). This allowed for robust control of flow while minimizing evaporative losses. The potential downside to the above technique is the need to have a non-limiting source of fluid to ensure complete wetting of the channel. Glavan et al. (2013) fabricated omniphobic channels and demonstrated flow control in them by folding the paper substrate (Fig. 4.1c).

4.3.1.3 Changing the Surface Wettability

As shown in Fig. 4.2, application of wax to the flow path has been employed to slow down the fluid flow rate by increasing the fluid-substrate contact angle and decreasing the surface wettability. Noh and Phillips (2010a, b) varied the wax concentration to control the delay times from a few minutes to a couple of hours

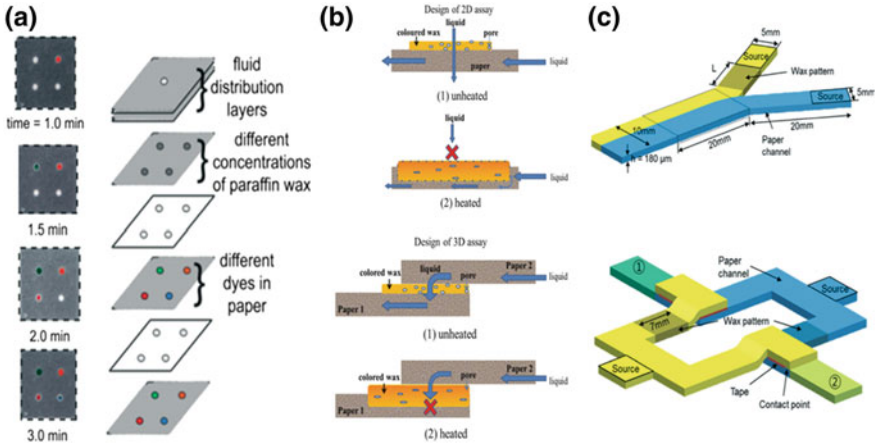

Fig. 4.2 Using wax to control fluid flow. **a** Wax deposition within the porous channels affects the surface wettability and alters the flow rates. Adapted with permission from the American Chemical Society (Noh and Phillips 2010a, b). **b** Schematic illustration of the fluid flow through wax barriers in 2D and 3D. Heating results in spreading of the wax and effective reduction in pore size and the number of pores. Adapted with permission from the American Institute of Physics (Weng et al. 2014). **c** Schematic of a paper-based Y-channel (above) and micromixer (below) which utilize wax patterns of varying lengths and brightnesses to control fluid flow. Adapted with permission from the Royal Society of Chemistry (Jang and Song 2015)

(Fig. 4.2a). Using the same principle, they incorporated fluidic timers into their device. These timers consist of a wax dye applied to the channels parallel to the actual assay to signify the end of an assay.

Along these lines, Weng et al. (2014) varied wax color and saturation during printing to control fluidic delays (Fig. 4.2b). Similarly, Fig. 4.2c shows controlled mixing of two dyes was demonstrated by adjusting the brightness of the printed wax and the length of the wax pattern (Jang and Song 2015). Presence of wax increases the resistance to flow without completely impeding it. This is controlled by varying the amount of the wax deposited and exploiting the changes in the porous structure. These methods increase the manufacturing time due to the inclusion of multiple perturbation steps associated with the differential deposition of wax. However, it is still compatible with the wax printing process which is commonly used to fabricate paper-based devices.

The sensitivity of a commonly used LFA chemistry was improved by using these delay hydrophobic barriers. The Merkoçi group (Rivas et al. 2014) fabricated devices by printing wax pillar patterns onto the nitrocellulose membrane in order to create pseudo-turbulence and introduce time delays in fluid flow. Human IgG was used as the model protein in this standard gold nanoparticle-based LFA. The introduction of wax-printed barriers led to a three-fold improvement in the sensitivity when compared with conventional LFAs.

The change in the surface wettability was also employed by Songok and Toivaka (2016) in their work. Open channels were fabricated using titanium dioxide-coated

paper which was UV-treated to decrease the wettability of open channels. In this method, fluidic delay in the channels was achieved by simply varying the surface area or the width of the treated portion. The potential pitfalls of this method include the possible fouling of the assay components with titanium dioxide and an increase in the fabrication complexity.

4.3.1.4 Changing the Properties of the Porous Substrate

The Biesalski group (Böhm et al. 2014) explored the importance of the physical properties of the porous substrate on capillary-driven transport in paper-based devices. The fiber source, grammage, and the channel width were found to differentially control the flow rates. Therefore, the physical properties of the substrate should merit consideration during paper-based device design. Flow control using changes in the channel dimensions on standard commercial filter papers was deemed too complicated to effectively reproduce due to confounding parameters such as fiber direction, porosity, chemical pre-treatment, etc. By varying the packing density and the alignment of the cellulose fibers, the porosity and the pore radius can be tuned. The Je-Kyun Park group (Shin et al. 2014) applied high pressure on the porous substrate in user defined regions. Fluid flow was shown to slow down due to a decrease in the average pore size in these regions. Fluidic delay times up to 7.4-fold were achieved using different amounts of pressure. While their observation is very interesting, a potential disadvantage of this technique lies in the use of expensive instrumentation for precise spatial positioning of the pressure head relative to the hydrophilic channel. The approach of using multiple paper substrates to perturb the flow rate has been Adapted in several devices. But this approach is burdened by the increase in fabrication complexity along with the great care that would be needed to produce reliable fluidic connections between the intertwining strips.

4.3.1.5 Increasing the Resistance to Fluid Flow Using Physicochemical Barriers

Another method to perturb the flow rates involves slowing down of the fluid front by introduction of dissolvable species on the flow channel (Fig. 4.3). Lutz et al. (2013) incorporated dissolvable sucrose barriers by drying them on the hydrophilic channels to introduce delays from a few seconds to fifty minutes (Fig. 4.3a). A folding card format, which employs manual activation to initiate contact between flow strips, and sample pads were used to initiate the flow. The concentration of the dissolved species was varied to obtain different time delays with low coefficients of variation (9–19%). The reduction in the flow speed was due to the increased viscosity of the sample after mixing with the dissolved species. The additional step of introducing dissolved species into the channel would increase the fabrication time and would require several validation steps to ensure the stability of the dissolved

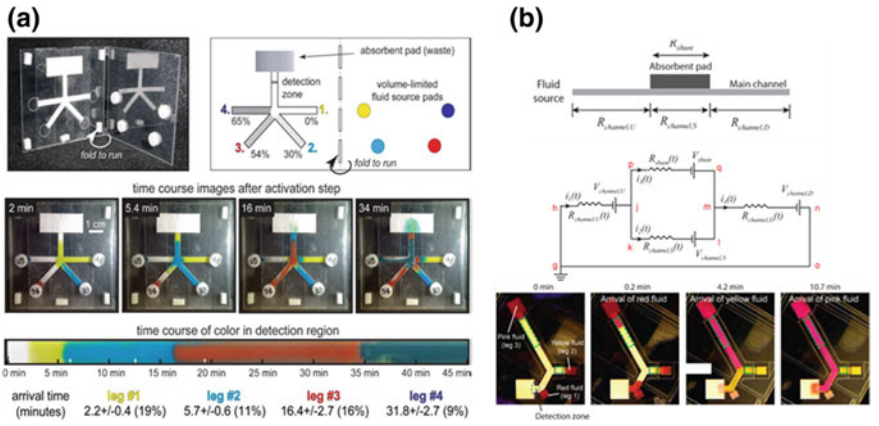

Fig. 4.3 Strategies to increase the fluidic resistance of the hydrophilic path by the introduction of a barrier to the flow. **a** Dissolvable barriers like sucrose increase the fluid resistance by increasing viscosity of solution. The time delay increases with an increase in sucrose concentration. Adapted with permission from the Royal Society of Chemistry (Lutz et al. 2013). **b** Absorbable shunts made from cellulose (green dotted areas) increase the fluidic resistance coupled with an increase in the path length to sequentially deliver analytes to a specific region. Adapted with permission from the American Chemical Society (Toley et al. 2013)

species within the confines of the flow channels. Another potential downfall of this technique is the possible undesirable interaction of the dissolved species with the downstream assay components.

The Yager group (Toley et al. 2013) developed a flow control tool consisting of absorbable sponge-like shunts parallel to the direction of flow (Fig. 4.3b). The fluid progression is slowed down due to the absorption of fluid by the shunt material. The capillary force exerted by the shunt was modeled using an analogous electric circuit and the flow dynamics were represented using the Washburn equation. The fluid delay was introduced by tuning the physical properties such as the cross-sectional area, the material and the thickness of the cellulosic shunts to produce delays between 3 and 20 min with <10% coefficients of variation. This method does not require introduction of any chemical species which can interfere with the downstream assay. Additionally, if dry reagents are deposited within the channel, the issue of reagent wastage can be circumvented. While this device is capable of being wetted by larger sample volumes, the addition of the dead-weight cellulosic shunts might make this device difficult to operate with assays using limited sample volumes.

4.3.1.6 Electrostatic Interactions Between Device Components

The Kubota group (da Silva et al. 2015) utilized the electrostatic interactions between the paper substrate and the analyte to delay fluid flow within paper

channels. The triboelectric effect was utilized to show that paper channels sealed with charged PET films slowed down the fluid flow by six minutes in comparison to paper channels sealed with uncharged PET films. The surface charges on the PET sheet promote an upward displacement of the aqueous solvent due to electrostatic attraction and significantly retard the capillary flow in the transverse direction. The reversible sealing of the paper substrate increased the capillary flow rate in comparison with open channels and reduced the effects of evaporation. The experimental conditions can be easily reproduced as friction-induced electrostatic charges are initiated by simply rubbing the PET sheet on another charged surface. However, the fluid utilized in most of these demonstrations was aqueous in nature and this might complicate the usage of this setup with assay reagents of other physicochemical properties.

4.3.1.7 Varying the Channel Dimensions for Specific Introduction of Reagents

The Yager and Lutz groups have incorporated multiple inlets in their device designs to allow for sequential delivery of fluid to the reaction zone. These configurations can be conceived as "valve like" as they have the same net effect of restricting the fluid flow to pre-determined regions in the paper network. One of the simplest ways to achieve this is to vary the length of each inlet channel to have the sample fluid sequentially arrive at the designated spots (Fu et al. 2010a, b; 2012). This approach (Fig. 4.4a) however requires careful design of the inlet channels to avoid unnecessary co-flows and it may not be practical for handling of large volumes due to an increase in the device footprint.

Liu et al. (2013) developed a two-dimensional slip layer-based device (SlipPAD) which was actuated to bring pre-dried test reagents in contact with the sample solutions flowing across a one-dimensional paper channel (Fig. 4.4b). The first slip brings the sample solution in contact with the test reagents and the second slip seals the connection between the two layers ensuring no co-flows are present to affect sensitivity of the multiplexed detection. A single SlipPAD can simultaneously perform four replicates of the sample and the standard controls.

Dharamraja et al. (2013) presented a simple one-dimensional design utilizing the electrical resistance equivalent of the fluidic resistance (Fig. 4.4c). Fluid-soaked source pads were spaced to avoid co-flows and allowed for sequential delivery of reagents to a desired location on the device. The device fabrication process is compatible with traditional lateral flow technologies and requires no further fabrication processes aside from the assembly of the device.

Another group (Apilux et al. 2013) utilized inkjet printing to vary the locations of the assay reagents and performed an ELISA to detect human chorionic gonadotropin (hCG) (Fig. 4.4d). Inkjet-printed barriers effectively increased the path length of the fluid flow by creating relative delays.

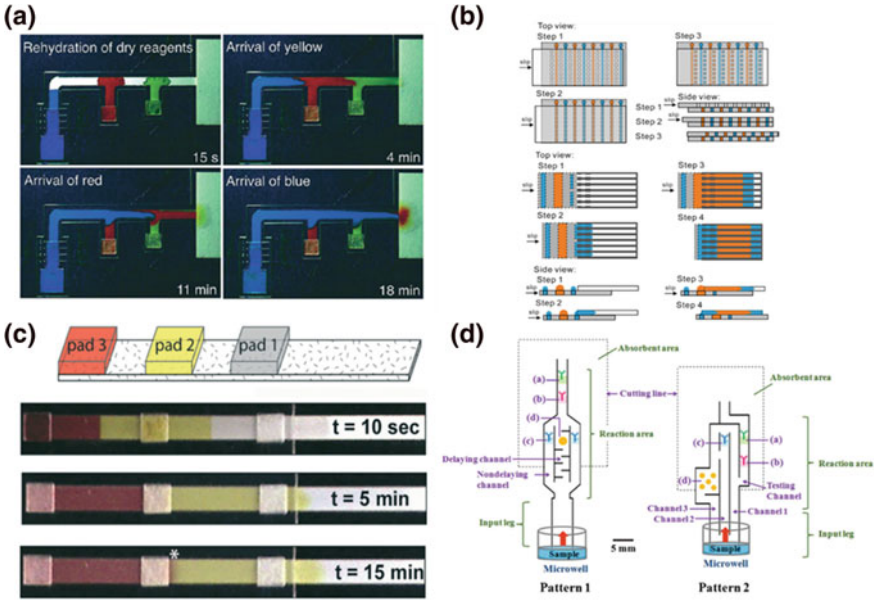

Fig. 4.4 Strategies to control fluid flow by increasing the net path length for sequential delivery of reagents. **a** Sequential delivery of reagents is achieved by varying the length of the inlet arms and tuning the distance from the detection zone. Adapted with permission from the American Chemical Society (Fu et al. 2012). **b** Schematic showing the operation of SlipPAD for parallel and sequential delivery of fluids. Adapted with permission from the American Chemical Society (Liu et al. 2013). **c** One-dimensional delivery of fluid achieved using sample pads at different distances. Adapted with permission from International Society of Photonics and Optics (Dharmaraja et al. 2013). **d** Schematic illustration of paper -based ELISA. Adapted with permission from the Royal Society of Chemistry (Apilux et al. 2013)

4.3.2 Techniques to Achieve Flow Control Utilizing Valve-Like Tools

4.3.2.1 Dissolvable Species

Dissolvable species have been used to by Houghtaling et al. (2013) to achieve fluidic delays in paper-based devices (Fig. 4.5a). Sugars (e.g. trehalose, sucrose and mannose) were used as connectors to bridge the gaps between disparate strips of nitrocellulose membrane. The flow could be completely stopped by dissolution of the barriers. The rate of dissolution depended on the constitution of the sugar and the dimensions of the bridge. A highly repeatable coefficient of variation (5–12%) in the volume of the transported fluid was achieved. Work along the similar lines was reported by Jahanshahi-Anbuhi et al. (2014). This involved the use of varying dimensions of pullulan film in contact with paper channels (Fig. 4.5b). Retardation of the flow was achieved by changing the film dimensions and the extent of the

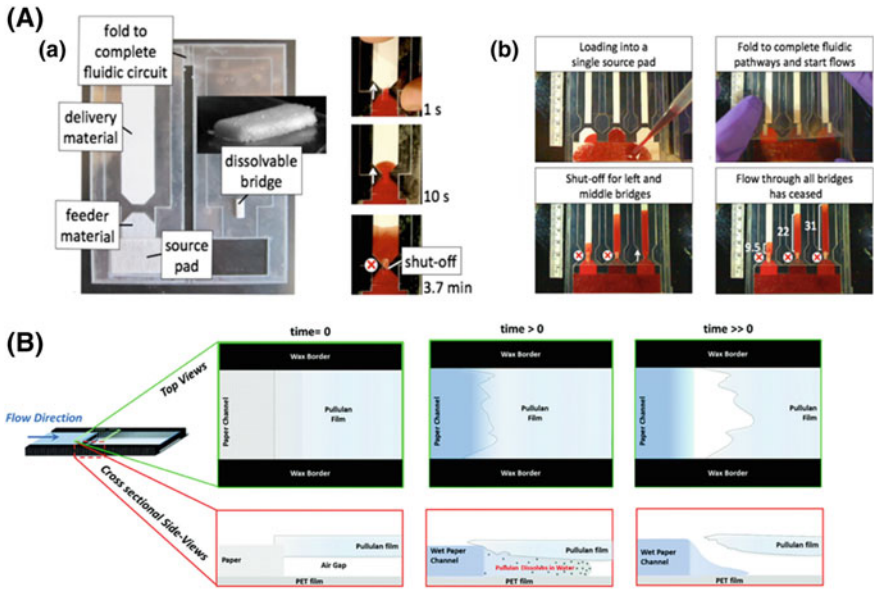

Fig. 4.5 Strategies to control fluid flow through the introduction of disconnectable dissolvable species. **a** Schematic of a fluidic circuit with a dissolvable bridge which is completed by card folding. Adapted with permission from the American Chemical Society (Houghtaling et al. 2013). **b** Schematic demonstrating delay in capillary flow through a channel until complete erosion of the dissolvable pullulan film. Adapted with permission from the Royal Society of Chemistry (Jahanshahi-Anbuhi et al. 2014)

contact with the paper channels. A potential pitfall of this method is the separate fabrication step required for making these barriers. Incorporating the barriers into paper-based devices can be tricky due their fragile nature. Additionally, the dissolved species are introduced directly into the flow path, which can potentially affect the downstream processes.

4.3.2.2 Mechanical Tools Which Connect or Disconnect Channels

The Lutz group (Lutz et al. 2011) demonstrated autonomous shutting off of the fluid flow in paper-based devices. The fluid flow was shut off when the level of the fluid in the reservoir dropped below a certain height, thereby cutting off contact with the main channel. The shut off time was controlled by playing with the dimensions of the fluid reservoir and the submerged length of the inlet. The repeatability of this process was confirmed by the low coefficient of variation (between 5 and 20%) of the shut off times. The main advantage of this method is the reduced evaporation rate due to the presence of the fluid reservoir. One needs to ensure proper storage to avoid triggering premature wetting of the channels.

As shown in Fig. 4.6a, Toley et al. (2015) achieved actuation with sponge-like materials that expand upon coming into contact with the incoming fluid. The initial configuration of the paper strips can be set to 'open' or 'closed'. Fluid flow triggers swelling of the polymer sponge, which either brings the two channels into contact or disconnects them. The dimensions of the sponges and their physical properties can be used to estimate the fluid volume to be withheld or delivered. As such, both volume and time-based switching can be achieved using this setup. Based on the developments in the area of switch-based fluid flow control and origami-based paper devices, Kong et al. (2017) used chromatography paper to reversibly control the fluid flow (Fig. 4.6b). The actuator is made of paper and requires no extra fabrication steps, coatings, materials or electrical/magnetic stimulation. The actuation principle relies on selectively wetting the crease of a previously-folded paper strip, which, in turn, relaxes the crease and increases its fold angle. The actuator responds within two seconds of wetting. Several switch configurations are realized using the folded paper actuator, such as single-pole single-throw (normally OFF and normally ON) and single-pole double-throw (with single and double break). A colorimetric assay was reported where the presence of glucose, nitrite and protein are detected in spiked artificial saliva. The assay integrated parallel operations of multiple actuators, delay timer strips, and flow channels in a portable enclosure. The advantages of this actuation method include easy preparation steps by paper cutting and folding, very fast actuator response time, choice between different switch

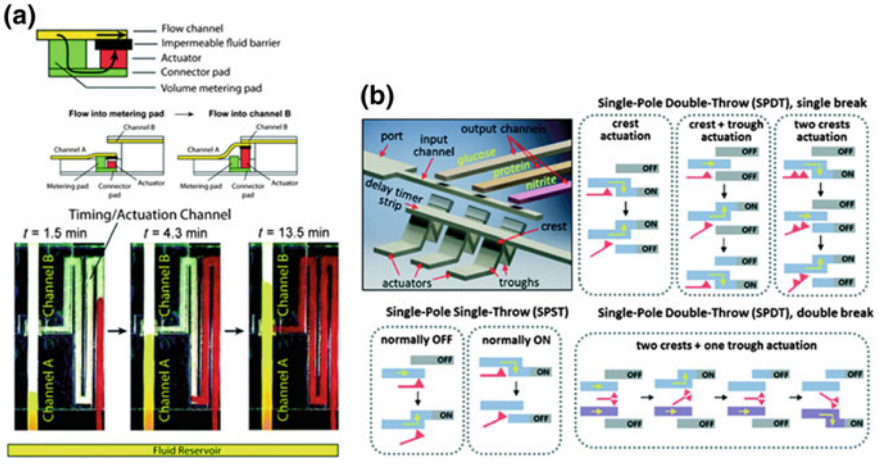

Fig. 4.6 Strategies to control fluid flow through the mechanical connection or disconnection of elements. **a** A disconnected fluidic pathway gets connected by the fluid-driven actuation of an expandable polymer element. The geometry and the material of the valve are varied to alter the valve actuation times. Adapted with permission from the Royal Society of Chemistry (Toley et al. 2015). **b** All-paper actuator which can be activated by selective wetting. Water is added directly on the folded strip or through a time delay strip to lift or lower the tip of the actuator. Adapted with permission from the Royal Society of Chemistry (Kong et al. 2017)

configurations, no observable contamination of the flow channels by the actuation fluid and reduced material costs. Some potential downfalls could be the reliance on the kinetics of paper folding.

4.3.2.3 Wax-Based Valves

The Linnes group (Phillips et al. 2016) demonstrated wax valves (Fig. 4.7a) that require minimal user involvement. These valves can be easily tuned to meet different incubation needs and can also be actuated multiple times. They used thin-film resistors to thermally actuate the valves and sequentially release liquids through a nitrocellulose membrane. The wax-ink valves can fully obstruct the fluid flow over a sustained time interval. They incorporated these valves into a silver-enhanced LFA demonstrating their ability to tune the assay incubation times and increase the signal intensity by six-fold. While wax printed time delays have been used by several other groups (Weng et al. 2014; Noh and Phillips 2010a, b), successful automation of multi-step assays has not been achieved yet.

Chen et al. (2019) used wax valves for distance-based detection of potassium iodate and glucose (Fig. 4.7b). The valves simplified multi-step reactions. The device led to short detection times (15–25 min) and signal linearity over a larger clinically relevant detection range (0.05–0.5 mM for potassium iodate; 1–5 mg/dl and 2.5–80 mg/dl for glucose). These delays can be easily incorporated into traditional wax printed microfluidic devices.

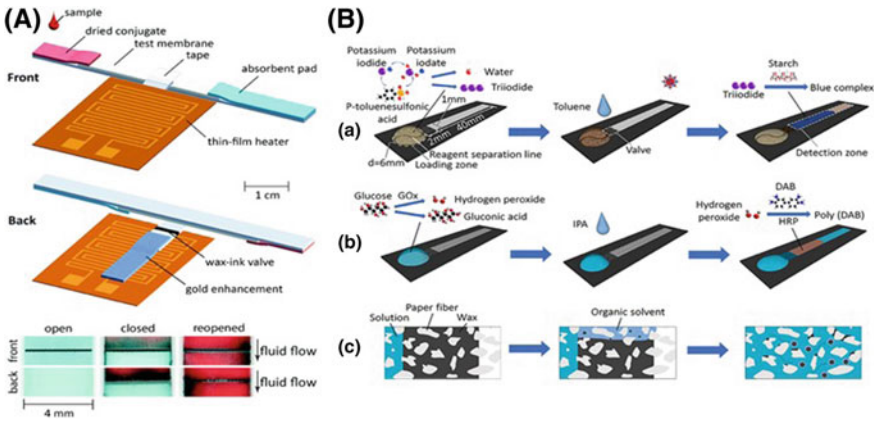

Fig. 4.7 Strategies to control fluid flow using wax valves. **A** Schematic of a multistep LFA using thermally actuated wax valves. Wax valves, which are normally kept open, can be closed via a heating element. These valves can be opened by reheating the waz. Adapted with permission from Royal Society of Chemistry (Phillips et al. 2016). **B** Schematic showing a wax valve with distance-based readout of Iodate (**a**) and glucose (**b**). The wax valves can be dissolved by introduction of a solvent (**c**). Adapted with permission from American Chemical Society (Chen et al. 2019)

4.3.2.4 Fluidic Diodes

Fluidic diodes (Fig. 4.8) are another class of valves. The Faghri group (Chen et al. 2012) exploited the wetting properties of surfactants to increase the wettability of hydrophobic areas within paper channels. Their valve consists of a disk trigger which is separated from an open ring (i.e. the connection to the rest of the channel) by a hydrophobic gap. The aqueous sample containing surfactants are released onto the trigger. It increases the surface tension of the hydrophobic barrier and releases the gated fluid. The same group (Gerbers et al. 2014) extended this work by creating a 3D paper-based device embedded with these fluidic diodes. Surfactants were pre-dried at several spots in the device. These spots were rehydrated to allow bridging of hydrophobic gaps. This innovative device design was used to perform a three-step alkaline phosphatase-based ELISA. Rabbit IgG was used as the model analyte with a detection limit of 4.8 fM. Fratzl et al. (2018) fabricated a two-way valve by combining preferential wetting with a magnetic mechanical valve. Hard

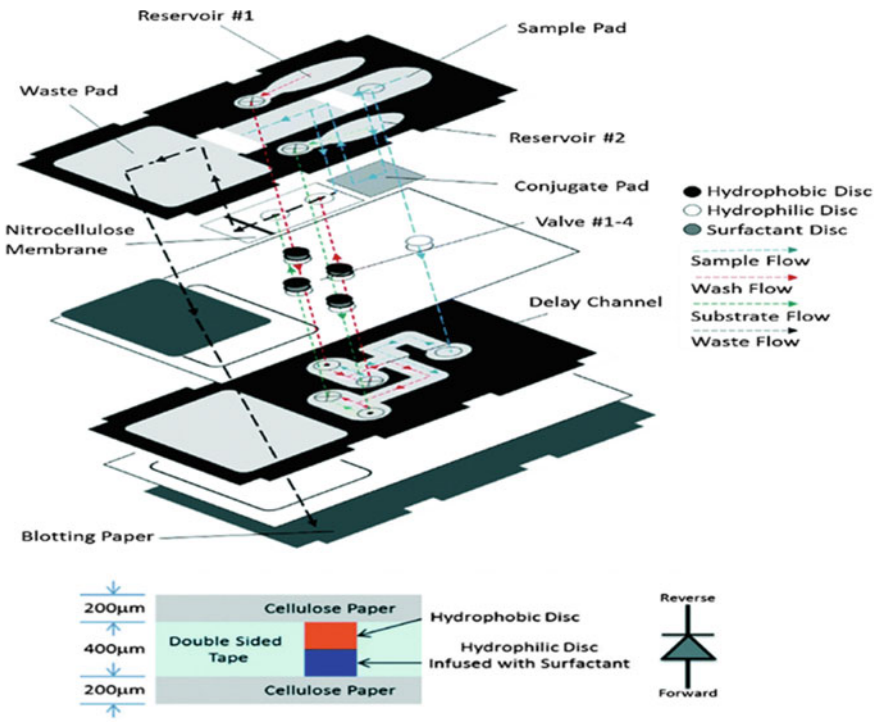

Fig. 4.8 Strategies to control fluid flow via fluidic diodes. The top panel shows the schematic of an automatic three-fluid ELISA with four valves. The bottom panel shows a fluidic one-way diode. It consists of a hydrophobic segment adjacent to dried surfactant spot. Surfactant-enriched sample crosses the hydrophobic region while simultaneously preventing reverse flow. Adapted with permission from Royal Society of Chemistry (Gerbers et al. 2014)

magnetic powders (e.g. NdFeB) were directly embedded into the paper substrate to create custom two-way valves. The paper surface could also be preferentially wetted. When coupled with the magnetization of the embedded powder, a cantilever was used to attract or repel the paper.

4.3.2.5 Automatically Actuated External Valves

A number of mechanical valves have been reported for switching on and off the fluid flow in paperfluidics devices. Mechanical valves typically consist of moving elements such as membranes, flaps or other micro-fabricated elements. The Liu group (Li et al. 2013) introduced a new design of programmable magnetic valves. A magnetized paper cantilever is driven by an electromagnet to connect or disconnect a paper channel (Fig. 4.9a). The device has a separate 'timing channel' which is connected to an ionic resistor that triggers an electrical circuit. Once the wicking of the fluid is completed in the timing channel and the liquid reaches the resistor, it activates the electromagnet. The timing period can be adjusted by varying the channel width and the wicking length. This device design allows for both open and closed valve configurations. Additionally, these valves can be operated autonomously with a single user intervention step to perform multi-step paper-based analytical tests. Kim et al. (2018) developed a bifunctional mechanical

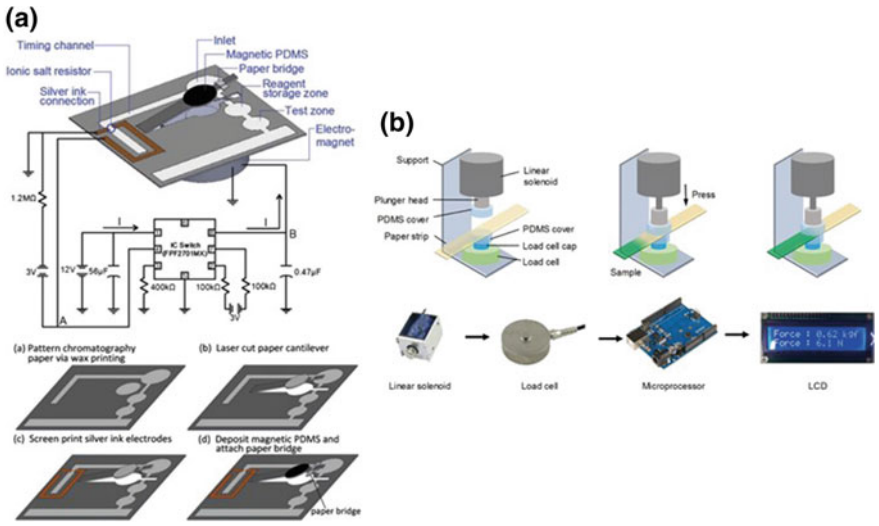

Fig. 4.9 Strategies to control fluid flow using automatically actuated valves **a** the top panel shows the schematic of a normally open magnetic valve and its triggering mechanism. The bottom panel shows the fabrication process of the valve. Adapted with permission from Royal Society of Chemistry (Li et al. 2013). **b** Principle of operation of a programmable valve using an external solenoid. A solenoid-driven press head applies pressure to the paper strip and delays the flow. Adapted with permission from American Chemical Society (Kim et al. 2018)

system for flow control (Fig. 4.9b). The solenoid pressure-driven valve (PDV) enables complete valve actuation. The solenoid coil can be pre-programmed to apply different magnitudes of pressure, allowing fluidic control to be performed under normal conditions. Therefore, the paper does not require any additional material modifications. This design reduces the risk of downstream assay contamination while also eschewing complex device designs as often required with devices using passive valves. A potential pitfall of these active control methods is their operational complexity and the requirement of external equipment.

4.4 Challenges to Translation of Flow Control-Based Paperfluidic Devices

The translation of paper-based devices from the laboratory to the end user can be facilitated by successful integration of flow control tools into these platforms (Akyazi et al. 2018). Two main issues need to be addressed for hassle-free adoption of these tools into existing diagnostic devices. First, the precision and accuracy of these flow control tools need to be characterized for different applications. Second, assays requiring minimal user intervention need to be developed in parallel. Reproducibility of the reagent volumes delivered to different parts of the device and the total assay time are two of the metrics that need to be characterized. A flow control tool which delivers a reagent for a downstream assay may lead to a variable detection signal if the reagent is delivered at different times.

Flow control methods should be developed in accordance with the needs of the end user. Often the user has minimal training and no access to instrumentation. Most of the reported methods, which seem easy to trained laboratory personnel, might not be suitable for field operators who perform these assays in low resource settings. It is in these settings where paper microfluidics has the greatest potential for creating a significant impact on global health. It is hence essential that device developers consider factors which will reduce the requirement of timed intervention on part of the end user for maximum repeatability of these high-performance assays. Additional constraints such as lack of support for device maintenance and instrumentation need to be considered. We believe benchmarking these devices in field and analyzing mechanisms of device failure and user compliance failure will be necessary to convince the diagnostic industry to license and adopt these devices (Kumar et al. 2015).

Finally, the development of flow control tools needs to compatible with high volume fabrication. Developers will need to assess if incorporation of their flow control tools will increase fabrication costs and ultimately increase the price of the final device. Consideration regarding cost of materials, reagents, and the processes need to be accounted for as these will influence the overall cost of the device. The fabrication of these flow control tools needs to be aligned with existing manufacturing processes. Use of complex fabrication techniques which require human intervention would drive up the device cost.

In summary, paper-based analytical devices are ideal low-cost microfluidic analytical systems which require minimal user intervention and can be deployed at the point of care. Flow control tools have been integrated into many paper-based devices to improve assay parameters such as LOD and assay time in the context of medical diagnosis and environmental monitoring. Lack of integration of the flow control tools into existing diagnostic devices and an absence of benchmarking for the processes they are designed for are the major roadblocks in their widespread adoption and commercialization. While researchers have been successful in utilizing these flow control tools for a myriad of analytical applications in research labs, challenges remain in creating appropriate assays for end users in low-resource settings. We believe a focus on these points will enable successful translation of these lab developed devices to the commercial space and be of aid to delivering healthcare in resource-limited settings.

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Chapter 5
Paper Microfluidic Based Device for Blood/Plasma Separation

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Abstract With the advent of research advances in the field of microfluidic devices, medical science is also progressing its way through fast and efficient micro-fabricated medical diagnostics. As most of the medical diagnosis involves blood test as the preliminary or necessary step, a smart micro-diagnostic for blood analysis can be considered as an essential. Blood being the most vital fluid, governing all the hemostatic and physiological conditions by acting as a carrier of all the essential minerals and vitamins throughout the body, makes it a potential analyte for diagnosis. But blood being a complex mixture of hematocrit, white blood cells and plasma along with other essential proteins, the analysis of the same is difficult. Especially, the presence of red blood cells (RBCs) and its agglutination causes interference in the procedure of analysis. For example the presence red blood cells can be a potential interfering factor during colorimetric detection of an analyte in the blood. Similarly, the presence of agglutination of RBCs and its count can interfere during a coagulation diagnosis. Thus, separation of plasma/serum from the erythrocytes is an essential step during a blood analysis. Conventionally, centrifugation and sedimentation were used to separate plasma/serum from the whole blood before its analysis. With the progressing microfluidic technology the micro-devices have been using numerous techniques like bifurcation (Zweifach-Fung effect), geometric obstructions, membrane filtration, and acoustic techniques for plasma separation. Thus, there exists several polymer based lab on chip biomedical micro devices performing the blood separation. Although these devices are portable, fast and efficient with small turnaround time, its fabrication procedure is mostly complex simultaneously involving integration of micro valves. It also requires a syringe pump or a voltage power supply for controlling the blood flow through its micro channels. Considering the complexity of the fabrication the paper based micro devices are steeply gaining its way through as a potential medium for micro diagnostics. Micro pads also doesn't has requirement for any external driving

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source for guiding the fluid flow through. The porous nature of the same performs the capillary action, allowing passive fluid transport. Thus, the respective chapter emphasizes on the paper based micro-devices tailored by the researchers for blood plasma separation with a brief enunciation of their respective working mechanisms. It will also discuss the different fabrication methods intelligently employed by the porous media platform for efficiently performing the separation to further cater to the queries of future researchers in the respective area.

Keywords Plasma separation · Paper based diagnostics · Point of care (PoC) · Hematocrit · Microfluidic devices · Microfluidic paper-based analytical devices (mPADs)

5.1 Introduction

Blood being the most vital clinical analyte, the diagnosis of which can provide a detailed information about the present physiological condition of a candidate, its accurate characterization is the key to perform efficient pathological diagnosis. Plasma, erythrocytes, leucocytes, thrombocytes along with several proteins, vitamins and minerals are the main constituents of the blood. A simple clinical diagnosis of blood and its constituents often requires a preliminary step of plasma/serum separation from the whole blood. Either, if the diagnosis involves erythrocyte related information (e.g. hematocrit count) or a routine diagnosis concerned with that of the other blood constituents excluding the erythrocytes (e.g. glucose/uric acid tests), both require a necessary step of plasma separation from that of the whole blood in general. Several analytical methods like colorimetric, fluorescence and chemiluminescence based diagnostics often face challenges in detection methodology when using whole blood as an analyte. The transduced signal for the respective is optical in nature which is often impaired by the strong colorimetric interference by the RBCs. The presence of erythrocytes in the whole blood also interferes with that of the intricate biochemical reactions resulting the transduction of chemical signal to optical in-case of color/optical based sensing platforms (e.g. colorimetric, fluorescence and chemiluminescence). Similarly, electrochemical detection methodologies face similar challenges as the presence of erythrocytes affects the rheological dynamics of the blood. The presence of cellular components results the blood to act as a complex dielectric medium with challenging biochemical/electrochemical interactions in an electrochemical sensing platform. The analytical methods based on mechanical techniques are challenged by the biophysical characteristics of the RBCs in fluidic environment like rouleaux formation and agglutination caused by complex rheological dynamics of blood. Thus, the respective challenges mandate the efficient separation of cellular components from that of whole blood for efficient characterization of blood analytes. There exists several efficient conventional laboratory based methods like centrifugation and magnetic separation to separate plasma from whole blood (Basu and Kulkarni 2014; Svoboda 2000). These table top blood separating

instruments often produced accurate results due to its intricate instrumentation which made the instrument costlier and often required trained personnel to operate the same. In addition to its installation, operating and maintenance charges, it was bulkier and required a proper clinical or laboratory setup environment for its efficient performance. Apart from the above listed shortcomings, the vital drawback was its higher turnaround time which necessitated the research advances in micro technology for blood analyzers or separators. Thus, the plasma separation using paper as a substrate provides an ideal platform for designing an inexpensive and efficient PoC diagnostic. Despite the presence of paper platform for several immunoassays, whole blood characterization is not preferred due to the optical and dielectric interference caused by the erythrocytes during PoC diagnosis. As PoC is a demanding assay methodology for emergency surgical/peri-operative situation or remote/developing area (out-patient) scenarios, the obvious analyte will be whole blood rather than plasma/serum. Thus, an embedded blood plasma separation unit is a must for any kind of PoC diagnostics. Some of the applicative areas of PoC diagnostics include hematoccrit testing, coagulation analyzer (PT, APTT etc.), blood metabolites analyzer (uric acid, glucose, cholesterol etc.), cancer biomarkers tester, pathogen analyzers (malaria/dengue diagnostics) and blood type analyzers. As evident the plasma separation is a necessary step for PoC diagnostics, several plasma separation membrane (PSM)/paper has been tailored by the researchers to cater the process of plasma separation in PoC immunoassays. These PSM types of paper include (i.e. Whatman LF1, MF1, VF1 and VF2) that are designed specifically to separate plasma from sample of whole blood (Songjaroen et al. 2012; Gong et al. 2014; Noiphung et al. 2013). The downside of these specially tailored membranes is that they are comparatively expensive (100 times of What man no. 1 and no. 4 filter papers), so there is good reason for more research to be done to find cheaper methods for plasma separation. Thus, it is evident that, it is important to engineer a low-cost, yet effective platform, which will have an integrated setup for separating RBCs and plasma from the whole blood and will accordingly detect the analytes of interest. In effort to obtain separation using low cost traditional filter paper (cellulose paper/chromatography paper), several microfluidic assays have been developed using agglutination, aggregation and physical separation methods. The detailing of the following mechanisms and its application with their separation strategies are detailed later in the following sections. A detailed enunciation of the several paper based plasma separation assembly using physiochemical mechanisms (aggregation/agglutination) and capillary driven flow dynamics in porous media is made to provide a clear idea for the respective domain. Thus, following chapter, discusses the mechanism of plasma separation in physiological domain along with that of its evolution of respective biomimetic concepts used in paper based microfluidic platform. Secondly, it discusses the recent advances made by the researchers in plasma separation methodology used by paper based substrates and its composites with pre designed polymeric plasma separating membranes along with is process of fabrication. The applicative areas of the designed devices are also simultaneously discussed. Lastly, a concluding section with future prospects in the following area is included.

5.2 Physiological Hemodynamics and Porous Media Hemodynamics

Blood is a circulating fluid with a complex combination of cellular components, proteins, ions, minerals and extracellular fluid, primarily responsible for transporting nutrients and waste from one part of the body to another (Silverthorn and Johnson 2010; Marieb and Hoehn 2007). It is largely responsible for several physiological conditions such as regulating body temperature, proper osmotic pressure, and pH of body fluids ($\sim$7.4), carrying the cells for defense against the invasion of pathogens, regulating clotting mechanisms etc. The main constituents of blood include erythrocytes (RBCs), leucocytes (WBCs), thrombocytes, and plasma/serum (which comprises the fluidic part of the blood along with several proteins and minerals). While performing clinical diagnosis either whole blood is used or preferably plasma/serum part is extracted out before the assaying. Serum is the fluidic component of the blood excluding the cellular hematocrit and clotting factors and plasma is serum along with clotting factors. Blood is generally characterized either for the presence of essential metabolites/bio-analytes (uric acid, glucose, cholesterol etc.) or hematocrit content. Excluding the cellular components of blood plasma/serum behaves as Newtonian fluid. But the presence of hematocrit make the flow dynamics complex. The normal range (Billett 1990) of hematocrit found in adult men is 40–54% and 36–48% for adult women. Prior to several clinical attempts to characterize blood, several efforts were made to understand the nature and flow dynamics of blood in actual in vivo physiological conditions. In the process, several biophysical phenomenon like Fahraeus effect and Fahraeus-Lindqvist effect were discovered. According to Fahraeus effect, an obvious decrease of hematocrit content can be noted if a flow occurs from a large vessel to small diameter vessel (Fåhræus 1929). Simultaneously a decrease in apparent viscosity was bound to occur in the smaller vascular network which was a consequence of Fahraeus-Lindqvist effect (Fåhræus and Lindqvist 1931). Upon further research, in the field of hemodynamics researchers observed that the RBCs share a tendency to migrate away from the micro-capillary walls caused by hydrodynamic interactions mediated by typical deformabilities of the RBCs. These type of cellular interactions leads to formation of plasma layer near the vessel walls. In attempt to mimic the biophysical environment and micro-capillary/vessel networking in on chip microfluidic technology to obtain plasma separation several other microfluidic phenomenon like electro-osmotic flow, (Fournier 2012), bifurcation (Zweifach-Fung effect), (Toksvang and Berg 2013; Secomb and Pries 2013) geometric obstructions, (Haynes 1960; Pries et al. 1992) were discovered. With the help of several aforementioned phenomenon polymer based microfluidic technology was able to develop potential blood analysis and separators which could produce efficient results at low volumes, low turnaround times at very low cost. However, the major challenges for respective devices were the need for an external force such as syringe pump or high voltage power supply to control fluid flow in the micro-channels. Additionally, it required complicated fabrication methodologies for integration of

valves and pumps. Whereas, microfluidic paper-based analytical devices (mPADs) doesn't require any external driving sources for capillary flow. The porous nature of the same results in self assisted passive flow of fluid media through the substrate. Thus, the extra costing of integration of pumps and complex fabrication is eliminated and the fabrication methods implemented for designing mPADs like ink-jet printing, wax-printing etc. are comparatively simpler but efficient and is capable of producing the device at large scale is very low cost. Thus, it provides a huge scope for exploration of different designs or geometries by the researchers to explore an efficient plasma separation platform in paper based assays. Apart from exploration in geometrical variation in shapes of the micro-capillaries or the flow channels the researchers have also tried reagent assisted methods. Separation by agglutination and aggregation are two main reagent assisted methods used in paper based platform. Hemagglutination is a biochemical phenomenon triggered by antigen specific antibody bonding of RBCs. It causes an agglutination of RBCs in presence of its matching antibody (Mitra et al. 2014; Mujahid and Dickert 2016). Hemagglutination triggers clumping of RBCs, thus resulting in an elevation of viscosity which can impede the flow of blood through the porous platform. This phenomenon is used by the researchers to create a differential flow dynamics between RBCs and plasma to wick out plasma from the whole blood. Similarly, clustering of RBC is achieved by another phenomenon known as erythrocyte aggregation. Erythrocyte aggregation is a resultant of face-to face stacking of RBCs which gets dispersed in an environment with high shear and reforms at low shear environment. Erythrocyte aggregation is primarily influenced by the composition of plasma proteins, the surface properties of RBCs, and the magnitude of shear force being experienced (Baskurt and Meiselman 2003). Thus, the following section will present a detailed enunciation of the advances in paper based plasma separation devices employing several working mechanisms along with its applicative area.

5.3 Recent Advances in Paper Based Blood Plasma Separation Devices

With the advent of growing research advances in PoC diagnostics, paper can be regarded as the most preferred and economic choice as a diagnostic platform in following domain. Several characteristics of paper like biocompatibility, easy disposability, low cost and easy availability are responsible for its increasing popularity as a substrate for the PoC diagnostics. Paper being naturally hydrophilic in nature also provides an easy actuation of flow of bio-analytes through its micro-channels (Sher et al. 2017). Thus, unlike other polymer based electromechanical micro-devices the paper based devices doesn't require any additional external pumps or sources to drive the flow. Capillary driven fluid flow in paper is governed by the intermolecular force between the fluid and the porous cellulose matrix (IET 2017). The hyrophyllic characteristic of paper makes it a viable choice

for an appropriate substrate for several micro-fabrication methods. The porous nature of the paper aids in efficient adsorbance of ink and adherence of any other functionalizing materials like nanoparticles. Thus, paper is regarded as one of the best choices for substrate for flexible diagnostics fabricated using screen printing, ink-jet printing or wax printing. In addition, to conventional cellulose/filter paper, there exists some paper like materials which are used alone or in combination with conventional cellulose paper in designing the PoC medical diagnostics which includes nitrocellulose, polysulfone, polyvinylidene difluoride, nylon and fiber glass. Apart from conventional cellulose paper, polysulfone and fibre glass are the most popular substrate used for plasma separation application. As discussed earlier, plasma separation is a mandate in characterization of blood especially in PoC diagnostics to overcome the interference caused by the cellular components during signal transduction. Plasma being the informative fluid rich in proteins and organic molecules containing the essential metabolites and biomarkers essential in easy and rapid diagnosis of internal pathological condition of a patient, its efficient separation is necessary. The following section discusses several paper based point of care plasma separators in chronological order which efficiently overcome the interference of hemoglobin from RBCs as well as the release of nucleic acids (Mielczarek et al. 2016) and other cellular components from blood cells to provide a secondary level of efficient characterization of plasma fluid for PoC diagnosis. It also includes enunciation of several geometrical patterns of microfluidic channels which assist the capillary driven flow via diffusion or filtration with or without the assistance of other reagents. The survey mainly emphasis on the separation of plasma from whole blood using chromatography or cellulose/filter paper as a separation platform following the separation assisted via agglutination, aggregation or physical separation. Physiologically, separation of erythrocytes from that of its counter medium plasma occurs either due to flow rate differences caused due to Fahraeus-Lindqvist effect inside the vascular network or due to RBC clumping inside the vascular system due to agglutination, aggregation or clot formation. Yang et al. reported (Yang et al. 2012) a hemagglutination based plasma separation in paper substrate. Although separation of RBCs can also be performed using paper substrates with pore size smaller than 2.5 um, agglutination is preferred methodology for separation. Reduction in pore size reduces the flow rate Q of separating plasma as scales with the forth power of the pore diameter ($Q \propto d^4$) (Beebe et al. 2002) and additionally the filtered RBC packs tends to block the flow plasma leading to complete cessation of plasma flow through the membrane as shown in Fig. 5.1c. Thus, hemagglutination is preferred over simple cross filtration or diffusion based separation mechanisms. In other words, the agglutination mechanism performs simple filtration where the effective size of RBC is increased using agglutination (Ludewig and Chanutin 1949; Zuk et al. 1985) which is further easily filtered using filter paper of much larger pore size consequently resulting in a significant increased rate of volumetric flow of filtered plasma as described in Fig. 5.1.

The paper based assay for plasma separation proposed by Yang et al. (2012) was fabricated by printing wax patterns in chromatography paper with hydrophobic

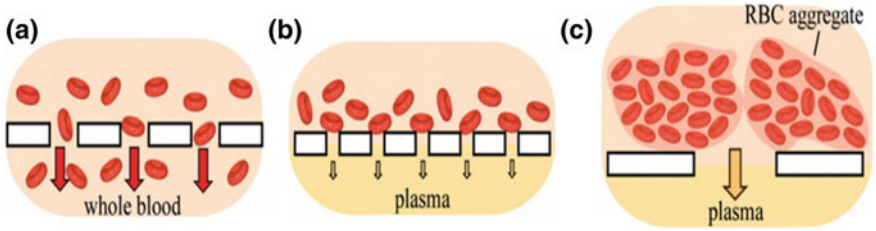

Fig. 5.1 Schematic illustration of blood flow through varying porous media and usage of agglutination to tailor the fluidic flow of plasma excluding the cellular clusters. Blood flow dynamics through filter paper of pore diameter of **a** ~2.5 um or larger **b** smaller than 2.5 um **c** blood flow dynamics accompanied by agglutination through filters with large-diameter pores. Reprinted with permission from Yang et al. (2012), Copyright, The Royal Science of Chemistry 2012

barriers surrounding the separation zone at the center and four separate detection zones at the periphery of the respective as shown in Fig. 5.2. RBC agglutinating antibodies of about 7 ul were spotted in the center of the device (separation zone) and the colorimetric assay reagents were functionalized and dried at the peripheral detections zones. While performing the test, the sample of whole blood (~7 ul) is pipetted at the center of the separation zone. Once the whole blood sample is in contact with that of the RBC agglutinating antibodies, the RBCs starts agglutinating and the plasma is wicked out towards the peripheral region (i.e. the detection zones) where it reacted with the reagents of the colorimetric assay as shown in Fig. 5.2. Prior to Yang et al., M. S. Khan et al. studied the transport mechanisms of agglutinated blood in paper platform for instantaneous blood typing (Khan et al. 2010). The authors observed the variation in transportation of blood droplets on paper based depending on the criteria that whether the blood agglutinates or not. Simultaneous agglutination and separation of plasma from that of the RBCs ensured the blood type matching. A schematic as shown in Fig. 5.2b enunciates the working of plasma separation using agglutination used for blood typing. Blood plasma separation using agglutination was also explored by some other authors like Jarujamrus et al. for blood typing and study of blood dynamics in paper platform (Jarujamrus et al. 2012).

With increased popularity of paper based sensing platform, multifunctional microfluidic paper based assays are constantly different PoC diagnostic are devised using origami techniques. In 2012, Ge et al. (2012) devised low cost 3D Origami-based multifunction-integrated immunodevice for simultaneous detection of four tumor markers with integrated plasma separation fold as shown in Fig. 5.3. The plasma separator (chromatography paper) fold was intelligently introduced into the chemiluminescence immunoassay using multiplexed sandwiched type assembly. The following device also implemented separation of plasma from whole blood using agglutination. The consequent agglutination and vertical filtration due to

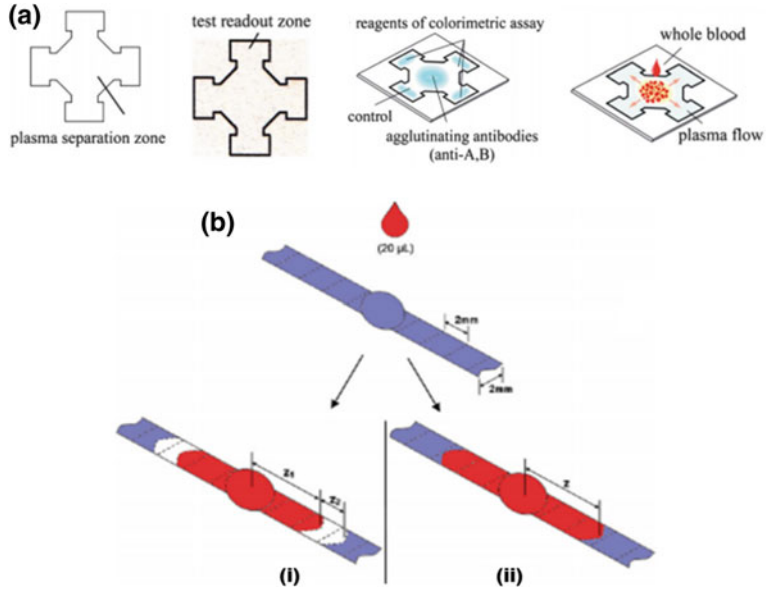

Fig. 5.2 **a** Design and enunciation of agglutinating and readout zones in a microfluidic paper-based analytical device Reprinted with permission from Yang et al. (2012). **b**. Schematic representation of the wicking of blood (i. AB+, ii. B+) on paper treated with a specific and a nonspecific antibody (anti-A). Reprinted with permission from Khan et al. (2010), Analytical Chemistry 2010

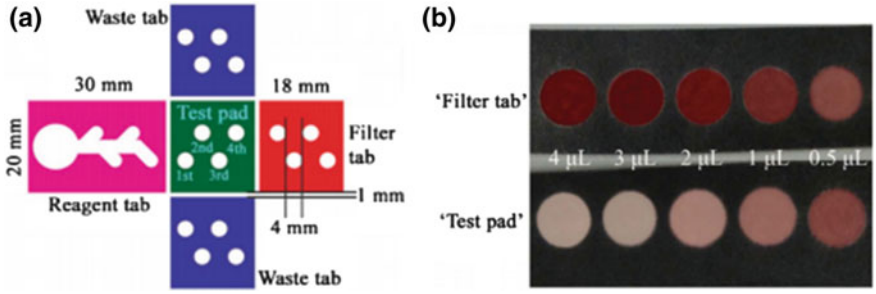

Fig. 5.3 **a** The schematic representation 3D origami-based device. **b** Images of the separation of red blood cells from the pretreated whole blood samples in the paper micro-zones. Reprinted with permission from Ge et al. (2012)

intelligently spatially attacked paper folds the efficient separation of plasma was achieved. The following assay used blood samples treated with Anti D were used to obtain separated plasma at the reaction fold of the device.

The paper based plasma separation assays discussed so far works on the basic principle of agglutination based separation which relies upon the existence of a

strong specific antibody–antigen interaction for its efficient working. In case of certain blood groups such as O-blood group, the assay fails to provide a strong specific antigen-antibody reaction using A, B and Rh antibodies. Additionally, this agglutination based method also requires preparatory blood typing to know whether antibody–antigen interactions are able to induce blood agglutination. Furthermore, antibodies used requires extra maintenance to retain its bioactivity resulting an increase in material cost. Thus, to overcome the limitations faced by the method of agglutination for blood plasma separation researchers preferred an alternative of implementing the method of aggregation of RBCs for plasma separation. Most recently, Li et al. used calcium chloride solution to aggregate RBCs in rabbit blood samples (Li et al. 2014). The following invention describes how calcium ions are able to destabilize the suspension of RBCs in blood by suppressing the electric double layer on the RBCs surfaces, reducing the charge repulsion between RBCs (Ataullakhanov et al. 1994). Following the concept of RBC aggregation using salt solutions introduced by Li et al., Nilghaz et al. also developed a plasma separation assay based on aggregation via salt solutions. Strong salt concentrations causes a hypertonic medium for the erythrocytes by increasing the osmotic pressure around the cell membranes and further resulting in a compression of cell's electric double layer (EDL). Loss of EDL of the respective cells results in close packing of RBCs. The close packing of RBCs with a fibrin network leads to formation of immobile RBC aggregates. Further, the immobility of RBC packs results in differential velocity of the cellular components with that of plasma. Thus, maintaining a mobile phase for separated plasma which further wicks and reaches the detection zone as shown in Fig. 5.4. The clinical validation of the respective method was verified by performing successful colorimetric detection of glucose from the separated plasma. The schematic blow describes the working of the saline functionalized uPAD developed by Nilghaz and Shen (2015).

Unlike, salt based differential osmosis based RBC aggregating methodologies, Kar et al. tried manipulating the hemodynamics using PBS solution and bifurcating geometry to separate plasma in paper porous media (Kar et al. 2015). They fabricated an H-shaped channel device where whole blood and PBS solution were simultaneously dispensed. During the simultaneous flow of the PBS and whole blood through the linear region of the device, the lighter molecules suspended in the bloodstream diffused into the PBS stream. This resulted in RBC-rich and plasma-rich components being separately collected in each of the two outputs of the device. A clear perspective of the working can be obtained from Fig. 5.5 enunciated.

In addition to cellulose paper (filter paper or chromatography paper) some researchers also tried amalgamating filter or chromatography paper with plasma separation membrane (PSM) to achieve efficient separation of plasma without the use of any RBC agglutinating or aggregating reagents in single step. A single step plasma separating device was engineered by Songjaroen et al. (2012) using an overlapped combination of PSM and chromatography as shown in Fig. 5.6a. PSMs made of polyvinyl alcohol-bound glass fiber filters like MF1 and LF1 were assembled with Whatman Grade 1 filter paper using wax dipping method.

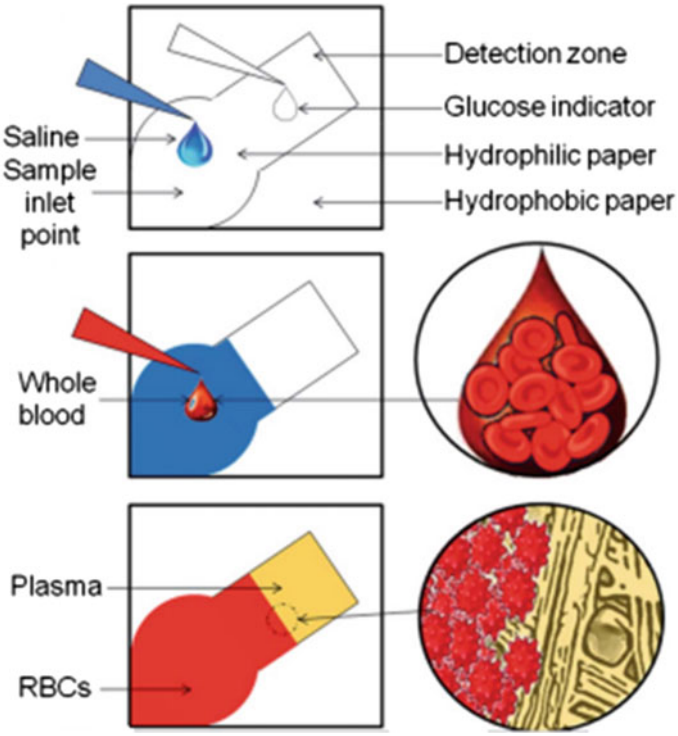

Fig. 5.4 Schematic illustration of the plasma separation method on saline functionalized mPAD. Reprinted with permission from Nilghaz and Shen (2015)

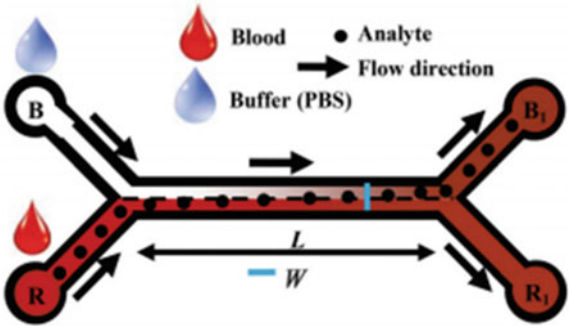

Fig. 5.5 Schematic representation of the H–type plasma separator with two reservoirs R and B at rear end for whole blood and PBS respectively resulting in separation of plasma and cellular components. Separated plasma is dispensed at reservoir B1 whereas the cellular components gets dispensed at reservoir R1. Reprinted with permission from Kar et al. (2015)

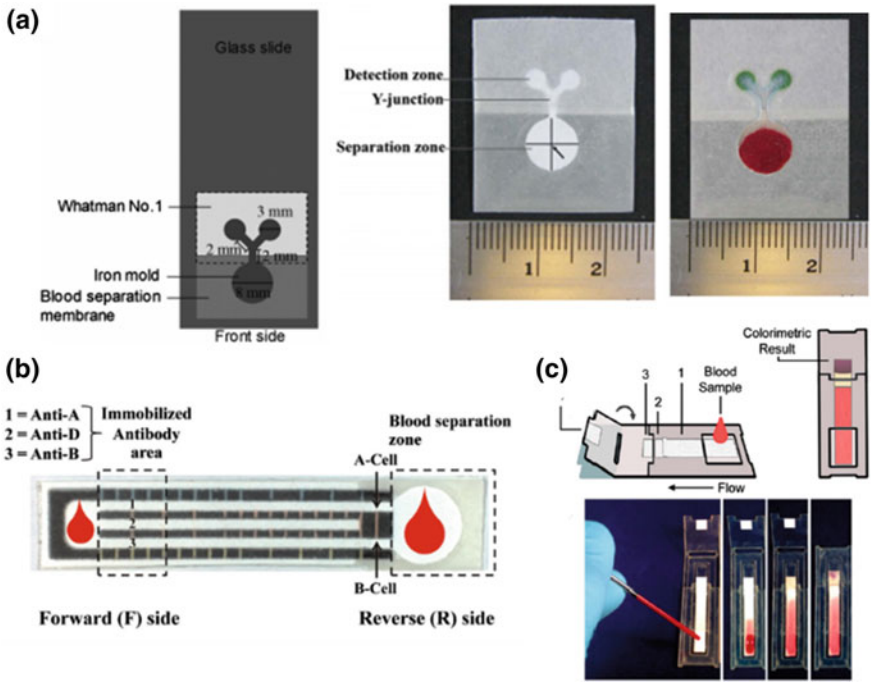

Fig. 5.6 **a** The assembly of the mPAD for plasma separation from whole blood using wax dipping method (top view) and the working mPAD for whole blood separation composed of separation zone and detection zone. Reprinted with permission from Songjaroen et al. (2012). **b** The PAD approach for simultaneous determination of Rh typing and the forward and reverse ABO blood groupings. Reprinted with permission from Noiphung et al. (2015). **c** Prototype of disposable paper-based device (Robinson et al. 2016 Copyright MDPI)

The assembly consisted the placing of filter paper over the PSM with a small overlapping area. The blood separation in the following device is achieved by combining two separate types of paper where the lateral fluidic flow is governed by Lucas-Washburn model. The following device was proven to be functional at higher hematocrit levels as well ($\sim$55% HCT). It was capable of separating plasma within 2 min of application of 15–22 ul of whole blood. In addition to efficient separation of plasma the device was capable of colorimetric detection of plasma protein concentration with high reproducibility [RSD = 2.62% (n =10)]. Similarly, Noiphung et al. (2015) used the combination of chromatography paper and MF1 blood separation paper for blood plasma separation in paper based assay for simultaneous determination of Rh typing and forward and reverse ABO blood group as shown in Fig. 5.6b. Wax printing and wax dipping were performed to fabricate the following device. A combination of PSM and traditional cellulose/ filter was also utilized by Robinson et al. (2016) for separating plasma for semi quantitative Phe (phenylalanine) detection in whole blood for PKU therapy

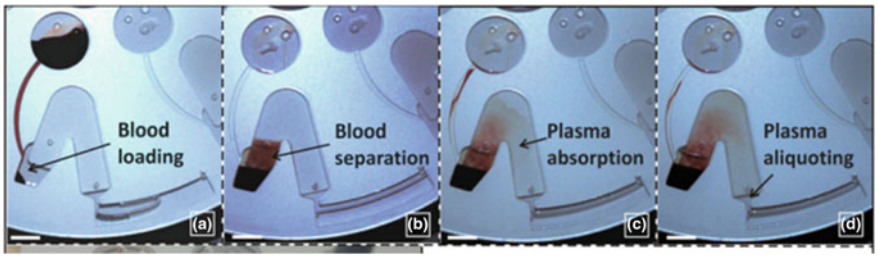

Fig. 5.7 Schematic illustrating the several steps in leading to blood separation process using paper siphon mechanism. Reprinted with permission from Godino et al. (2014)

monitoring as shown in Fig. 5.6c. The separation was executable due to the layering of PSM over the porous substrate with an addition of Mylar folding housing with a small overlap of 1.5 mm.

In contrast to previously discussed hybrid devices for plasma separation working on differential flow in lateral flow assays, Godino et al. engineered a novel hybrid paper-polymer device using paper siphon. The respective device relies on two main interplaying forces to create unique valving and liquid sampling under centrifugal microfluidics (Godino et al. 2014). The author has utilized the differential behavior of centrifugal force to obtain paper siphoning. At elevated speed the fluid is found to accumulate at the edge whereas at sufficiently low speeds the fluid tends to wick inwards along the curved geometry. Thus, distinct modes of flow modes can be accounted for different fluid with varying viscosity. The device mimics the conventional method of centrifugation for plasma separation in paper-polymer composite platform. The two main physical forces responsible for separation action are capillarity action and the centrifugal field. The assurance of efficient interplay of the forces is obtained by deciding the optimized frequency for centrifugal force required for efficient siphoning. The stepwise separation through siphoning is enunciated using Fig. 5.7 shown.

5.4 Summary and Future Perspectives

The chapter describes several paper based plasma separation assays developed in last few years (2010 onwards). It also enunciates several assisting methodologies such as agglutination and aggregation used to achieve efficient separation. Apart from reagent assistance the porous media structural architecture of paper which affects the flow dynamics of blood fluid through the same is also enunciated. The detailed explanation of the devices developed along with its working mechanisms is elaborated. These paper based devices are often found to follow Darcy's Law in porous media resulting in dynamics defined by Lucas Washburn to undergo passive capillary driven flow through the porous paper substrates. Further the separation

efficiency of the cellular components from plasma is either assisted using reagents causing agglutination/ aggregation of RBCs or some geometrical modification achieved by tailoring the shape of the paper substrate. Often the non-reagent based separation physical separation is achieved by creating a hybrid or creative composite of paper with that of the PSMs or by tailoring the geometrical shape of the platform itself. The intelligent tailoring of the substrate shape leads to manipulation of the flow dynamics (e.g. flow rate, centrifugal forces or other driving forces) of the blood itself as performed for the device with H-channels or the device developed by Godino et al. to act as paper siphon. Thus, the following chapter presents the researchers with detailed advances in the domain of paper based assays for plasma separation to present a clear understanding and knowledge about the advances. The researchers may further use the following knowledge to tailor new and efficient devices with added modified characteristics. The future researchers should understand that to achieve the maximum working and separation efficiency of the engineered device, the following must be exclusive or independent of the variations in hematocrit content of the blood as hematocrit content largely affects the rheological dynamics or properties of the blood. Efforts should also be made to explore various geometrical shapes which would be capable of manipulating the flow of blood and effective in separating out plasma from the same without any usage of reagents which are costly and require challenging preservation platforms. Scientists may also explore the domain of developing paper based composites capable of separating plasma but comparatively cheaper to the existing PSMs (LF1, MF1, VF1 and VF2). Methods of improvements in the existing fabrication methodologies (ink-jet printing, wax-printing etc.) to develop more intricate and narrow micro-capillaries in paper platform is also suggested. Narrower and intricate micro-capillaries will lead to usage of small sample volume for characterization/ analysis resulting in maintenance of the homogeneity of the sample and reduction in errors caused due to hematocrit variations. Simultaneously, attention should be paid in dimension of the micro-capillaries to avoid edge effects. Conclusively, there exists a huge scope of future developments in the following domain to obtain a fully functional/ efficient and low cost paper based plasma separation device which can further assist several other PoC diagnostics.

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Chapter 6
Evolution of Paper Microfluidics as an Alternate Diagnostic Platform

Shantimoy Kar and Suman Chakraborty

Abstract Since the inception of paper microfluidics, there has been a significant leap in developing microfluidic assays for wide range of applications, particularly in healthcare. Considering the average economic status of the population and epidemic outbreaks of diseases within territory of developing nations, it is indeed an essential demand to have an alternate diagnostic plat-form which is ideally expected to serve the purpose at faster and affordable means. In this context, introduction of the paper devices started with qualitative assays and thereafter it has subsequently evolved for semi-quantitative and quantitative platforms. Conventional diagnostic procedures involve time consuming multi step processes that are rather elaborate and expensive, necessitating the involvement of highly trained personnel for analysis. Paper based diagnostic procedures; on the other hand, normally deploy tiny amounts of physiological fluids and consumables, to achieve the same end objective at much reduced expenses and time. Towards this, colorimetric assays have shown significant promises, but are limited by the sensitivity of the diagnostic method. In an effort to improve the sensitivity of the detection, other approaches like fluorescent, electrochemical, luminescence have been progressively explored. In this Chapter, the discussion is systematically organized from fabrication of paper-based devices to their different applications in the diagnostic arena. Starting from the fabrication fundamentals and working principles, we delve deep on different colorimetric detection of bio-molecules, bacteria etc. We also focus on the critical aspect of plasma separation from the whole blood, which is a key for most colorimetric detections. Finally, we summarize the key findings about the advancements of these devices for practical use in resource-limited settings and bring out some new and unaddressed questions as well as challenges in the field.

Keywords Paper-microfluidics · Immunoassay · Colorimetric · Diagnostics

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6.1 Introduction

Why do we need to think of alternate diagnostic strategies? Since last two decades, point-of-care testing strategies have emerged as an alternate diagnostic tool. Before we discuss the necessity of the point-of-care testing platforms, it is important to understand the strategy and limitations involved in current day's standard clinical laboratory protocol (Yager et al. 2006; Gubala et al. 2012; Chin et al. 2013; Sharma et al. 2015). In the developing world, when someone develops a symptom or sets of symptoms, he/she usually walks into a local pharmacy where the patient explains about the symptoms, and then takes the medicines with the consultations with the pharmacy personnel. In most of the cases, the preliminary symptoms are resolved, however; the actual reason remains undiagnosed until it turns to its next phase. This specific approach is certainly cost-effective (as it does not involve the clinical laboratories and doctors in overall decision-making process) and thus affordable to mass population. However, this practice has significant adverse effects in terms of patient management in the long run, particularly, in the context of developing drug resistance, in addition to being non-specific to the cause. This practice has been well accepted to the mass population as the standard clinical laboratory facilities are not only expensive but also cumbersome and limited as per the needs. Furthermore, these gold standard stereotype laboratory practices have other disadvantages like involvement of trained personnel, requirement of high reagent volume and sophisticated laboratory instruments; which essentially adds up to the overall cost of the diagnosis. Above all, the centralization of these laboratory practices within the urban and semi-urban areas makes it more difficult for accessing from rural sectors. Considering the limitation of current standard pathological laboratory scenario, it is essential to adopt point-of-care (POC) testing technologies as an alternative diagnostic tool; with the aim of bridging the gap between the need and affordability.

Emergence of 'microfluidics' as a subject, over the last two decades, demonstrates the potential of several alternate diagnostic strategies. Conventional microfluidic systems involve silicon, poly-dimethyl-siloxanes (PDMS), poly-methyl-methacrylate (PMMA), glass as substrates (Becker and Locascio 2002); which can be categorized as the first-generation microfluidic devices. These platforms certainly offer significant advantages in terms of liquid handling, flow control, and precise investigations across different domains (Mao and Huang 2012; Chin et al. 2013; Volpatti and Yetisen 2014; Kar et al. 2016). These advantages offer significant deployment of these devices in different biological applications and especially in the domain of healthcare technologies. However, the overall expenses restrict its wide usage in the diagnostic world, particularly, within the territory of resource limited settings. In the diagnostic domain, avoidance of contamination is the utmost priority. Thus, in most cases, the re-use of same device is not recommended. This restriction of not using the device for repeated use contributes towards increasing the overall diagnostic expenses. In this context, paper serves as a pretty handy,

inexpensive, flexible and easy disposable substrate; which makes it most appealing towards meeting the current day's demand of point-of-care testing devices.

'Paper microfluidics' is essentially a combination of two different words namely 'paper' and 'microfluidics', which effectively deals with the microfluidic systems on paper, i.e., paper is used as the main substrate for device. Paper has been used in different analytical experiments, especially for chromatographic assays. Recent advancements in microfluidics have led the researchers to realize the potential of the paper substrate as the purpose of utilizing it as low-cost disposable substrates for the preparation of different devices (Oyola-Reynoso et al. 2015). On the other hand, microfluidics is the subject which specifically deals with the fluid flow through miniaturized devices where the working length scale remains within the range of microns to sub microns. Since last decade, paper microfluidics captures significant attention across the globe due to easy handling, flexibility, and inexpensive alternative as compared to its traditional microfluidic counterparts.

In this chapter, we will focus our discussion of paper-based systems in the context of point-of-care diagnostics.

6.2 Point-of-Care (POC) Diagnostics

An ideal point-of-care diagnostic platform is something on which a drop of physiological fluid (i.e. the sample of interest) will be put and thereafter the results will be analyzed with a smartphone. Most importantly, the entire system (including result analysis) will be operated by the user itself (Martinez et al. 2010; Cate et al. 2015). Schematic in Fig. 6.1 depicts the essential steps involved in POC diagnostics systems.

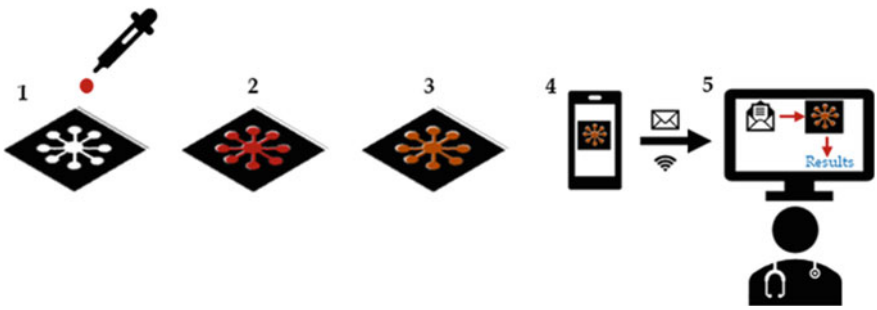

Fig. 6.1 Schematic representation of a standard paper-based point-of-care diagnostics: Step 1: Sample fluid is put on top of the device; Step 2: Fluid is distributed into the device towards the detection regions; Step 3: Colorimetric changes occur on the detection pads; Step 4: Images taken on a smartphone for analysis; Step 5: Image is sent through wireless connection to the expert clinicians for further analysis and subsequent advices

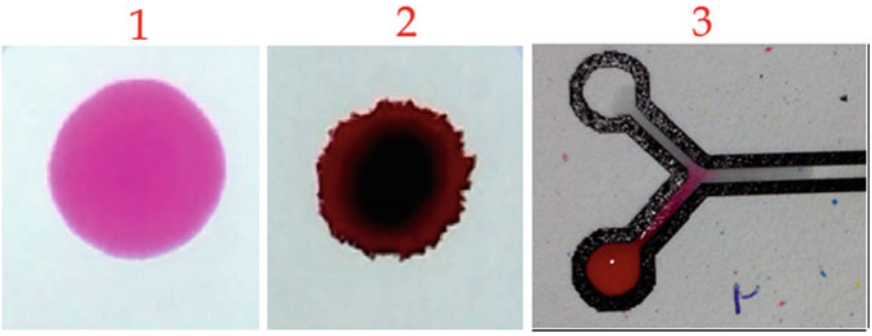

Fig. 6.2 Images of fluid spreading on paper surfaces: (1) Aqueous solution of dye; (2) Blood drop (without any hydrophobic barrier) and (3) Fluid flow on Whatman filter paper is guided by the printed hydrophobic barriers

To understand the fluid flow through paper or any other paper-like porous matrix, consider the examples shown in Fig. 6.2; which depicts the spreading of a liquid drop on paper matrix. From the figure, it is evident that fluid starts spreading immediately in every possible direction randomly while none of the directions was preferred over the other possible flow circuits. In this regard, to guide the fluid flow in a desired direction, it is essential to restrict the undesired flow to the other directions; which is often done by creating a hydrophobic barrier (Ballerini et al. 2012). Thus, by creating a hydrophobic barrier on paper, offer a definite direction to the fluid flow on these substrates.

6.3 Fabrication of Paper-Based Devices

In this section, we will understand the different fabrication techniques and the corresponding working principles of the paper-based devices as well.

Paper is a porous hydrophilic matrix, composed of random distribution of cellulose fibers as shown in the Fig. 6.3. Creation of a hydrophobic barrier on the paper substrate offers directionality to the fluid transport on these devices. There are many different fabrication techniques have been exploited for such device fabrication which can be broadly categorized into four types as summarized in Table 6.1.

Photolithography: The following schematic in Fig. 6.4 depicts the standard photolithographic based fabrication protocol (Mandal et al. 2012; Das et al. 2018). In this technique, paper is first soaked with photoresist followed by pre-baking step. Thereafter, the photoresist soaked paper is exposed under UV and subsequent post-baked. In the final step, the unexposed photoresist is washed out with acetone leaving the desired pattern on paper surface.

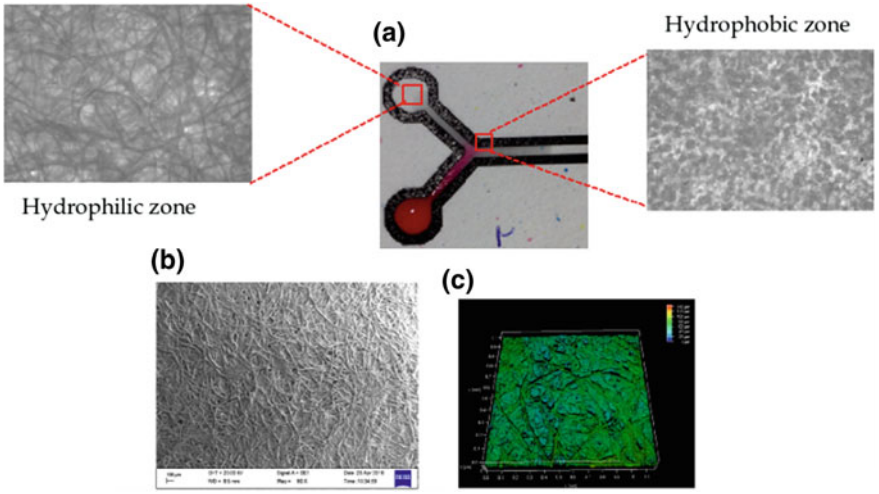

Fig. 6.3 **a**. Microscopic view of the hydrophilic and hydrophobic zones of the paper-based devices; **b**. scanning electronic microscopy (SEM) image; and **c**. confocal view of the hydrophilic zone of the paper matrix depicts the random distribution of the cellulose fibers

Table 6.1 Summary of the different fabrication techniques

Patterning principle	Fabrication technique
Physical blocking of pores	Photolithography (Bruzewicz et al. 2008), PDMS printing (Bruzewicz et al. 2008)
Physical deposition of reagent on paper	Inkjet printing (Abe et al. 2008), Wax printing (Carrilho et al. 2009), Wax screen printing (Carrilho et al. 2009), Flexographic printing (Carrilho et al. 2009), Toner printing (Dey et al. 2015), Writing through correction pen (Mani et al. 2019)
Chemical modification of paper	Plasma treatment, Printing using alkyl ketene dimer (Li et al. 2010)
Two-dimensional shaping/ cutting	CO_2 laser (Arun et al. 2014), Cutter plotter

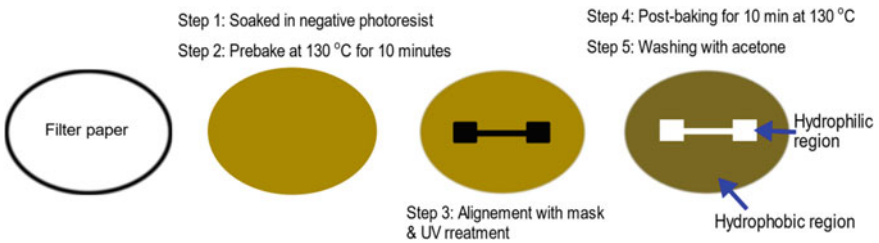

Fig. 6.4 Above schematic depicts the associated key steps involved in photolithographic fabrication protocol. Reproduced from Das et al. (2018) with permission from The Royal Society of Chemistry

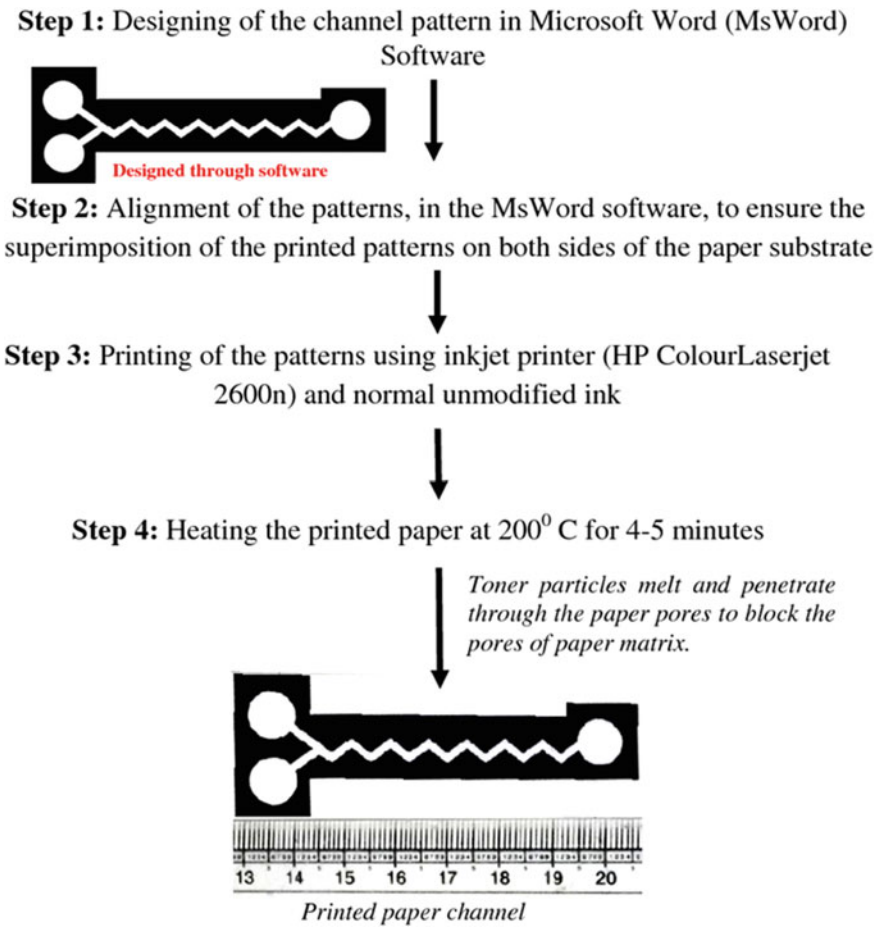

Fig. 6.5 Flow diagram of the toner printing protocol using a standard office printer and hot plate. Reproduced from Dey et al. (2015) with permission from © 2015 Springer-Verlag Berlin Heidelberg

Toner printing: An in depth look into the recent literature delineates that there are different inkjet printing and toner printing protocols have been followed. All of these use either a specialized setup or modified ink for this purpose. To overcome these challenges, Dey et al. (2015) reported a simple technique for fabrication (Fig. 6.5), which prints the desired structure on both sides of the paper while one side is completely superimposed to other. In the subsequent step, printed paper is heated for 4-5 min; which allows the toner particles to melt and thereby blocks the paper pores. This specific technique apparently requires nothing (except office printer and a heating plate), thus can be very suited for resource limited settings.

Apart from the aforesaid two techniques, few other techniques like wax printing, laser cutter (CO_2 laser) and chemical modifications based techniques have been adopted in recent reports. Amongst all these available techniques, wax printing is most used for fabrication purposes.

Depending on the potential usage of the device, one particular fabrication technique is usually preferred over the others. To make this point very clear, photolithography technique provides better precise width structures preferably in few microns (100–500 μm) as the crosslinking of the polymer is mainly dictated by the accuracy of the photomasks. On the other hand, the inkjet/wax/toner printing has an inherent key step of heating the substrate after the structure is printed. During this heating process, the ink/wax/toner particles get melted and thereby block the hydrophilic pores of the paper matrix. While blocking through wax/ink/toner particles, it diffuses through equally in all three possible dimensions, which may reduce the effective width of the structures than that of the intended measurements; thus duration of heating plays a key role in determining the precision of the desired structures.

For the purpose of paper-based device fabrications, till date, the most commonly used substrate is Whatman laboratory grade filter paper. These filter papers are usually manufactured for the general purpose of filtration. Thus, it is important to understand that these papers were not manufactured for the very specific purpose of microfluidic experiments; which often leads to the common understanding of non-uniform and random distributions of cellulose fabrics and the pores too (see Fig. 6.3). It is important to understand the fundamental differences between the underlying flow mechanism (Chaudhury et al. 2016) between paper-based devices and conventional microfluidic devices. In conventional microfluidic devices, fluid flow is actuated through external actuations (like in most cases a syringe pump is used for developing a pressure gradient across the two ends of the device). On the other hand, in case of paper, fluid flows through the paper matrix not flowing on top of the paper. This flow mechanism is primarily guided by the capillarity action of the porous paper network; thus does not require any external pumping/actuation arrangements; thus often called as ‘pump free’ flow. The random structures of the paper matrix often offer rudimentary control over the fluid flow. In this regard, researchers have delineated the usage of other external actuation mechanisms like electric field (Dey et al. 2015), surface acoustic waves (Rezk et al. 2012) in tandem. However, considering the diagnostic applications, this rudimentary control may not have significant impact in terms of qualitative diagnostic assays.

6.4 Diagnostic Assays

To realize the diagnostic assays, different principles have been followed according to the specific target of detection. Among many diagnostic principles, colorimetric assay is the most common one. Within the scope of the current chapter, we will now discuss about colorimetric detection of different bio-analytes e.g. biomolecules (like

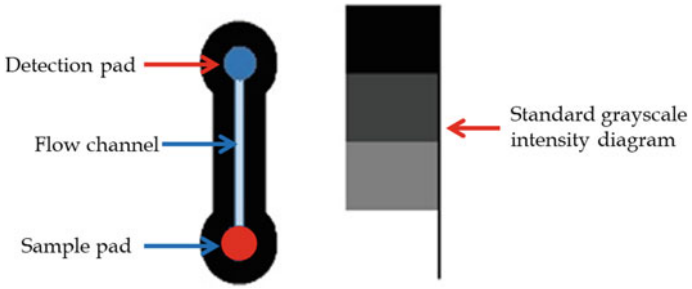

Fig. 6.6 Schematic of the paper-based diagnostic platform

glucose, uric acid), proteins (e.g. antigens), bacteria (e.g. different pathogens present in foods and drinking water). In depth look of these detection protocol involves an underlying chemical assay which is specific to the target bio-analyte. In majority of the cases, specific dried reagent was kept on the detection pad of the paper device (Fig. 6.6); which demonstrates chromogenic changes when the particular target analyte is available from the sample fluid. These colorimetric changes provide the necessary indications about the presence of specific chemicals; which is again quantified using considering linear variation of target analyte concentration.

Schematic in the Fig. 6.6 explains the different parts of the device; sample pad (where the sample was put), flow channel (through which the sample flows to the detection region), and detection pad (where the sample reacts with the embedded dry reagents). In addition, a standard grayscale intensity diagram has been printed alongside of the paper channel. This grayscale bar takes care about the relative intensity corrections during result analysis due to the non-uniform illumination and imaging conditions. It is important to note here that if the post-processing step does not involve grayscale intensity analysis; in that case other type of standard color chart e.g. macbeth chart needs to be printed.

This particular diagnostic analogy uses the similar chemical assays as used in standard clinical laboratory assays. Depending on the target analyte especially chemical nature of the target molecule, a suitable diagnostic assay is followed. In this section, we will systematically discuss few key strategies e.g. chemicals-based assays, enzymatic assays, immunoassays and DNA hybridization which have been adopted on paper-based systems towards successful implementation of the diagnostic assays.

6.4.1 Chemical-Based Assays

Till date, paper-based test strips have been successfully exploited for the detection of many different bio-analytes e.g. glucose, protein, lactate, uric acid, cholesterol and to name a few. These colorimetric assays are quantified by analyzing the color

intensity of the unknown concentration against the calibration curve generated by measuring the same analyte of known concentration, following the same protocol. In 2010, (Dungchai et. al. 2010) the group of Henry et. al. demonstrated the fact of utilizing multiple analytes for the detection of same analyte; thus providing a readout which could be easily distinguishable by bare eye. Usage of the same colorimetric indicator provides shades of the same color which is hard to realize using bare eye; but could be done simply through the processing of the digital image. During this detection of multiple analytes using a specific enzymatic reaction (glucose oxidase for glucose detection, uricase for urea detection, lactate oxidase for lactose detection) which involves the corresponding oxidase that produces hydrogen peroxide which in turn oxidizes the embedded chromogenic substrates i.e. the indicators, thus creating a color change in the respective detection pads. Dungchai et. al. delineated the usage of different colorimetric indicators together; mixture of 4-aminoantipyrine (AAP) and 3,5-dichloro-2-hydroxybenzenesulfonic acid (DHBS) in 1:2 ratio, o-dianisidine dihydrochloride (OD), potassium iodide (KI), acid yellow (Y), and acid black (B). Colorless indicators (AAP+DHBS), OD, and KI turn into red, green-brown, and yellow-brown respectively upon oxidation. On the other hand, Y and B become colorless from yellow and black color respectively. It is important to note here that these devices were explored for multiplexing purposes i.e. simultaneous detection or quantification of multiple targets from the same sample fluid on a single detection platform following similar principle.

6.4.2 Immunoassays

In case of detecting any antigens on these devices, most relied on immunoassay technique. Enzyme linked immunosorbent assays, i.e. ELISA, is one of the most common and regular practices used in diagnostic industries in regular basis. In standard laboratory environment, this practice is usually exercised in micro-titer plates. Like the conventional 96 well based standard, ELISA protocol works similarly on paper substrate (Cheng et al. 2010), while the requirement of chemical volume is significantly reduced. The signal detection can be easily performed using a desktop scanner without necessitating the standard plate reader. In 2010, Cheng et al. delineated the ELISA study following a colorimetric approach (schematically shown in Fig. 6.7). This study demonstrates the quantification of antibodies specific to the HIV-1 envelope antigen gp41 in human serum with the aid of anti-human IgG antibody conjugated with alkaline phosphatase (ALP) following colorimetric readout.

Jokerst et al. (2012) reported a detection methodology of three different pathogenic bacteria namely Escherichia coli, Salmonella spp., and Listeria monocytogenes from food samples. This detection methodology utilizes an enzymatic assay based colorimetric detection protocol, where an enzyme specific to the target bacteria reacts with embedded chromogens and a colorimetric change is noticed

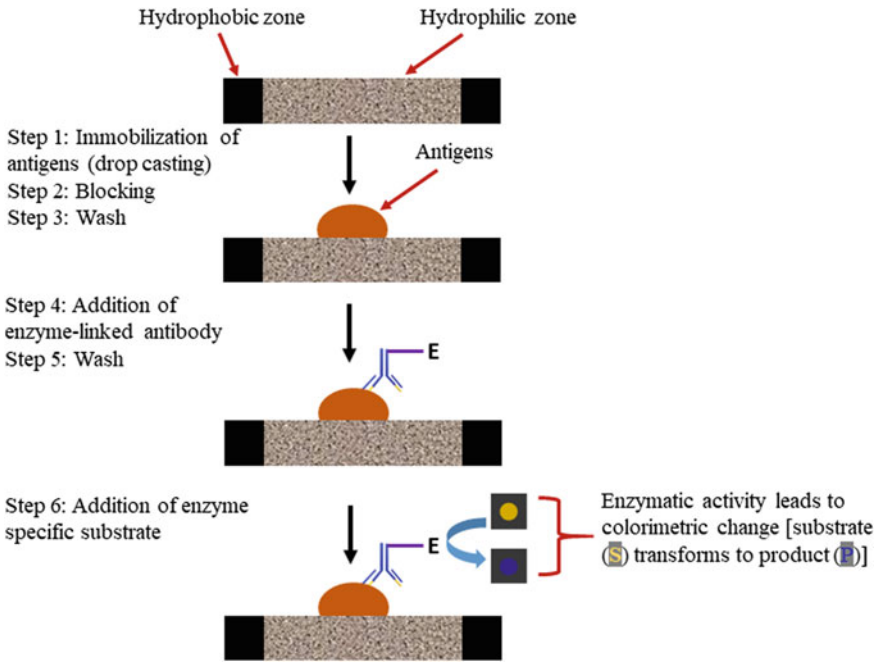

Fig. 6.7 Flow chart depicts the involved steps in paper-based ELISA. Adapted from Cheng et al. (2010) Copyright © 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

[specific enzyme chromogen pairs are: Escherichia coli (β-galactosidase with chlorophenol red β-galactopyranoside), Salmonella spp. (esterase with 5-bromo-6-chloro-3-indolyl caprylate (magenta caprylate)), Listeria monocytogenes (phosphatidylinositolspecific phospholipase C (PI-PLC) with 5-bromo-4-chloro-3-indolyl-myo-inositol phosphate (X-InP)].

6.4.3 DNA Hybridization on Paper

It is now realized that the colorimetric detection method works fine within the certain range of target analyte concentration. In particular, detection of very low concentration of analyte often does not offer any detectable signal; thus, an alternate tool is also required. In this regard, detection using nucleic acids-based assays is attracting the attention of the researchers. Nucleic acid-based assays offer much higher sensitivity than any other reported techniques. These nucleic acid-based assays are mainly relying on the fundamental principle of DNA hybridization technique. In this section, we will address the DNA hybridization methodologies on paper-based devices (Araújo et al. 2012). DNA hybridization is nothing but coupling of single stranded DNA to its complementary counterpart.

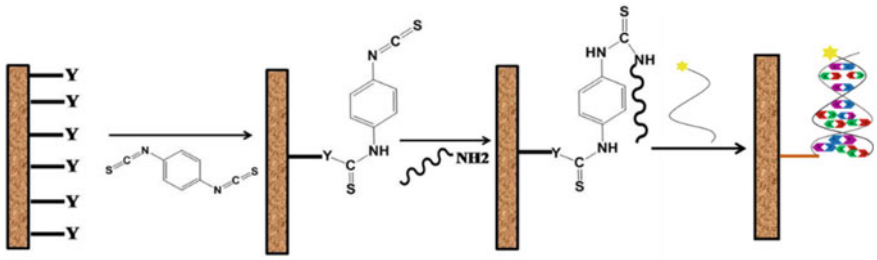

Fig. 6.8 DNA hybridization on chemically modified paper surface. Adapted from Araújo et al. (2012), Copyright © 2012 American Chemical Society

For capturing the DNA on paper devices, initially the paper surface is functionalized with chemical treatment with p-Phenylene diisothiocyanate (PDITC); which essentially generates chemically active functional group on cellulose fabrics. Chemically activated paper surface (Tian et al. 2012; Araújo et al. 2012) facilitates the binding of free amine groups of aminated single stranded DNA (ssDNA). This binding of ssDNA probes allows the binding of corresponding complementary DNA strands; which is tagged with the fluorophore thus generates a detectable fluorescent signal (Fig. 6.8).

6.5 Blood Plasma Separation

From the preceding discussion, it is evident that most of the diagnostic methodologies completely rely on the colorimetric detection approach; which also dictates the choice of the sample fluid, thereby ensuring the fact that the sample's original color should not interfere with the colorimetric analysis. Thus, in most of these cases, usage of whole blood directly on these devices often interferes with the colorimetric detection; thus needs separation of blood plasma from whole blood prior to the detection. In this regard, we try to summarize different techniques which have been adopted for blood plasma separation on paper-based devices (Songjaroen et al. 2012; Noiphung et al. 2013; Nilghaz and Shen 2015).

In the context of blood plasma separation, centrifugation technique stands out as most standard and efficient tool. However, this centrifugation method does not fit well with paper-based micro-devices which usually handle fluid in relatively lower volume of fluid (typically 50 μl). In this regard, there are many attempts to integrate the blood plasma separation on to the chip itself. Exiting commercial platforms utilize red blood cell (RBC) specific membranes which specifically allow RBCs to adhere (Songjaroen et al. 2012), and thus only plasma elutes through the downstream part of the devices for detection. Furthermore, some devices use the agglutination reagents for the purpose of agglutinating the cells in desired locations of the device and thereby allow the plasma to flow through for subsequent

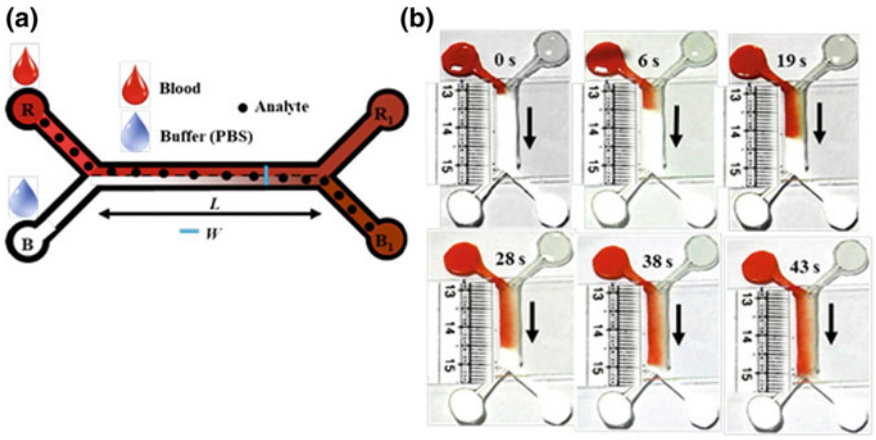

Fig. 6.9 **a**. Schematic representation of paper-based 'H-filter'; **b**. real time image sequences show the diffusion of blood stream into the buffer stream. Reproduced from Kar et al. (2015), with permission from The Royal Society of Chemistry

experiments. Application of these RBC-specific membranes and agglutination reagents performs efficiently, but makes the device relatively expensive. In this context, paper-based 'H-filter' (shown in Fig. 6.9a) has been exploited which shows ~75% of separation efficiency. In the 'H-filter', two arms were loaded with blood (reservoir 'R') and buffer (phosphate buffer saline in 'B' reservoir) respectively. While the co-lateral streams of blood and buffer flows through the device (triggered by capillarity-driven wicking), low molecular weight small molecules (e.g. glucose) diffuses into the buffer stream from blood stream. Small bio-analytes have higher diffusivity ~ (O ~ 2) than its cellular counterpart; thus this diffusion enriches the buffer stream with small molecules while red blood cells are retained within the blood stream. This diffusive transport of the small molecules leads to an effective separation in the downstream part of the channel and B1 reservoir as well. Paper-based H filter demonstrates fast (~4 min) and efficient on-chip separation protocol which could be adopted for designing the POC devices to detect the bio-analytes from whole blood.

6.6 Limitations of the Assays

The utilitarian scope of the paper devices has been explored in different aspects of detections like detection of bio-analytes from different physiological fluids like blood, serum, urine etc. Furthermore, its scope has been expanded for other detections too like detecting pathogenic or metallic contaminations which has significant impact for developing quality control measures for drinking water and food samples. These qualitative colorimetric assays have been deployed

successfully for many different targets over the years. Colorimetric chemicals-based assays are semi-quantitative in nature; applications of which is limited when the presence of target analyte is significantly low; thus cannot be fitted for monitoring early stage infections. To overcome the challenges of the colorimetric assays, other techniques like fluorescence-based assay (He and Liu 2013), electrochemical assays (Dungchai et al. 2009; Noiphung et al. 2013), electrochemiluminescence (Li et al. 2013) have also been exploited.

6.7 Three-Dimensional (3D) Paper Devices

From the previous discussion, it can be noted that majority of these paper devices are fabricated on Whatman filter paper (grade 1); which has typical thickness ~110 μm; hence the devices can be considered as two dimensional (2D) platforms. Though for qualitative/semi-quantitative/quantitative detection, 2D devices made substantial impacts; the necessity of 3D devices is yet to be realized in full extent. With the increasing applications of the paper-based devices, demand of 3D devices is more realized. 3D devices are mostly fabricated by following origami-based paper folding method (Liu and Crooks 2011; Scida et al. 2013); which essentially leads to the stacking of multiple paper layers which essentially creates a 3D paper device. In this layer-based 3D paper device, fluid could easily get transported into the other subsequent layers which are kept underneath to the dispensing layer. In the context of multistep protocol, these 3D devices offer a better platform than 2D devices while minimizing the chances of cross contaminations. The layer-by-layer structure offers flexibility to flip and clamp it as needed. Furthermore, effective chemical concentration is more in layered-based 3D devices than 2D platforms, as the loss due to the fluid transport is minimized.

A representative example of 3D paper device is shown in Fig. 6.10, which has been deployed to develop the understandings for antimicrobial susceptible assays following a colorimetric assay. In this particular device, the top layer 1 contains the

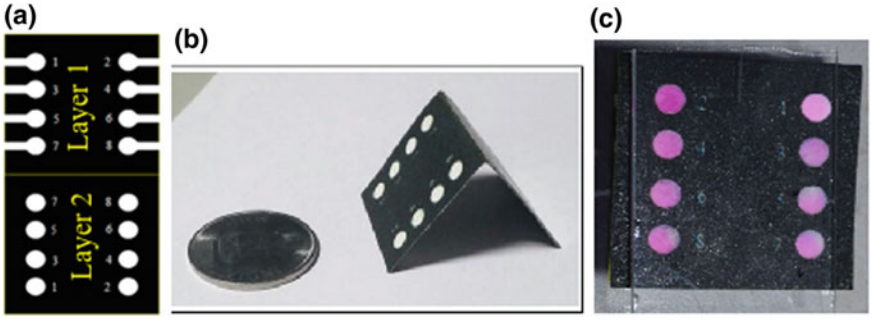

Fig. 6.10 Two layers based 3D paper device: **a**. device design; **b**. printed device; **c**. colorimetric reactions on the device

bacterial suspension while layer 2 contains antibodies; thereafter both layers were stacked together for incubation. After the incubation stage, the bacterial susceptibility was monitored by following resazurin chemical assay (Sarker et al. 2007).

6.8 Conclusions and Outlook

In this chapter, we systematically discussed the different aspects of the paper-based devices; which essentially covers it fabrication, flow mechanisms and detection of different bio-analytes following colorimetric detection approach. From the entire discussion, it is important to note that all the necessary clinical practices i.e. enzymatic assays, chemical assays, ELISA can be successfully implemented over a simple paper-based matrix; thus, would certainly serve the purpose of clinical diagnostics. While comparing the cost of diagnosis with conventional diagnostics; it comes down to $\sim$40 times lesser without compromising the sensitivity of the overall diagnosis. Recent advancements of paper-based microfluidics have indicated that these devices could be adopted for many diagnostic protocols. These types of substrates always provide superior flexibility in terms of designing multiple assay systems within the affordability of the masses; thus, certainly will deliver the need of the resource limited settings. Considering the recent developments in this specific domain, still there are few challenges which need to be addressed in near future. Till date, there are attempts to make the sample pre-processing step and multi-step protocol easier; but those are not yet materialized in true sense.

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Chapter 7
Paper-Based Microfluidic Devices for the Detection of DNA

Geeta Bhatt and Shantanu Bhattacharya

Abstract Microfluidic paper-based analytical devices are an attractive tool for point of care diagnostics as they facilitate fast detection without the need for any sophisticated instrumentation and skilled professional. These devices are disposable, portable, and affordable; hence, they are utilized in almost all the diagnostic domains for carrying out the detection. There are various aspects associated with the paper-based devices, namely working principle, reaction mechanism, fabrication schemes (2D/3D), detection sensitivity, and readout mechanism. Over the period, continuous progress is envisioned in all these domains to enhance the sensitivity of the detection and several variants, namely miniaturization, the inclusion of nanoparticles, multi-functionalization, etc. are also explored to make the detection more efficient. This chapter provides a state of the art review of the various aspects of paper-based microfluidic devices, including their fabrication scheme, sensing methodology, and their several applications in DNA detection domain. Also, advantages, disadvantages, and future aspects of these devices are also discussed.

Keywords DNA based sensing · Dipstick · LFAs · μPADs · POC · Detection · Microfluidics

7.1 Introduction

DNA being the most basic entity in the biological world, there exist several schemes for carrying out its detection. There can be enormous ways to detect the DNA, but the basic principle of detection remains the same. DNA analyte is taken close to the

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sensory surface, some biochemical change is observed near the surface, and a corresponding transduction signal is generated through this biochemical change, which helps in identifying the analyte characteristics. Hence there are several components related to the detection aspect, namely substrate, detection methodology, detection chemistry, etc. Substrate and detection chemistry are mostly responsible for sensory surface design, while the type of transduction signal helps decide the detection methodology. There exist a variety of substrates on which DNA detection can be carried out, and they are silicon (Bhatt et al. 2019), polymers (Bhatt et al. 2017), glass (Zou et al. 2013), nanoparticles (Zhao et al. 2003), paper (Kumar et al. 2018) and carbon nanotubes (Li and Lee 2015), etc. There are majorly four types of detection methodologies which are utilized for DNA detection, namely colorimetric detection (Kumar et al. 2018), fluorescence detection (Bhatt et al. 2017), electrochemical detection (Bhatt et al. 2019) and mass-based detection (Kumar et al. 2019). Sometimes colorimetric detection and fluorescence detection are considered under the same head, optical detection. The different type of detection methodologies requires a different set up in terms of requirement of detection electrodes, detection chemistry, or some combination of both. A variety of detection entities/structures are carved on to the different substrates to facilitate the various detection schemes like fluorescence-based, electrochemical-based, and mass-based. Interdigitated electrodes (Bhatt et al. 2019), crenelated electrodes (Nakano et al. 2011), cantilevers (Nakano et al. 2011), microarray dot electrodes (Yafouz et al. 2013), etc. are fabricated at various times to facilitate the detection.

Mostly detection schemes are based on either hybridization based chemistry or amplification-based chemistry. Hybridization-based chemistry requires adhesion of the sensing entity on the sensory surface, and in case of DNA detection, it can be carried out through various means, like physical adsorption, covalent immobilization and streptavidin-biotin immobilization (Kant et al. 2017). The amplification-based detection is mostly dependent on the amplification of the analyte to the detectable limit and its quantization in terms of a detectable signal. In this line, the polymerase chain reaction (PCR) is the most deployable technique for detection. PCR was discovered years back in 1893 by Mullis (Mullis et al. 1987), and since then, it has evolved in many directions to prove its utility in different aspects. Hence there are various combinations of substrates, electrode geometries, and detection schemes that can be clubbed together to achieve sensitive detection. Various methodologies possess several advantages and some disadvantages as well. Some schemes are very sensitive while some facilitate easier and affordable detection aspect. Out of various existing stated schemes, paper-based detection devices are gaining attention these days, due to their easy and fast detection, biodegradable and handy nature and easier and affordable availability.

Paper-based microfluidic devices, as the name suggests, utilize paper as the substrate, on which some sensory elements are coated. These sensory elements are responsible for showing some color change corresponding to the analyte. Hence mostly colorimetric detection technique is utilized for observing the detection event which has happened. This is the most basic detection device, which is used in day to day life, as it does not require any minimum set up and today, many handheld

paper-based detection devices are available in the market. This chapter will deal in detail various modules related to paper-based devices, their evolution, their detection principle, fabrication schemes, and their applications.

7.2 Evolution of Paper-Based Devices

The paper-based devices are the most deployable detection systems due to their easy and affordable availability and easy readout mechanisms. There exist several variants to paper based-microfluidic devices, namely, dipstick assays, lateral flow immunoassays (LFAs), and microfluidic paper-based analytical devices (μPADs). Dipsticks assays are the most preliminary and initial time paper-based devices that mainly referred to as urine test strips initially, which were developed by Jules Maunmene in 1850. Later it got transformed to pH test strips (Foster and Gruntfest 2009), which are widely accepted pH measurement platform even today. Further latex agglutination assay laid the basic foundation for LFAs (Wong and Tse 2008), and now it is widely used for the detection of various diseases. μPAD technology (Martinez et al. 2010) has been invented recently by inducing paper-based microfluidics to the paper-based devices, and it was also listed as one of the emerging technologies in 2009 by *Technology Review* magazine.

Paper-based devices mainly utilize hybridization/conjugation schemes on the sensory surface for carrying out the detection. Several conjugation schemes are developed for enhancing the detection sensitivity, which is discussed in detail in the forthcoming sections. As a substrate, a variety of paper materials, like filter paper and chromatography paper are used for devising paper-based devices, mostly in μPADs and dipstick assays. At a very basic regime, mainly for dipstick assays, the detection strips are simply fabricated by soaking the paper in a certain concentration of acid-alkali indicator mixture. For detection purpose, the strip is directly dipped into the analyte solution and color change that warrants the presence of the analyte is recorded. The other variety, namely LFAs, utilize a nitrocellulose membrane for making the devices. LFA test strip comprises of a sample pad, nitrocellulose membrane, conjugate pad, absorbent pad, and backing pad. The purpose of the absorbent pad is to provide a capillary-based driving force while the backing pad is to provide mechanical support to the device. Various capturing antibodies are deposited on the nitrocellulose membrane to form control and test lines capable of carrying out the detection. The techniques utilized for depositing these antibodies can be a hydrophobic force, a hydrogen bond, or electrostatic interaction. The sample pad is pretreated with buffer solution to help make analyte compatible with the other components of the device (Wong and Tse 2008). The analyte makes conjugate with particles already loaded in the conjugate pad and is further detected through sandwich or competitive mode of detection (Wong and Tse 2008). Figure 7.1 presents one such device based on LFA principle (Kumar et al. 2018), which is used for the detection of dengue NS1 antigen. The authors utilized gold decorated graphene oxide sheets on a tapered nitrocellulose membrane for

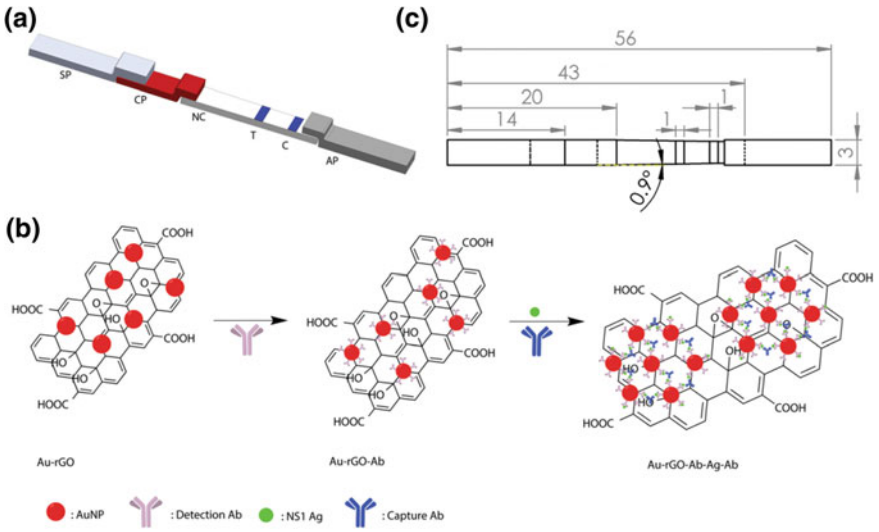


Fig. 7.1 Lateral flow immunoassay device for the detection of dengue NS1. **a** Schematic (SP: Sample pad, CP: Conjugate pad, NC: Nitrocellulose membrane, T: Test line, C: Control Line, AP: Absorbent pad); **b** Detection scheme via nanoparticle aggregation; **c** Dimensional representation (in mm). Reprinted with permission from Kumar et al. (2018) Copyright (2018) AIP

achieving a detection limit of 4.9 ng/mL. 0.9° taper was used in the device for efficiency enhancement.

The most recent development, μPADs utilize capillary force in paper to drive the movement of analyte in the device. These devices can be 2D or 3D in nature. 2D μPADs comprises of formation of microchannels on the paper surface by patterning. Physisorption, mediator based deposition (some nanoparticles) or chemical coupling are used for immobilizing chemical/biological molecules on the paper surface. 3D μPADs are fabricated by stacking 2D μPAD layers one above another in a way to keep connection amongst each other (Martinez et al. 2008).

7.3 Principle of Detection/Reaction Mechanism

There are several reaction mechanisms that can be utilized in paper-based sensing devices (Hu et al. 2014). These are mostly detected through colorimetric detection schemes through chemical/biochemical reactions, as observed in pH test strips, biological reactions, and electrochemical reactions. In the case of chemical reactions, there are acid-alkali combinations which indicate the color change as in case of pH test strips. In the case of biological reactions, there come antibody-antigen chemistry, nucleic acid hybridization, and functional nucleic acid-based reactions. Antibody-antigen chemistry deals with detection via biological interaction of antigen with its respective antibody as coated on the test strip. This scheme is used

in home pregnancy, tumor marker detection, and AIDS detection kits, etc. Nucleic acid hybridization is a more effective method for early detection of diseases, and here the detection is carried out through immobilizing a nucleic acid probe along with some kind of nanoparticles on the test strip and detecting the complementary target probe. Functional nucleic acids like aptamers, DNAzymes, and aptazymes can also be used as immobilization probe for detecting various ions, proteins, or some electrochemical molecules.

Along with colorimetric detection, there are also some modifications like fluorescence (Scida et al. 2013), chemiluminescence (Yu et al. 2011) and electrochemiluminescence detections which are currently being used in paper-based devices. In recent years, electrochemical detection is also being utilized through both redox and non-redox reactions (Han et al. 2013) for sensitivity enhancement of paper-based devices. Redox reactions are based on the electron transfer between the analyte molecules or particles while non-redox reactions deal with change in electrical properties of the analyte molecules like resistance, impedance, conductance or potential (Han et al. 2013).

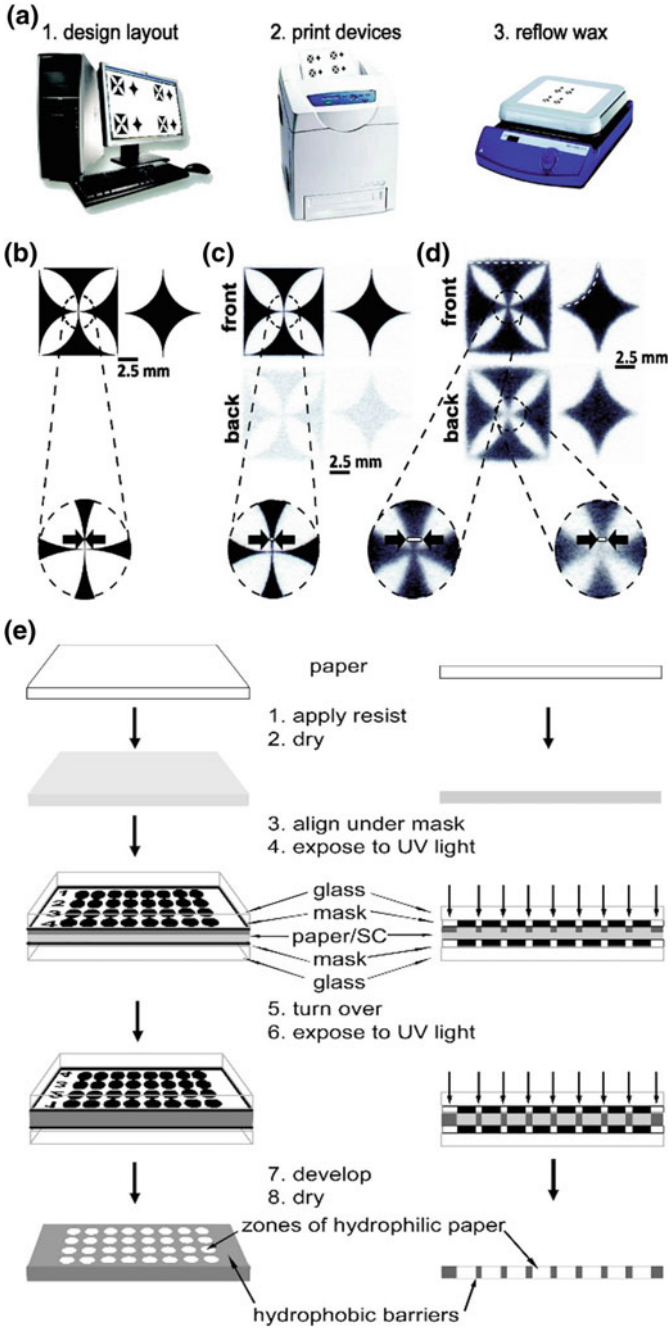
Mostly μPADs report a color change which is observed through naked eyes, but this kind of measurement is not optimum, especially in clinical diagnostics, as a quantitative analysis is required to explain the sensitivity. The color change perception can also be different for different persons depending on the light availability as well. Hence some mode of quantification is required. Hence for application of these devices to the clinical trials, some camera or scanner is also utilized to express the exact color intensity, which can be further related to the concentration/amount of the analyte that can be detected. Zhu et al. have also explained the usage of mobile phone for detection signal quantification in point of care diagnostics (Zhu et al. 2013). Electrochemistry based techniques are further emerging well in terms of signal quantification. Also, the barcode detection scheme is also used for quantifying the analyte concentration (Cho and Paek 2001).

7.4 Fabrication Schemes of Microfluidic Paper-Based Devices

As stated in the earlier section, μPADs can be 2D or 3D in nature (Xia et al. 2016). Various techniques like plotting, photolithography, cutting, wax printing, screen-printing, plasma/inkjet etching are proposed for fabricating microchannels/geometries on the μPAD devices. Some of them are explained in detail here:

7.4.1 Wax Printing

Wax printing (Carrilho et al. 2009a) is the easiest, most economical, and eco-friendly method to achieve hydrophobic barriers in μPAD devices. There are different ways of wax printing, namely, painting through a wax pen, inkjet printing,


(a)
1. design layout
2. print devices
3. reflow wax
(b)
2.5 mm
(c)
front
back
2.5 mm
(d)
front
back
2.5 mm
(e)
paper
1. apply resist
2. dry
3. align under mask
4. expose to UV light
glass
mask
paper/SC
mask
glass
5. turn over
6. expose to UV light
7. develop
8. dry
zones of hydrophilic paper
hydrophobic barriers

◂**Fig. 7.2** Wax printing (Reprinted with permission from Carrilho et al. (2009a) Copyright (2009) American Chemical Society); **a** Schematic representation 1–3; **b** Test image digital design; **c** Printed design on Whatman no. 1 chromatography paper using solid ink printer; **d** Test design after heating. **e** Photolithography steps for fabricating μPAD devices (Reprinted with permission from Carrilho et al. (2009b) Copyright (2009) American Chemical Society)

and subsequent tracing by painting through wax pen or direct wax printing. The wax can be easily absorbed in the paper surface through heating. Figure 7.2a shows the basic schematic of the process. It is observed that test design can be printed and heated to obtain hydrophobic barriers. It can be observed from the schematic (Fig. 7.2d) that there is some deformation in the actual design after heating, the dashed lines present actual geometry while the carved geometry is a bit broader than the actual design. Hence while fabricating a μPAD device, this should be kept in mind while designing the mask.

7.4.2 Photolithography

Photolithography mainly deals with patterning paper sheets in a hydrophilic zone while they are surrounded by hydrophobic polymeric areas. Photolithography takes very less time for making microchannels on the surface in an inexpensive way by using photoresist (Carrilho et al. 2009b). Figure 7.2e shows the schematic of the fabrication steps which shows that hydrophilic zones can be created by coating a resist layer onto the paper surface both sides and subsequently exposing it from both sides through UV exposure and further developing and drying it.

7.4.3 Inkjet Printing

Inkjet printing is a new emerging cost-effective technique, which is a more precise printing technique to deliver biomolecules/indicator reagents into the microfluidic patterns for completing the sensing device. It utilizes direct design printing through digital inkjet printers to attain the device designs.

7.4.4 Laser Treatment

Laser treatment uses polymerization of photopolymers to guide the flow of fluids in the device. This process is mostly a one-step process for cutting the paper according to the designed pattern (Nie et al. 2013) through laser, hence is a quick process for cutting a variety of paper-based devices.

7.4.5 Plasma Treatment

In this technique, the paper is initially hydrophobized via octadecyl trichlorosilane (OTS) salinization, and further, the silanized paper is plasma treated via a mask with a channel network. The plasma exposed portions turn hydrophilic (Yan et al. 2015) to make distinct hydrophobic/hydrophilic areas.

7.4.6 Wet Etching

The fabrication of hydrophobic filter paper-based μPADs through selective wet etching comprises of two steps, first is hydrophobic patterning of hydrophilic filter paper through a patterning agent, trimethoxyoctadecylsilane and the second step is the alignment of paper mask penetrated with glycerol contained NaOH solution onto the hydrophobic filter paper to allow selective etching of silanized filter paper using an etching agent (Cai et al. 2014).

The mentioned techniques are mostly capable of fabricating 2D μPAD devices. Further, 3D μPAD devices are mostly fabricated by stacking the 2D μPAD devices in some manner to retain the fluidic connections. The stacking can be carried out by some adhesive joints or some other joining techniques. As 3D devices have higher utility concerning the sample handling and performance; more than one sample can be detected simultaneously through 3D devices, but these devices cost higher as compared to the 2D devices. The research is being carried out to enhance the simplicity and cost-effectiveness of the 3D devices. Philips et al. proposed a simple methodology for assembling the 3D μPAD devices, by using spray adhesive to permanently join the 2D films and removing time-taking alignment and assembly task (Lewis et al. 2012). Some authors have also used a toner as the thermal adhesive to reduce the equipment requirement (Schilling et al. 2013). The researchers have also utilized craft-cutting and lamination scheme (Cassano and Fan 2013) and also simple folding of fabricated 2D μPAD devices to attain 3D structures (Liu et al. 2012) to make these devices more robust and cost-effective. Table 7.1 presents the application of various fabrication schemes for μPAD devices and the corresponding sensitivity of the reported devices.

Substrates like filter paper, nitrocellulose membrane, and glass fiber membrane, etc. are used for making μPAD devices. These are used for detection of various analytes such as bovine serum albumin, glucose, uric acid, H_2O_2, and also ions through a variety of detection modes. Different combinations have reported the different level of sensitivity to make them usable in various modalities.

Table 7.1 Comparison table of various fabrication schemes for detection on μPADs

Fabrication scheme	Substrate	Detected entity	Assay	Detection scheme	Limit of detection	Authors
Dip coating	Nitrocellulose membrane, glass fiber membrane	Human Serum Albumin	Competitive immune-chromatographic	Membrane strip bar coding (colorimetric quantization)	30–120 mg/dL	Cho and Paek (2001)
Photolithography	Paper	Bovine serum albumin		Fluorescence	125 fmol	Carrilho et al. (2009b)
CO_2 laser cutting	Paper	Glucose and Bovine serum albumin		Colorimetric	25 μm/12 mM	Nie et al. (2013)
Cutting method	Whatman chromatography paper	Uric acid		Chemiluminescence through Computerized ultraweak luminescence analyzer	1.9 mM	Yu et al. (2011)
Wet etching	Filter paper	Glucose		Colorimetric	0–20 mM	Cai et al. (2014)
Movable type printing	Filter paper	H_2O_2	Equipment-free quantitative assay	Colorimetric	10 mM	Zhang et al. (2014)
Digital inkjet printing	Whatman filter paper	NO_2^-		Colorimetric	5 mM	Li et al. (2010)

7.5 Applications of μPADs in DNA Sensing

The μPADs technology is growing very fast and is being used in various modalities for carrying out the detection. Although conventional devices do not have that level of sensitivity, the signal enhancement techniques are continuously being incorporated in the detection devices. The enhancement is mostly enzyme-based or metal ion based. μPAD technology is being used for detecting a variety of analytes, namely, bacteria, cells, ions, proteins, DNA, enzymes, antigens, etc. As DNA is the most basic entity for point-of-care diagnostics, this section further discusses the application of μPAD technology for DNA detection. Both kinds of assays, sandwich and competitive can be used for detection of proteins and nucleic acids.

Scida et al. utilized hybridization-based detection of DNA on an origami-based PAD. A competitive assay was designed where analyte, single-stranded DNA (ssDNA) and quencher labeled ssDNA competed to hybridize to a fluorescence-tagged ssDNA. The fluorescence growth was recorded to confirm the detection (Scida et al. 2013). Lu et al. proposed a 3D folded paper device for sensitive DNA detection (Lu et al. 2012). A folded electrochemical DNA detection device was fabricated, which comprised of gold nanoparticles/graphene modified screen-printed electrodes. Complementary DNA-thionine tagged dsDNA-nanoporous gold bioconjugate served as an efficient amplification label for detection of DNA. Along with the extracted DNA, the device was further tested for its performance for human serum assay and is found to perform well with that also. Liu et al. proposed an aptamer-based μPAD device for electrochemical detection of adenosine (Liu et al. 2012). The device is printed on a single piece of paper and folded to 3D configuration and laminated in a plastic casing. An aptamer immobilized on microbeads is utilized to bind to analyte target and further releasing the glucose oxidase-labeled DNA. The connected capacitor shows an increase in instantaneous current to express higher sensitivity.

Wei et al. proposed a DNA hydrogel mediated μPAD device for the detection of multiple target samples (Wei et al. 2015). The device was also capable of providing a signal-off readout in the case when the target is present. It is done by making hydrogel flow in the channel and further stopping the flow in the channel. When the target is present, no hydrogel formation is reported, and a smooth sample flow was recorded to express signal-on readout. Multiple targets like Pb^{2+}, cocaine, and adenosine is simultaneously detected by the fabricated device. Cunningham et al. fabricated a paper-based electrochemical device for detecting DNA and thrombin by target induced conformational switching (Cunningham et al. 2014). The SlipChip concept was used for fabricating e-sensor. For ssDNA detection, stem-loop functionalized on 5' end with thiol group was used as an electrode-bound receptor. 3' end was containing methylene blue to act as an electroactive reporter. While for thrombin detection, thrombin aptamer was immobilized on the sensory gold surface, which allows redox reporter (methylene blue) to approach the working electrode. Gong et al. proposed a lab-on-paper device for Hepatitis B virus DNA detection (Gong et al. 2015). The device was designed in a way to be used for three different operations, namely pre-concentration, separation, and detection.

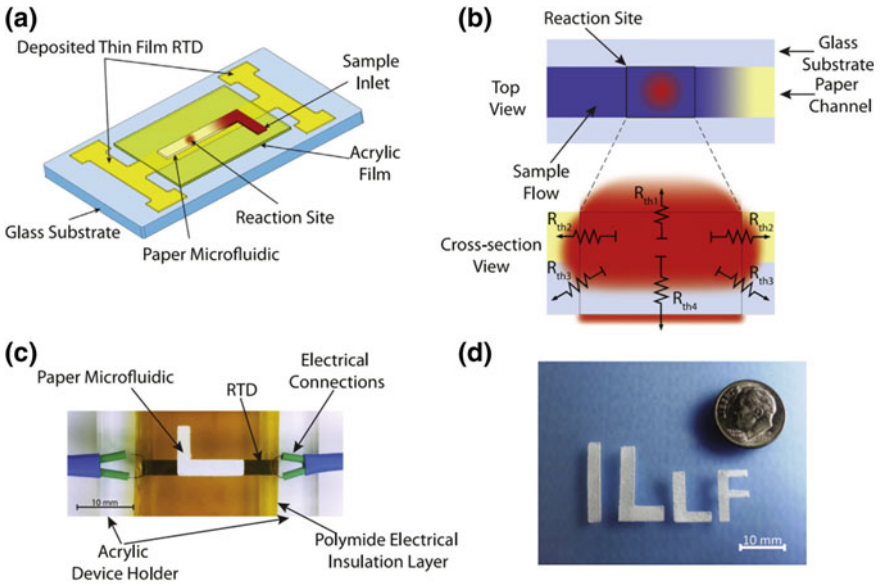


Fig. 7.3 **a** Schematic of the fabricated paper-based device; **b** Top and cross-sectional view of the reaction site (along with equivalent model); **c** Fabricated device; **d** Knife plotter cut paper strips as a reaction substrate and a microfluidic channel. Reprinted with permission from Davaji and Lee (2014) Copyright (2014) Elsevier

Davaji and Lee proposed first of its kind paper-based micro-calorimetric bio-chemical detection scheme for glucose and DNA (Davaji and Lee 2014), to accommodate the variability of temperature through various reaction for sensitive detection. The device presented detection through colorimetric scheme and Fig. 7.3 shows the schematic of the detection device along with the equivalent model of the reaction scheme. The device has shown temperature changes in biotin and strep-tavidin reaction.

Table 7.2 further lists various fabrication schemes, substrate selection, assay type, and limit of detection for the discussed μPAD devices for DNA detection.

7.6 Conclusions

As it is ubiquitously accepted that the paper is the most affordable entity, and this has appealed most researchers to make it usable for mankind through variable research schemes. Lab-on-paper is the most basic devices, which are readily available in the market for point-of-care diagnostics of many diseases. These devices are affordable and mostly provide reliable results for preliminary detection. Keeping the popularity of these paper-based devices in mind, this chapter discusses

Table 7.2 Comparison table of various DNA detection scheme concerning other parameters

Fabrication scheme	Substrate	Detected entity	Assay	Detection scheme	Limit of detection	Authors
Wax printing and controlled heating	Origami paper	ssDNA	Competitive assay	Fluorescence-based	<5 nM	Scida et al. (2013)
Plotter cut-RTD based	Paper	Glucose, DNA		Colorimetric and electrochemical detection	0.9–7.3 mg/mL	Davaji and Lee (2014)
Printing and lamination	Origami paper	Adenosine	Redox couple	Electrochemical detection	11.8 μm	Liu et al. (2012)
Wax printing combined with screen printing	Whatman chromatography paper	ssDNA		Electrochemical	2×10^{-16} mM	Lu et al. (2012)
Wax patterning	Whatman grade I chromatography filter paper	Pb^{2+}, cocaine, and adenosine	DNA hydrogel mediated assay	Colorimetric	Pb^{2+}: 200 nM; Cocaine: 50 μm; Adenosine: 100 μm	Wei et al. (2015)
Double-sided printing of a wax pattern-SlipChip concept	Chromatography paper	DNA, thrombin	Target induced conformational switching	Electrochemical	DNA: 30 nM; Thrombin: 16 nM	Cunningham et al. (2014)
Wax patterning	Nitrocellulose paper	Hepatitis B virus DNA		Fluorescence	150 copies/mL	Gong et al. (2015)

various aspects of μPAD devices. The chapter at first discusses the evolution of paper-based devices and the way they have moved through all these years. The various reaction schemes, fabrication methods, and detection methodologies are then discussed concerning the mostly μPAD devices. DNA being the most basic entity for detection is of preliminary importance for detection domain. Hence, the chapter further organized to discuss the application of μPADs for detection of DNA and related entities. The chapter further shows a comparison of various fabrication schemes to develop μPAD and their corresponding application for detecting various entities. As can be observed through table also, mostly colorimetric type of detection is used in μPAD devices but with time, various modifications like fluorescence, chemiluminescence, etc. are also being these days. Electrochemical μPAD devices are the most recent evolution in μPAD technology.

There are also some disadvantages associated with the paper-based devices, like dependence of external environmental conditions, humidity, availability of surrounding lights, and mostly dependent on the analyte concentration. These parameters may result in the false readout, as the perception of color varies for person to person depending on the surrounding light and also a lower concentration analyte can lead to negative results. Hence these devices are being refined more and more to make the detection easier and reliable.

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Chapter 8
Nucleic Acid Amplification on Paper Substrates

Priyanka Naik, Riddha Manna and Debjani Paul

Abstract Paper is a versatile platform for several biological assays. One of the most popular assays performed on paper substrates is the nucleic acid amplification test (NAAT). Compared to other diagnostic techniques, NAATs are specific, sensitive and fast. While the polymerase chain reaction (PCR) is still the most widely-used technique for amplifying nucleic acids, isothermal DNA amplification techniques have emerged as viable alternatives to PCR on paper. Many innovative detection techniques have also been employed to detect the amplified DNA on paper. There are three main aspects of nucleic acid analysis on paper, namely (a) sample preparation to extract the nucleic acids from cells, (b) amplification of DNA, and (c) detection of the amplified DNA. In this chapter, we will review various amplification techniques in detail. We will also briefly cover the mechanisms to detect the amplified nucleic acids on paper. Finally, we will discuss the potential of these technologies to be translated to the point of care.

8.1 Introduction

Microfluidic technology is widely used in a number of fields including environmental sensing, food safety and diagnostics. Much of the exploratory research in biological applications of microfluidics has been performed using the elastomer poly(dimethylsiloxane), otherwise known as PDMS, as the chip material. But these devices are not robust enough to put to use in a point-of-care (POC) set-up due to incompatibility with certain reagents, adsorption of small biomolecules due to inherent polymer hydrophobicity, and 'aging' (Sia and Whitesides 2003). These drawbacks of polymer-based conventional microfluidics have spurred the need for substrates which are more robust and compatible with biomolecules.

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Paper has been used in the field of healthcare in the form of dipstick and lateral flow assays since the 1950s (Free et al. 1956; Leuvering et al. 2006; Kumar et al. 2018). Apart from the obvious advantages of using paper as a substrate, such as easy availability, cost-effectiveness, and eco-friendliness, paper offers perks in terms of the fluid rheology. Paper, which is inherently a porous network of cellulose fibres, provides the 'micron-sized' channels for performing microfluidic assays. Also, small pore size reduces the diffusion distances and enhances the reaction significantly (Yetisen et al. 2013). It is easy to modify paper substrates by performing various kinds of printing and structural modifications to suit the experimental requirements. Its white color provides sufficient contrast to enable easy colorimetric detection (Yetisen et al. 2013). A renowned example of a paper-based assay which has been a huge commercial success is the lateral flow immunoassay (LFIA) for conducting at-home pregnancy tests (Stephen et al. 1998). The use of paper to perform more complex microfluidic applications was first described in 2007 by the Whitesides group for multiplexed detection of glucose and protein on a patterned chromatography paper (Martinez et al. 2010). Their results renewed the interest of the microfluidics community in paper-based analytical devices or μPADs.

Much of the interest in transposing solution-based assays to a paper substrate has also been for the development of point-of-care (POC) devices. POC devices are diagnostic devices which aim at providing a timely diagnosis in low-resource settings. Nucleic acid analysis is one of the most reliable, sensitive, and specific diagnostic methods. It involves the identification of specific sequences in the genome of the organism of interest (Niemz et al. 2011). This technology is applicable in a wide variety of fields ranging from environmental sample testing to disease diagnosis. As shown in Fig. 8.1, typical nucleic acid analysis consists of three major steps, namely, extraction and purification of nucleic acids from the cell, amplifying them, and finally detecting the amplified nucleic acid (Fig. 8.1).

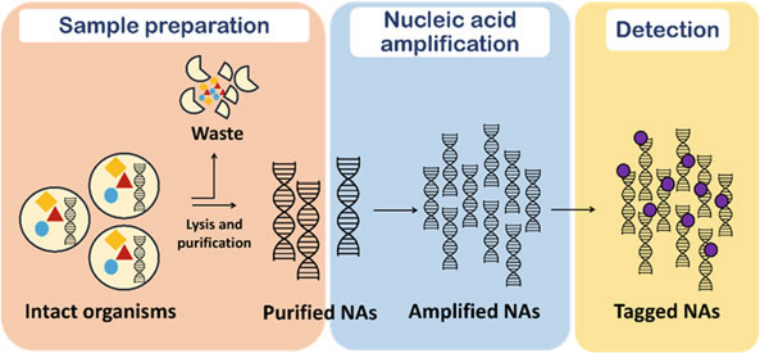

Fig. 8.1 Different steps involved in a typical nucleic acid (NA) analysis process. Sample preparation involves lysis of the intact organisms, followed by purification of the NAs from the cellular debris. The purified NAs are subjected to amplification. The amplified NAs are detected in the final step

8.2 Nucleic Acid Extraction

Nucleic acid extraction, also known as "sample preparation", is the first step in molecular biology assays. It involves lysing the cell to release the nucleic acids (DNA and RNA) and purifying them from the rest of the cellular gunk such as the proteins, lipids, etc. (Tan and Yiap 2009). Cell lysis can be achieved by physical or chemical methods. The method to disrupt the cell membrane is chosen based on the type of the clinical sample. Lytic enzymes such as achromopeptidase, chaotropic agents such as guanidium thiocyanate, and different types of detergents such as Triton X100 or sodium dodecyl sulfate are the reagents of choice for performing chemical lysis. Mechanical lysis relies on disrupting the cells by physical forces such as shearing and bead beating (Ali et al. 2017). The purpose of the sample preparation step is to make the nucleic acids as much accessible as possible to enzyme molecules (e.g. polymerase) in the amplification step. The physical barriers to accessibility such as the cell membrane, nuclear membrane, or the nucleic acid-associated proteins should be removed to enable maximum interaction with the nucleic acids. It eliminates the cellular components other than the nucleic acids, also termed as 'contaminants' from the mixture. Once a sample of satisfactory purity is obtained, the next step is to subject it to amplification.

Use of cellulosic substrates for storage and purification of samples has gained popularity due to the need for transposing the entire nucleic acid analysis platform on paper. The high degree of hydroxylation on cellulose generates a strong polar attraction to bind nucleic acids under specific conditions (Ali et al. 2017). The commercially available Fast Technology for Analysis (FTA) paper can purify and store DNA. FTA cards are impregnated with a proprietary mix of reagents containing Tris (pH 8), SDS, and EDTA. Once the sample is added to the paper, the detergent SDS lyses the cellular membranes. The role of EDTA is to chelate the metal ions and make the nucleases inactive (Smith and Burgoyne 2004). In their dried form, nucleic acids can be stored stably on the FTA card. The FTA technology comes with a proprietary purification reagent which is then used to elute the nucleic acids from the paper fibres. Even though this technology is highly advantageous and seemingly straightforward, post-processing the FTA cards to extract good yields of nucleic acids is complex, especially for samples that are highly diluted (Milne et al. 2006).

Zou and others reported a 30-s DNA extraction protocol using a cellulose dipstick constructed using Whatman No. 1 paper (Zou et al. 2017). The protocol involves tissue homogenization in lysis buffer. Ball bearings are added to this solution for maceration. The dipstick is then dipped into the tube to capture nucleic acids, followed by washing steps to get rid of contaminants. Finally, the dipstick is dipped directly into the amplification mix thrice to elute the trapped nucleic acids (Fig. 8.2a). They demonstrated that their protocol could extract 0.1 ng/μL DNA from plant, animal, and microbe samples. The authors report the efficiency of their assay to be comparable to that in silica-based Whatman Fusion 5 and FTA cards.

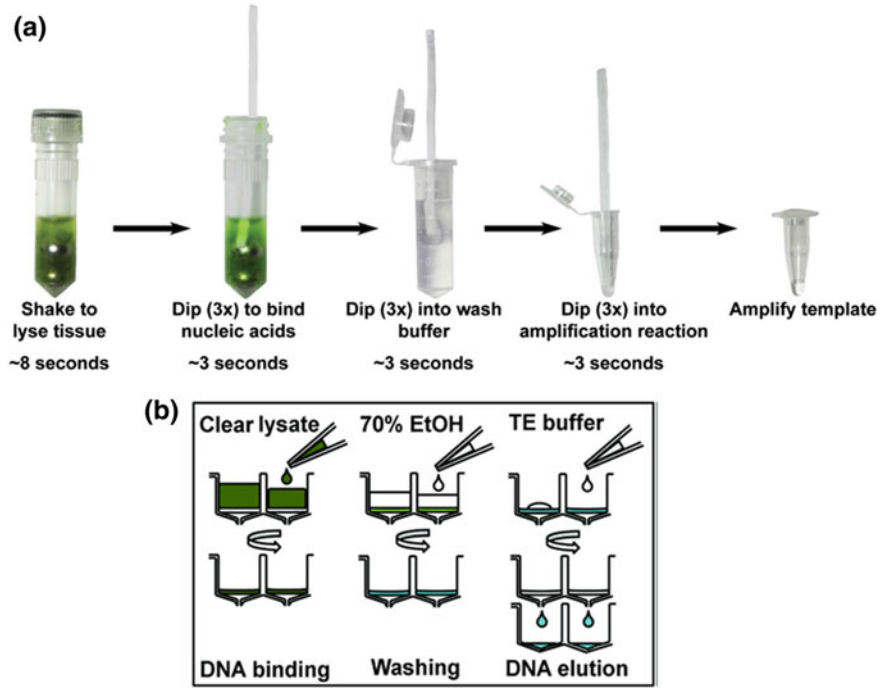

Fig. 8.2 Sample preparation on paper substrates. **a** Cellulose dipstick based 30-s DNA extraction protocol. Reproduced from Zou et al. (2017) with permission from PLoS. **b** Protocol of DNA extraction using filter paper spin plate. Reproduced from Shi and Panthee (2017) with permission from Springer

Another study by Shi et al. reported a low-cost filter paper-based 96-well spin plate method for high throughput plant DNA extraction using home-made lysis buffers (Shi and Panthee 2017). The authors compared the DNA yield from filter paper and glass fibre substrates of different grades. Whatman filter paper Grade 3 yielded 7.5 μg DNA, which was higher compared to the rest of the substrates tested by the authors. The spin plate was constructed by drilling holes at the bottom of a 96-well V-bottom storage plate and placing filter paper discs at the center of the wells. The plant samples were lysed using the CTAB extraction protocol, followed by incubation at 65 °C for 30 min. The lysate was then pipetted onto filter paper discs kept in the spin plate. Underneath the spin plate, a fresh 96-well plate was kept to serve as the collection plate. The spin plate was subjected to centrifugation followed by washing steps. Finally DNA was eluted using Tris-EDTA buffer (Fig. 8.2b). They report extraction of 5–10 μg DNA from the lysate of 25 mg tomato leaves, which is commensurate with the commercial kit-based extraction.

8.3 Nucleic Acid Amplification

Nucleic acid amplification (NAA) has been synonymous with polymerase chain reaction (PCR) for several decades. Kary Mullis developed PCR in 1983 for exponential amplification of the template nucleic acids (Mullis 1990). It involves three major steps at three different temperatures: (i) denaturation at 95 °C, (ii) annealing at 50–65 °C (Mullis and Faloona 1987), and (iii) extension at 70–74 °C. One thing to note is that even if the template provided at the beginning of the reaction is RNA, the amplification does not progress without synthesizing a complementary DNA (cDNA) copy of the template with the help of the enzyme reverse-transcriptase (a process known as 'reverse transcription'). This scheme of PCR is repeated over 25–30 cycles, resulting in the accumulation of billions of copies of the template DNA within 3–4 h.

The extracted DNA sample often has to be eluted prior to PCR to get rid of contaminants. Successful integration of DNA extraction from lysed whole blood and in situ PCR on nanoporous aluminum oxide membrane has been reported previously (Kim and Gale 2008). These studies have established that PCR can be performed without an elution step. This observation further suggests that DNA extraction can be directly integrated with PCR on paper substrates. Excluding the elution step could potentially improve the detection sensitivity due to the usage of the entire extracted volume, some of which might have lost during elution. The Liu group reported a plastic microfluidic device using a filter disc for automated DNA capture and demonstrated in situ PCR amplification from various raw samples without an elution step (Gan et al. 2014). The device was tested using samples such as dried blood spots, buccal swabs and saliva. The authors could extract 21.8 ng of DNA using their device, which is higher than the 13 ng of DNA obtained using the QIAamp® kit. They experimentally proved that the quality and concentration of the extracted DNA is sufficient for more sophisticated molecular analyses.

Despite the apparent success in integrating PCR on a paper-based device, PCR exhibits some major disadvantages when it comes to the development of POC devices. The main problem is the requirement of thermal cycling and the bulky thermal cycler used for this step. Even if this problem is obviated by development of a portable instrument, such instruments are too sophisticated, and consequently too expensive, to be put to use in a resource-poor setting. Apart from this, repeated exposure to high temperatures for denaturation could compromise the mechanical integrity of the paper substrate. PCR is sensitive to certain classes of contaminants and inhibitors found in crude samples (Schrader et al. 2012). Bearing these constraints in mind, various research groups have developed isothermal techniques which amplify nucleic acids at a single temperature. These techniques replace the temperature-assisted denaturation step with enzymatic denaturation (e.g. helicase) for generation of ssDNA. These techniques can be performed with simple and inexpensive heating instruments, such as hand/toe warmers, hot plates and heat baths, which are compatible with the requirements of low-cost POC devices. Some of the most common techniques for DNA amplification on paper are loop-mediated isothermal amplification (LAMP) (Notomi

et al. 2000), thermophilic helicase-dependent amplification (tHDA) (Vincent et al. 2004), recombinase polymerase amplification (RPA) (Piepenburg et al. 2006), rolling circle amplification (RCA) (Fire and Xu 1995) and isothermal strand displacement amplification (iSDA) (Walker et al. 1992).

8.3.1 *Loop-Mediated Isothermal Amplification (LAMP)*

Loop-mediated isothermal amplification (LAMP), as shown in Fig. 8.3, is a type of strand displacement amplification. The amplification cycle proceeds by strand displacement by the *Bacillus stearothermophilus* (Bst) DNA polymerase. It employs four primers targeted to six distinct regions on the double-stranded template DNA. An amplification temperature of 60–65 °C ensures optimal activity of the Bst polymerase. Double-stranded DNAs are held at a dynamic equilibrium at this temperature to enable primer annealing without denaturation (Notomi et al. 2000). The LAMP reaction can be split into two steps: (i) a non-cycling amplification step and (ii) a cycling amplification step. During the non-cyclic amplification step, primer annealing and extension forms a dumbbell-shaped "seed" structure,

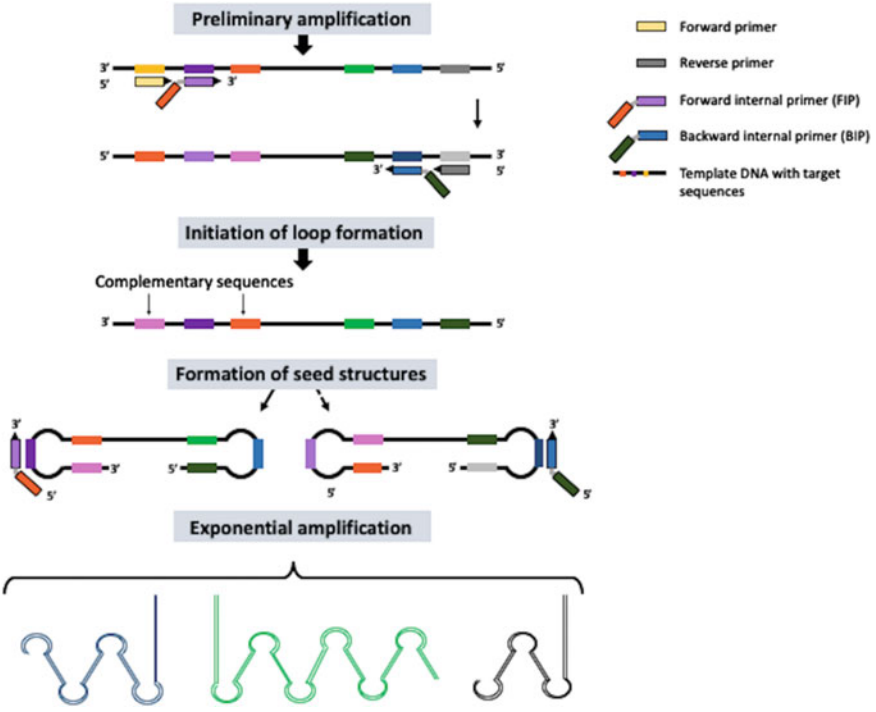

Fig. 8.3 Loop-mediated isothermal amplification

which is needed for the next step. The cyclic step is marked by annealing of the four primers to specific regions of the template and strand extension by the polymerase. This step leads to the formation of amplicons of varying sizes. This is unlike PCR, which produces inverted repeats of the target sequence on the same strand. LAMP produces 10^9 copies of DNA in an hour (Notomi et al. 2000).

Whatman FTA membranes would be the most obvious choice for integration of the different nucleic acid analysis modules. Liu and others were the first to report an integrated paper-based LAMP platform using a polymer cassette for detection of HIV-1 in oral fluids (Liu et al. 2011). Whatman FTA membrane served as the substrate for nucleic acid entrapment, purification, and amplification using reverse transcriptase LAMP (RT-LAMP). The authors demonstrated a sensitivity of 105 particles/mL (in the absence of the FTA card) and 102 particles/mL (with the FTA card). They attributed the decreased sensitivity in the absence of FTA card to the presence of inhibiting substances in the saliva. The authors also demonstrated real-time fluorescence detection using a portable fluorescence reader. Detection sensitivity of 102 particles/mL was achieved in 40 min. In another study, the Whitesides group developed a "paper machine" for integrated sample preparation, LAMP, and detection on FTA cards using a hand-held UV-source camera phone (Connelly et al. 2015). The performance of their device was demonstrated using Escherichia coli spiked blood plasma samples with a detection sensitivity of 500 cells/mL.

They also show the ability to concentrate target DNA in the reaction disc by applying sample volumes up to 60 μL. At higher volumes washing adversely affects the final yield (Fig. 8.4a). This underlines yet another point entailing washing of the paper substrates. Washing is important to ensure the removal of the unbound target and contaminants if any. However, excessive washing might lead to decrease the yield of the desired product, thereby indirectly affecting the sensitivity of the assay. Figures 8.4 and 8.5 show the results of different LAMP reactions on paper.

Most of the studies on LAMP have used glass fibres as their substrate of choice. These pads consist of extremely fine fibres of glass, unlike paper substrates which are made up of cellulose fibres. Glass fibres have better resistance to chemicals and are inert, which makes them ideal substrates for reactions. The fibres also possess strength, flexibility, and stiffness (Wallenberger et al. 2001). Their large pores enable efficient reagent storage and provide room for diffusion. These are biocompatible and do not contain any water-soluble material that might affect the amplification efficiency (Rohrman et al. 2012; Choi et al. 2015).

The first study reporting the use of glass fibre as the amplification substrate was published by the Xu group. They demonstrated LAMP in a two-layer device for detecting synthetic dengue viral DNA. Once LAMP had occurred on the glass fibre, the DNA was thermally denatured followed by contacting the glass fibre pad with the lateral flow strip (LFA). The strip had single-stranded gold nanoparticle detector probes deposited on it to capture the complementary single-stranded DNA product from the glass fibre pad. They compared the detection sensitivity of their assay with that of the tube-based reaction. Both assays could detect 3×10^3 copies of the viral

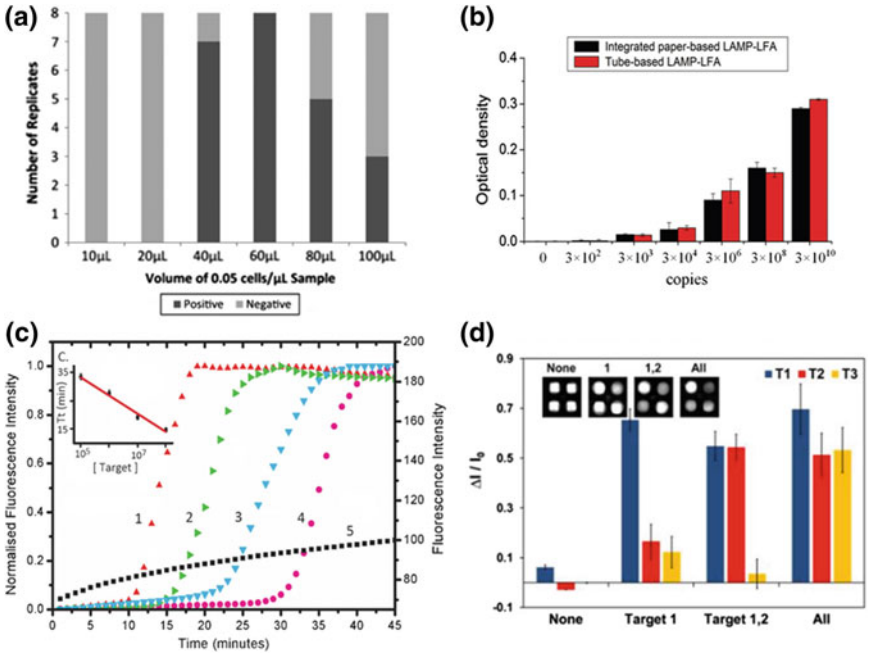

Fig. 8.4 Paper-based devices for LAMP. **a** Effect of sample volume in "paper machine". Reproduced from Connelly et al. (2015) with permission from ACS publications. **b** Comparison of detection sensitivities in tube-based and paper-based LAMP. Reproduced from Choi et al. (2016a, b) with permission from ACS publications. **c** Real time amplification curve of *Plasmodium pan* with serially diluted target DNA Adapted from Xu et al. (2016). **d** Simultaneous detection of 3 targets. Adapted from Seok et al. (2017)

DNA (Fig. 8.4b). They developed a handheld battery-powered system with two pockets, one for incubation at the amplification temperature and the other for holding a tube that contained the LFA reagents. This design ensured self-contained execution of amplification and detection steps in order to prevent false positive results (Choi et al. 2016a, b). The same group developed a paper-based biosensor by adding a FTA card for nucleic acid extraction at the bottom of the previous two-layer design (Choi et al. 2016a, b). During amplification, the strips were sealed using a tape to prevent evaporation loss. This zone was placed in the handheld battery powered system for amplification, followed by detection as previously mentioned. The authors dried the FTA cards for different durations for best extraction efficiency and ascertained 15 min to be the optimal duration. *E. coli* spiked in drinking water, milk, blood, and spinach showed detection limits of 10, 10, 10^3, and 10^2 CFU/mL, respectively. Finally, the device was shown to detect *Streptococcus pneumonia* in clinical blood samples, emphasizing on its use as a POC diagnostic device.

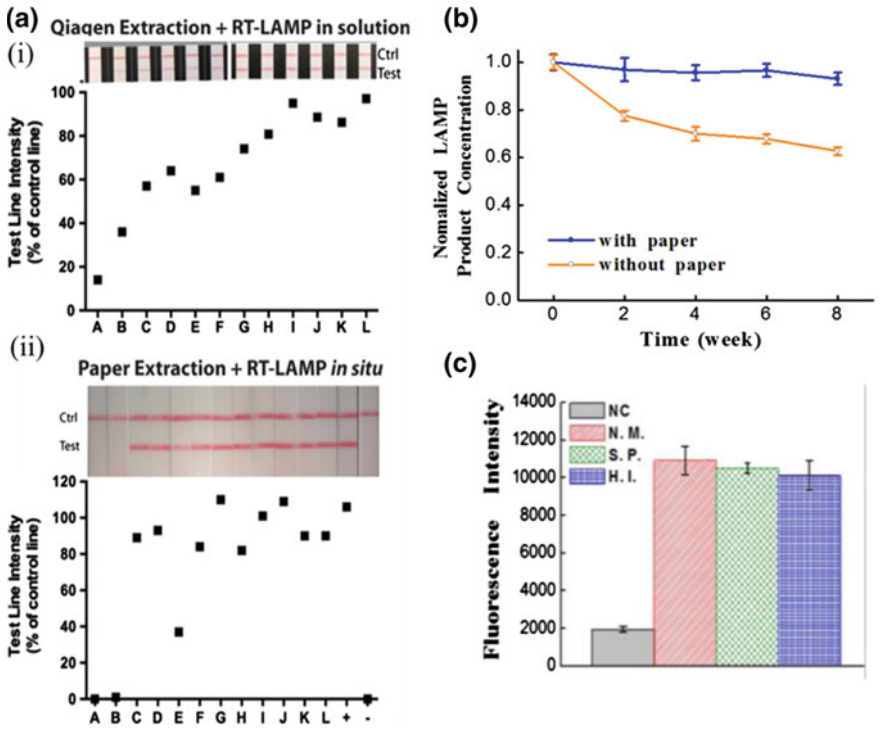

Fig. 8.5 Paper-based devices for LAMP. **a** Comparison of assay efficiency of (i) standard procedure versus (ii) the paper-based protocol. Reproduced from Rodriguez et al. (2015) with permission from ACS publications. **b** Performance comparison between devices with primer immobilised on paper and without paper over a period of 2 months. Reproduced from Dou et al. (2014) with permission from ACS publications. **c** Multiplexed detection of *N. meningitidis* (N.M.), *S. pneumoniae* (S.P.) and *Haemophilus influenzae* B (H.I.) Reproduced from Dou et al. (2017) with permission from Elsevier

Subsequently, the Cooper group reported a paper origami device for multiplexed diagnosis of malaria from a finger-prick of blood using LAMP. The device performed each step of the analysis sequentially by folding the paper in pre-determined directions (Xu et al. 2016). Human blood samples were mixed with the lysis buffer containing guanidium thiocyanate. Subsequent folds ensured rehydration of the printed LAMP reagents on the glass fibre prior to amplification. Real-time detection was done using calcein with the fluorescence being visible to the naked eye. The authors showed that higher the concentration, sooner the amplification begins as demonstrated by the exponential increase in the fluorescence. They acclaim that this method of observation is analogues to real-time PCR (Fig. 8.4c) The assay was multiplexed to detect *Plasmodium falciparum, Plasmodium vivax,* and *Plasmodium* pan-species from a whole blood sample. The detection sensitivity was shown to be 10^5 IU/mL for *P. falciparum* DNA.

In another study, Seok and others developed a paper-based device for simultaneous detection of *Streptococcus agalactiae*, *Streptococcus pneumoniae*, and *Staphylococcus aureus* using LAMP (Fig. 8.4d) (Seok et al. 2017). The amplification reagents must be stabilized to ensure their efficacy after drying. The authors compared polyvinyl alcohol (PVA), polyvinylpyrrolidone (PVP), BSA, gelatin, and agarose, and determined PVA to be the most suitable reagent for this purpose. The device was placed in a petri plate containing moistened toilet paper for maintaining constant humidity to prevent evaporation. Each reaction square stored a different set of LAMP primers, which enabled multiplexing. Detection was performed using hydroxynaphthol blue (HNB). The fluorescence intensity of HNB was directly proportional to the magnesium pyrophosphate concentration, which enabled real-time detection. The authors demonstrated the detection sensitivity to be 4.1×10^2 copies of genomic DNA.

Yang et al. reported multiplexed paper-origami tests for the detection of three bovine infectious reproductive pathogens (e.g. bovine herpesvirus-1, *Brucella* and *Leptospira*) spiked in semen samples using LAMP (Yang et al. 2018). The detection sensitivity was estimated to be 50 for *Leptospira* organisms, 50 CFU of *Brucella*, and 1 TCID50 of bovine herpesvirus-1. The device operation involved a series of pipetting steps for the addition of reagents required at each step. The Klapperich group developed several polyethersulphone (PES)-based devices for DNA amplification. They reported an integrated assay for DNA extraction and in situ reverse transcriptase LAMP (RT-LAMP) for detection of influenza A (H1N1). Leftover nasopharyngeal swab specimens were mixed with the lysis buffer containing guanidium thiocyanate. These were then spotted onto a PES filter pad. The reaction mixture was pipetted on the same PES membrane and the membrane was placed in a 0.2 mL tube for RT-LAMP. The PES membrane was placed on a lateral flow strip to allow colorimetric readout (Rodriguez et al. 2015). They compared the efficiency of their paper-based protocol with the standard protocol comprising of a Qiagen kit-based extraction and tube-based amplification to demonstrate comparable efficiencies (Fig. 8.5a). In an extension of this work, a modular and foldable microchip for integrated nucleic acid analysis of human papilloma virus (HPV) DNA from patient cervical samples was developed (Rodriguez et al. 2016a, b). The device consisted of a PES filter paper disc for extraction and amplification, and an lateral flow strip for detection. Loss of the aqueous phase due to evaporation causes a change in the sample concentration. In either case, this loss adversely affects the efficiency of the reaction. This device was covered with laminating sheets to avoid evaporation losses during amplification on the hot plate. Direct contact between the sample port and the lateral flow strip underneath it was established by peeling off the laminating sheet.

More recently, an integrated device for detection of HIV-1 spiked in whole blood samples using reverse transcription loop-mediated isothermal amplification (RT-LAMP) was reported (Phillips et al. 2019). The "microfluidic rapid and autonomous analytical device" (microRAAD) analyzed whole blood samples spiked with HIV-1. Among several features, one unique feature of this device is that it leverages the wicking abilities and pore sizes of the paper substrates to isolate

HIV viral particles from human blood samples. RT-LAMP amplicons were subjected to restriction digestion with SphI and PstI to verify product authenticity. The authors report a detection sensitivity of 4×10^6 virus copies/mL, which is below the reported HIV-1 concentration at the peak of infection in clinical samples.

A somewhat less popular substrate for LAMP is chromatography paper. Dou and others reported a PDMS-paper hybrid device for detection of *Neisseria meningitidis* spiked in artificial cerebrospinal fluid (ACSF) (Dou et al. 2014). They used chromatography paper for immobilizing the oligonucleotide primers. They claimed that this step improved detection sensitivity by improving the stability of the primers. They tested the stability of the primers with and without the paper present in the LAMP zone over a period of eight weeks. A steep decrease in the product concentration was seen in just two weeks in the sample without the paper. It exacerbated to a 40% decrease, while the sample with paper showed stable amplification until the 8th week (Fig. 8.5b). An extension of this work was later published in which three meningitis-causing microbes, e.g. *N. meningitidis*, *S. pneumoniae*, and *Haemophilus influenzae* type b (along with a negative control), were detected in a multiplexed manner (Fig. 8.5c) (Dou et al. 2017). In this design, the authors integrated the sample preparation step on the chip, unlike their previous work.

In another study, Naik and others reported direct DNA amplification from unprocessed *Mycobacterium smegmatis* and *E. coli* cells on chromatography paper discs using LAMP. They demonstrated a detection sensitivity of 10^2 CFU/mL after 30 min of amplification. This was the first report to demonstrate lysis and amplification of mycobacteria on a paper substrate (Naik et al. 2019). The authors also highlighted the extreme sensitivity of LAMP to cross-contamination from previous rounds of amplification and suggested some measures to prevent non-specific amplification.

8.3.2 Helicase-Dependent Amplification (HDA)

Helicase-dependent amplification (HDA) (Fig. 8.6) employs the helicase enzyme to denature the double-stranded DNA instead of the conventional thermal denaturation. It mimics the replication fork created during DNA replication in vivo. When first introduced, the technique utilized *E. coli* UvrD helicase along with its accessory protein methyl-directed mismatch repair protein (MutL), and cofactor adenosine triphosphate (ATP) to unwind the double-stranded DNA (Vincent et al. 2004). It also requires single strand binding proteins (SSB) to stabilize the single strands of DNA, while an exo-Klenow fragment of DNA polymerase I synthesizes the complementary strand. The reaction was performed at 37 °C, and it suffered from problems, such as, limited speed (20 bp/s) and processivity (less than 100 bp/binding) of the *E. coli* UvrD helicase (Vincent et al. 2004). A newer mesophilic HDA was later developed to replace the *E. coli* UvrD helicase with *Thermoanaerobacter tengcongensis* (*Tte*) UvrD helicase which is more thermostable. This allowed amplification to occur at a higher temperature (60–65 °C)

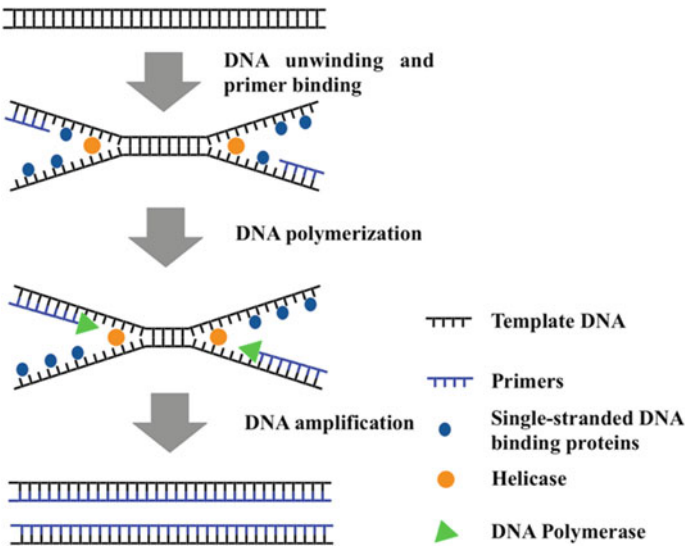

Fig. 8.6 Helicase-dependent amplification

with better sensitivity and specificity (An et al. 2005). Also, the *Tte*-UvrD helicase was reported to be the best partner for *Bst* DNA polymerase in HDA. This improved technique is the more widely used form of HDA, also known as thermophilic HDA (tHDA).

Based on the existing reports on tHDA, there seems to be no apparent preference towards any particular substrate (Fig. 8.7). Different substrates such as, chromatography paper, glass fibre and PES membranes have all been used for tHDA. Linnes and others compared the flow rate of the sample fluid on polymer micro-solid phase extraction (μSPE) columns and paper substrates to show that the fluid flow was slower on paper (Fig. 8.7a). Slower flow rates allow longer durations for efficient DNA extraction from the cells. Sample preparation is a time-consuming operation. Increased efficiency results from ensuring adequate incubation times of the sample with the required reagents. This group reported detection of *Chlamydia trachomatis* cells spiked in synthetic urine by in situ tHDA on chromatography paper. The amplicons were later eluted and spotted on lateral flow detection strips for visual detection. Evaporation was prevented by overlaying the tips with mineral oil. The detection sensitivity was 10^4 cells/mL (Linnes et al. 2014).

Subsequently, the Paul group reported HDA of an 84 bp DNA fragment of *Mycobacterium tuberculosis* spiked in artificial sputum on chromatography paper discs in 10 min. The authors compared amplification efficiency of a standard thermocycler with inexpensive heating sources like hot plate and hand warmers to show that HDA could be performed equally well on all the studied heat sources (Fig. 8.7b). They further compared the signals from different DNA intercalating fluorescent dyes such as SYBR green, PicoGreen, DAPI, and ethidium bromide and

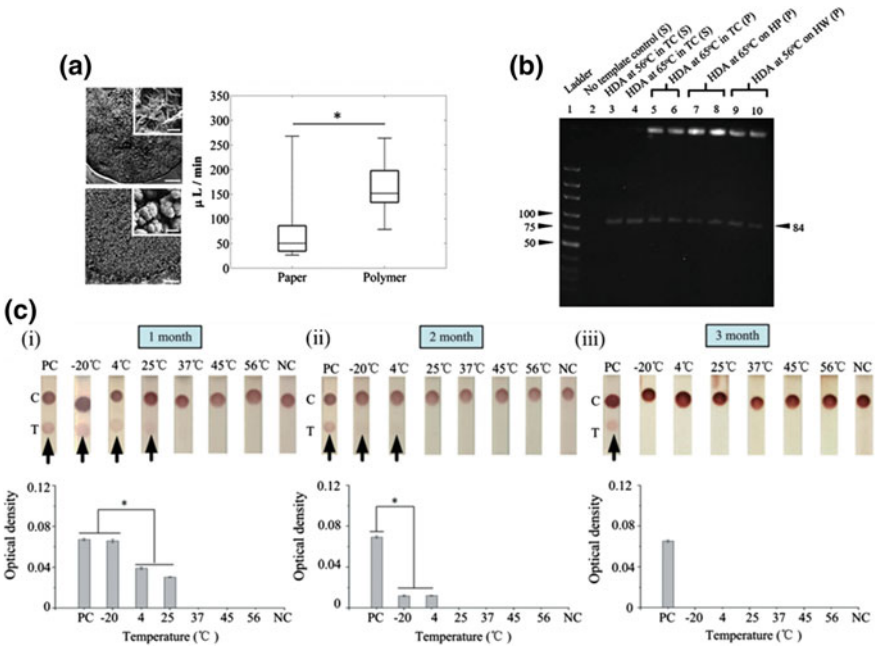

Fig. 8.7 Paper-based devices for HDA. **a** Comparison of fluid flow on paper and solid phase extraction polymer. Reproduced from Linnes et al. (2014) with permission from the Royal Society of Chemistry. **b** Gel electrophoresis data depicting the use of thermocycler (TC), hot plate (HP) and hand warmers (HW) in solution-based (S) and paper-based (P) HDA reactions. Reproduced from Shetty et al. (2016) with permission from the Royal Society of Chemistry. **c** Dry storage of assay reagents on paper at various temperatures over a period of 3 months. Reproduced from Tang et al. (2017) with permission from the Royal Society of Chemistry

propidium iodide on paper to determine that PicoGreen generates the least background fluorescence. They also tested the shelf-life of the dried HDA enzyme mix at room temperature over a period of 34 days and reported that the reaction efficiency remains unaffected over this period (Shetty et al. 2016).

A study by the Xu group reported a fully disposable and integrated device for extraction, tHDA and LFA detection of *Salmonella typhimurium* spiked in wastewater, egg, milk and juice samples (Tang et al. 2017). A glass fibre pad served as the amplification substrate. The authors demonstrated a detection sensitivity of 10^2 CFU/mL in wastewater and egg, and 10^3 CFU/mL in milk and juice. The authors also tested the stability of the dried reaction components in the vacuum-packed devices stored at −20, 4 and 25 °C as well as higher temperatures (37, 45, and 56 °C) for 1, 2 and 3 months. They reported that the devices stored at −20 and 4 °C showed stable results for up to 2 months, after which no amplification was seen. Temperatures higher than 25 °C led to extremely faint results after one month of storage, which they attributed to the reduced protein activity with increase in temperature (Fig. 8.7c). This is an important observation as dry storage

conditions of the reaction components affect the assay efficiency. NAATs are expensive partly because of the cold-chain costs involved during transportation and storage of the reagents.

A paper-based platform for diagnosis of *Neisseria gonorrhoeae* (Horst et al. 2018) was reported as an extension of the group's previous work (Rodriguez et al. 2016a, b). PES membrane served as the amplification substrate. The assay began with addition of urethral/vaginal swab samples mixed with the guanidium thiocynate lysis buffer followed flow of the extracted DNA to the tHDA reaction chamber, followed by lateral flow strip detection. This modified design also included an internal control to differentiate between negative results and invalid tests, unlike their previous device (Rodriguez et al. 2016a, b). Inclusion of internal controls is of high importance in diagnostic assays. They authenticated their test results by ruling out the possibility of false positive results (Sharma et al. 2014). They demonstrated a detection sensitivity of 500 genomic copies per reaction with purified DNA. A sensitivity of 95% (detecting 19 out of 20 positive samples) and specificity of 100% were also reported in this study.

8.3.3 Recombinase Polymerase Amplification (RPA)

Recombinase polymerase amplification (RPA), shown in Fig. 8.8, was first reported by Piepenburg et al. (2006). It is similar to HDA in that it employs enzyme-based DNA denaturation. The scheme of RPA is analogous to homologous recombination as it uses recombinase enzyme bound to the oligonucleotide primers to scan the template sequence for homology. Once the homology is located, strand exchange reaction commences and the new complementary strand is synthesized. RPA can take place at incubation temperatures between 37 and 42 °C.

The first study demonstrating the use of an unmodified paper substrate for RPA was reported by the Richards-Kortum group (Rohrman et al. 2012). They developed a two-fold paper-plastic device for detection of HIV DNA using RPA. They screened multiple paperlike substrates such as, glass fibre, cellulose, GF/DVA, MF1, VF2, and Fusion 5 to demonstrate that RPA was most efficient on glass fibre (Fig. 8.9a). After the reaction, the device is peeled open, the sample wick strip is torn off the device and immersed in a buffer solution. The diluted products are pipetted on a lateral flow strip. The authors achieved a detection sensitivity of 1 copy/μL of HIV DNA within 15 min of RPA.

Since then, all reports, except one study using glass fibre, have used chromatography paper as the substrate for RPA. An extension of the work done by Rohrman and others was reported to detect synthetic *Plasmodium* DNA (Cordray et al. 2015). The device design remained similar in that different paper substrates were employed for different purposes. The amplification reaction was carried out on Whatman number 1 paper. However, the new device was more sophisticated since the detection was done using an integrated lateral flow assay. Also, the sample pad was mobile and could be moved across the device for sample delivery by passive

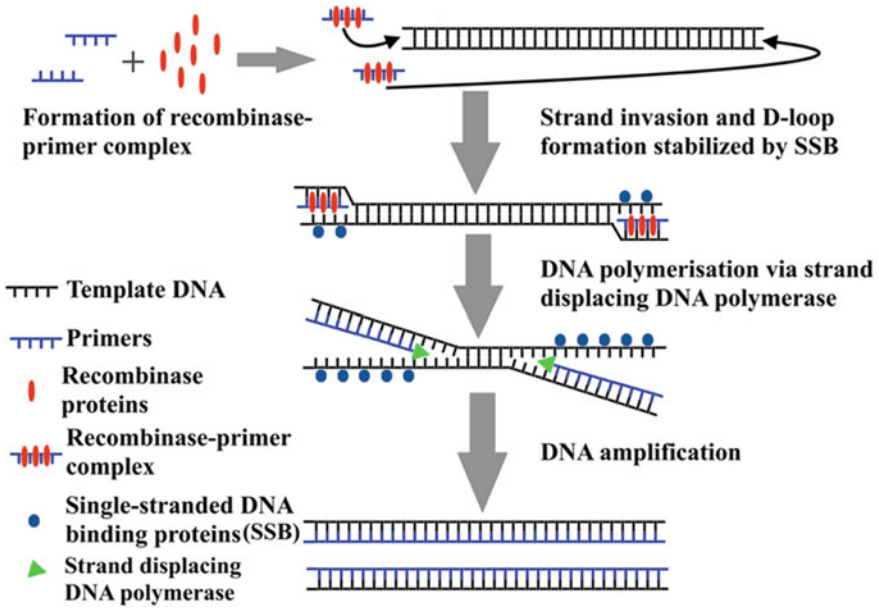

Fig. 8.8 Recombinase polymerase amplification

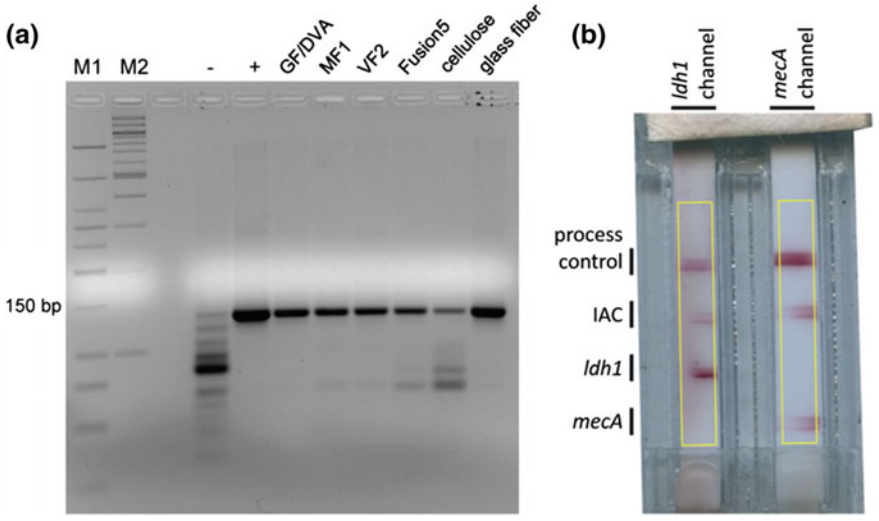

Fig. 8.9 Paper-based devices for low temperature amplification by RPA, RCA and SDA. **a** Gel electrophoresis data depicting the efficiency of different paper substrates for RPA. Reproduced from Rohrman et al. (2012) with permission from the Royal Society of Chemistry. **b** Lateral flow strips with various control lines along with a test line for detection of S. aureus using SDA. Reproduced from Lafleur et al. (2016) with permission from the Royal Society of Chemistry

diffusion. The reagents were loaded onto the respective zones and device was sealed shut for incubation on a hot plate. The entire process occurred over a period of 55 min and it could detect 5 copies/μL of the target DNA.

Detection of ebola virus on paper using reverse transcriptase RPA (RT-RPA) was reported (Magro et al. 2017). RNA samples extracted and purified from patient blood were used as the template for amplification. The amplification substrate was chromatography paper with wax printed hydrophobic barriers. The RT-RPA reaction components were freeze-dried on the reaction zones and rehydrated at the time of experiment. The fluorescence intensity of the oligonucleotide probes complementary to the target was measured. This test demonstrates a sensitivity of 90% compared to the gold-standard RT-PCR on 43 patient samples. Even though the template for amplification was purified RNA, this was the first study to report the use of patient samples. Long-term stability of the dried components was tested by conduction reactions at various intervals over a period of 30 days. The sensitivity of RT-RPA remained unaffected, however, the fluorescence intensity decreased significantly by the end of the 30-day time point. This emphasizes on the importance of dry storage conditions. The authors also demonstrated prospective multiplexing by developing a five-layer paper origami device. They showed that it requires minimal manual intervention due to the valve-like functions induced by the folding of the paper to establish sequential contact between surfaces.

Tsaloglou and others developed a "universal mobile electrochemical detector" ($uMED^{NA}$) to detect small concentrations of DNA present. Detection sensitivity of genomic DNA from *M. smegmatis* and *M. tuberculosis* was estimated to be $\sim$0.040 ng/μL (Tsaloglou et al. 2018). A number of groups are currently trying to develop integrated nucleic acid tests. However, the detection strategy employed in each of these assays is either developed specifically for that assay or involves the use of another accessory equipment.

8.3.4 Rolling Circle Amplification (RCA)

Rolling circle amplification (RCA) emulates in vivo replication of circular DNA templates such as plasmids. It employs the strand displacement activity of Phi29 bacteriophage DNA polymerase (φ29DNAP) for the synthesis of tandem repeats of the template DNA. The resultant amplicons can be digested by restriction enzymes into short oligonucleotide parent sequences. RCA requires incubation temperature of around 23 °C (Fig. 8.10) (Fire and Xu 1995; Liu et al. 1996). While RCA is a valuable technique, amplification takes place over time in a linear manner compared to other techniques (e.g. HDA) which exhibit exponential amplification.

One of the early reports of RCA employed DNA-conjugated microgels embedded in paper as the substrate (Ali et al. 2009). The use of fluorescent probes for detection of DNA led to a sensitivity of 100 pM. They observed that the fluorescence intensity is proportional to the amplified DNA. In another study, Liu and others demonstrated RCA in a microzone plate fabricated on a paper substrate

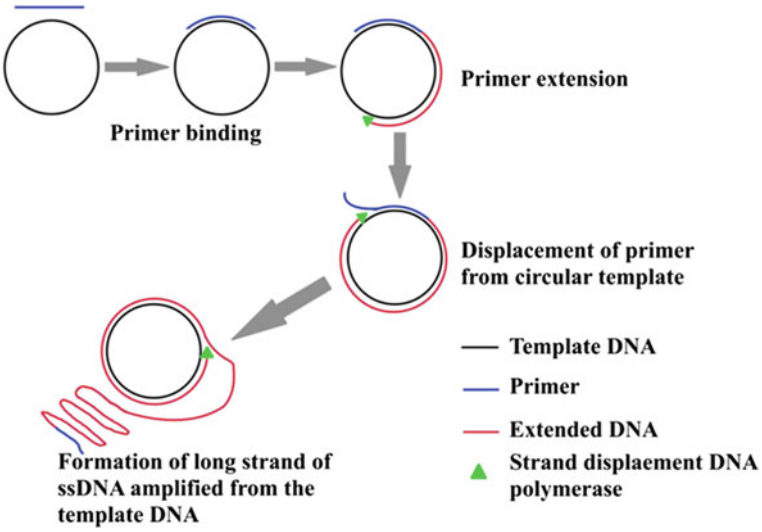

Fig. 8.10 Schematic of rolling circle amplification process

by wax printing. They improved the storage of RCA reagents by first encapsulating them in a polymeric sugar film (pullulan) and then integrating it with the paper device (Liu et al. 2016). They demonstrated that the entrapped reagents retained 91.5 and 66.8% of their initial activity after storage at 4 °C and room temperature respectively for 15 days. Furthermore, they reported that the RCA efficiency on paper is enhanced relative to the solution due to increased local concentration of reagents. The authors also claimed that the immobilization of reagents on paper substrate improves the thermal stability of the primer-template duplex, which enhances the RCA efficiency on paper. There are not many reports on paper-based RCA. This is because it requires circular nucleic acid templates which have to be first generated by a ligation event.

8.3.5 Strand Displacement Amplification (SDA)

In strand displacement amplification (SDA) (Fig. 8.11), the template DNA is nicked by a restriction endonuclease. The exo-Klenow polymerase performs the downstream amplification. Unlike the other techniques, the double-stranded DNA needs to be thermally denatured in SDA. The rest of the reaction takes place at 37 °C. Similar to LAMP, SDA also needs a set of four primers. It amplifies DNA in an exponential manner (Walker et al. 1992).

The first fully integrated device was introduced by Lafleur and others from the Yager group. The two-dimensional paper network (2DPN) device was a portable prototype named MAD NAAT ("multiplexable autonomous disposable nucleic acid

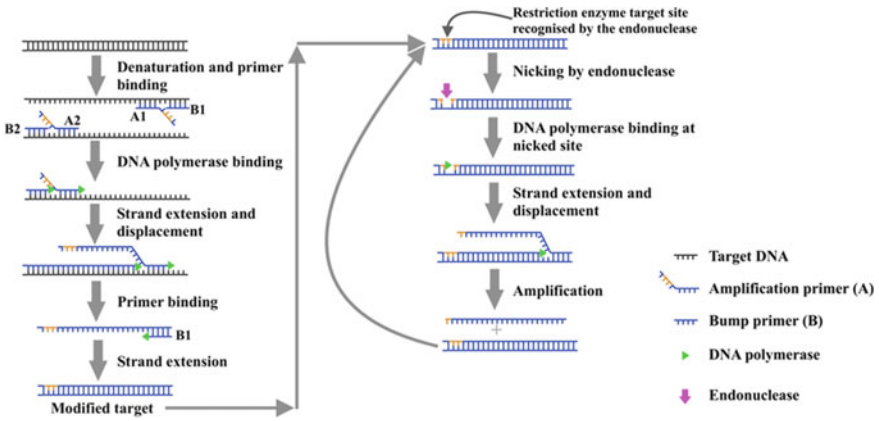

Fig. 8.11 Strand displacement amplification schematic

amplification test") (Lafleur et al. 2016). This was a noteworthy invention because it was the first NAA prototype which did not require any user inputs or equipment after the first actuation step. They employed strand displacement amplification (SDA) to detect methicillin-resistant *Staphylococcus aureus* (MRSA) in patient nasal swab samples. After introducing the sample from the nasal swab into the inlet, the sample housing was closed to start the reaction. First, the sample was lysed using achromopeptidase (ACP) and the DNA was extracted chemically. Then the processed sample was introduced into the 2DPN, where it was divided into two channels for detection of ldh1 (universal *S. aureus* gene) and mecA (methicillin-resistant gene found only in MRSA). Each channel contained four capture lines (Fig. 8.9b). Target lines for ldh1 and mecA were used to confirm the presence of the target in each channel. The internal amplification control (IAC) line was present to confirm amplification and flow of reagents into the strip. The process control line had bound gold nanoparticles to confirm successful execution of the process. The IAC line and the process line were independent of the target lines. The reaction took place at 49 °C for 30 min, following which, the sample flowed into the nitrocellulose strips for detection. The SDA reagents were pre-mixed with LFA reagents and dried onto Standard 17 glass fibre pads before the start of the process. Adding the sample rehydrated the reagents. The authors tested the stability of the lyophilised reagents on paper and reported efficient functioning of the assay for a minimum of six weeks at ~23 °C, and for 15 days at 40 °C, at a relative humidity of 2–3%. They claim that the reagent stability can be improved at higher temperatures by using stabilisers. The detection sensitivity was estimated to be $\sim 5 \times 10^3$ genomic copies.

8.4 Detection of the Amplified DNA

The final step in nucleic acid analysis is the detection and quantification of the amplified DNA. Though a large number of papers have independently reported detection of DNA on paper, only a few of them have integrated both nucleic acid amplification and detection steps in the same paper device. We limit our discussion to those reports that have performed both DNA amplification and detection on paper substrates. We focus on colorimetric and fluorescence detection as these are the two most popular detection techniques.

8.4.1 Colorimetric Detection

Lateral flow assays are the most common format integrating amplification and colorimetric detection on paper. Frequently, the detection method relies upon aggregation of tagged nanoparticles (NPs) (mostly AuNPs) on a detection line in the LFA device. Immunochromatography, based on a sandwich assay, is another common technique for the same. The doubly-tagged amplicons bind to antibody-tagged-NPs via the first tag. The detection line captures this DNA-NP conjugate using antibodies against the other tag (Rohrman and Richards-Kortum 2012; Cordray and Richards-Kortum 2015; Rodriguez et al. 2016a, b). Using NPs tagged with ssDNA probes complimentary to the amplicons gives a better specificity compared to immunochromatography. This is because the complementarity of the DNA strand provides a higher specificity than antibody-antigen binding (Choi et al. 2016a, b).

Unmodified AuNPs tend to aggregate in high salt solutions, changing the solution colour from red to blue. When single stranded DNA molecules attach to the AuNPs, the NPs cannot aggregate. A solution containing unmodified AuNPs and detection ssDNA (i.e. a single DNA strand complimentary to a particular target DNA) can therefore be used to detect the target DNA. In the absence of the target DNA, the ssDNA continues to protect the AuNPs from aggregation and hence the red color of the solution is retained. When target DNA is present, the detection ssDNA preferentially hybridizes with the target DNA, and, therefore, cannot prevent the aggregation of the AuNPs. This leads to a change in color of the solution from red to blue, which can be detected by UV-vis extinction spectroscopy. This detection scheme can be transposed to a paper substrate and used in conjunction with a mobile camera. The average pixel intensity of the spotted regions correlates almost linearly with the target DNA concentration. (Tsai et al. 2013). Teengam et al. uses the same approach, but with citrate anion-stabilized silver nanoparticles (AgNPs) and (1S,2S)-2-aminocyclopentanecarboxylic acid (acpcPNA) probes. The lysine at the C-terminus provides the acpcPNA probe with a single positive charge. This leads to aggregation of the AgNPs. In the presence of a target DNA complimentary to the acpcPNA, an anionic DNA-acpcPNA duplex is formed, leading to

the dispersion of the AgNPs due to electrostatic repulsion. This leads to a detectable color change (Teengam et al. 2017). DNA targets bound to tagged micron-sized beads can be made to aggregate under the influence of a force field (e.g. surface forces, electric field, etc.). This phenomenon has been utilized for visual detection of DNA (Song et al. 2014). Crystal Violet, a commonly available dye, is used for detection of DNA in Roy et al. (2017). The amplified DNA forms a stable complex with the Crystal Violet stain on paper due to electrostatic interaction between the negatively charged phosphate group of the DNA and the positively charged quinoid of Crystal Violet. This leads to recoloration of Crystal Violet that is then bleached in the presence of Sodium Sulfate (Roy et al. 2017). Neither of these reports two have performed nucleic acid amplification on paper, but we have discussed them as they represent promising techniques for integration with amplification devices.

From the point of view of diagnostics, colorimetric detection provides the most robust and easy-to-read format, and, therefore, is the most commonly utilized strategy. The main advantage of colorimetric detection is that the change in color can be easily detected by the naked eye without any instrument. This feature makes it suitable for integration into various device design formats like lateral flow devices, μPADs, etc. Due to ongoing research in the fields of photography and imaging, many sensitive colorimetric sensors (e.g. CMOS, CCD, flatbed scanners, etc.) are also relatively inexpensive and easily available. Colorimetry is particularly compatible with smartphone camera-based detection and quantification. These advances have made colorimetric detection devices much more popular compared to those based on traditional technologies such as spectroscopy.

8.4.2 Fluorescence Detection

Amplified DNA can be detected using fluorescence by intercalating it with different fluorescent dyes such as ethidium bromide [$\lambda_{\text{absorption max}}$: 210 nm, 285 nm (UV); $\lambda_{\text{emission max}}$: 605 nm (orange), calcein ($\lambda_{\text{absorption max}}$: 495 nm (blue); $\lambda_{\text{emission max}}$: 515 nm (green), SYBR Green I ($\lambda_{\text{absorption max}}$: 497 nm (blue); $\lambda_{\text{emission max}}$: 520 nm (green) and picoGreen ($\lambda_{\text{absorption max}}$: 485 nm (blue); $\lambda_{\text{emission max}}$: 528 nm (green)]. These dyes fluoresce only after binding to dsDNA on paper devices (Connelly et al. 2015; Xu et al. 2016). Fluorescently-tagged nucleic acid probes are also used to detect amplified DNA. RPA employs DNA probes with tetrahydrofuran (THF) spacers and exonuclease in the reaction mixture to cleave the double stranded DNA-probe hybrid at the THF region. The exonuclease has no effect on the ssDNA or the probe molecules. A fluorophore molecule and a quencher molecule are attached to the probe on either side of the spacer. When the probe hybridizes with the template DNA strand to generate a double stranded DNA, the exonuclease cleaves the DNA at the THF spacer region. This action separates the quencher and the fluorophore molecules, which then fluoresce upon photo-excitation at specific wavelengths (Magro et al. 2017). Ali et al. (2009) demonstrate an interesting way of detecting DNA in paper devices. Here RCA is

used as a method of signal amplification for detecting the target DNA. First, microgel (MG) coupled with an oligonucleotide (DNA1) is spotted on paper. It is then used to detect the DNA target (DNA3) in the following manner. DNA3 acts as a template for ligation of the primer (DNA2) to DNA1. After washing, RCA is carried out using DNA2 as the primer and a circular template DNA in the solution. This creates long ssDNA molecules at the microgel spot. The RCA product is visualized through hybridization with a fluorescently tagged complementary DNA probe. The authors report a detection limit of 100 pM.

Fluorescence detection has a much higher sensitivity compared to simple colorimetric detection. A major drawback of fluorescence detection on paper is the background fluorescence due to the presence of whitening additives introduced during the paper manufacturing process. The background fluorescence leads to a low signal to noise ratio. Shetty et al. (2016) compared the background fluorescence of several common fluorescent dyes and found picoGreen to be the most suitable dye for use with chromatography paper. Scattering of light from heterogenous paper substrates also leads to a loss in sensitivity and reproducibility, thereby making it unsuitable for detecting sub-nanomolar DNA concentrations. For these reasons, fluorescence detection works best when it is combined with DNA amplification on paper. The need of a low-bandwidth light source to excite the fluorophores at a specific wavelength, and the specific nature of the emission spectra of the excited fluorophore, does not allow fluorescence detection to be an equipment-free technique. In one example (Xu et al. 2016), the group used a small and portable UV lamp for exciting the fluorophores, and the fluorescence signal was read by the naked eye.

Apart from colorimetric and fluorescence detection, other detection techniques used in paperfluidics include electrochemical, luminescence, chemiluminescence, and electrochemiluminescence. Some groups have also performed gel electrophoresis after extracting the amplified DNA from the paper substrate, or directly loaded the paper substrates into the loading wells of electrophoresis gels. This is done to verify that the amplification of the correct target has occurred. Gel electrophoresis is also used to initially optimize the reaction conditions on paper.

8.5 Factors Affecting the Efficiency of DNA Amplification on Paper

A number of factors can affect the success of DNA amplification on paperfluidic substrates, including the efficiency of sample preparation and the sensitivity of the detection method. These include diffusion of the reaction components across the substrate (Rohrman et al. 2012), adsorption of enzymes to the surface (Pelton 2009), enhanced amplification of non-specific products due to inhomogeneous diffusion resulting from an absence of the template molecules in certain areas, and the leaching of inhibitory chemicals from the paper surface. One group increased

the amplification efficiency by reducing the flow rates, thereby allowing for better diffusion of reagents (Linnes et al. 2014). Surface passivation of paper substrates using BSA was shown to improve HDA efficiency (Shetty et al. 2016). Here we limit our discussion to the roles played by (a) the choice of the specific amplification technique, (b) the choice of the substrate, and (c) the loss in amplification efficiency due to the storage, transport and rehydration of the reagents.

8.5.1 *Choice of the Amplification Technique*

We searched the existing literature for reports on paperfluidic DNA amplification to understand which substrate and which amplification technique are the most widely used by the amplification community. We plotted the data as histograms without any normalization. As shown in Fig. 8.12a and Table 8.1, LAMP appears to be the most popular method for DNA amplification in paperfluidics due to its high specificity and high amplification efficiency. It has used for the detection of a variety of pathogens. The abundance of cellular proteins in complex crude samples can potentially compromise the assay specificity (Tian et al. 2014). LAMP is less sensitive to the presence of inhibitory substances compared to PCR (Mori and Notomi 2009). Most of the publications reporting the use of intact cells or crude samples as the starting material have identified LAMP as their amplification method of choice.

Despite its popularity, LAMP also suffers from a major disadvantage due to its high sensitivity. Amplification in the negative control of LAMP due to

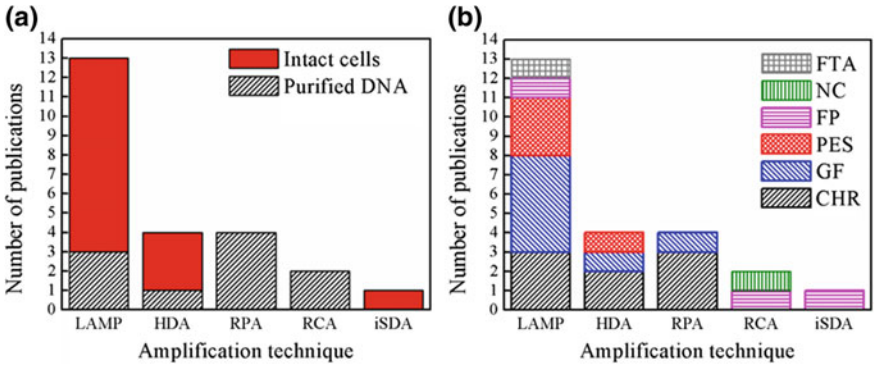

Fig. 8.12 Choice of the amplification techniques and the substrates reported in the paperfluidic literature. None of the histograms are normalized. **a** Amplification techniques reported with intact cells (i.e. integrated devices) and purified DNA as the respective starting material. LAMP is the most widely used technique with intact cells. RPA and RCA have not been demonstrated with intact cells yet. **b** Use of different substrates for different amplification techniques. *NC* nitrocellulose; *FP* filter paper: *PES* polyethylsulphone; *GF* glass fibre; *CHR* chromatography paper. LAMP appears to be the most versatile technique in terms of the choice of substrates

Table 8.1 Paper-based assays employing isothermal NAATs for nucleic acid analysis

Technique	Target	Amplification template	Sample	Substrate	Lysis	Purification needed?	Detection	Limit of detection	Complete assay time (min)	Reference
LAMP	*N. meningitidis*	Nucleic acids	Spiked in artificial CSF	Chromatography paper	–	–	Fluorescence	3 copies	47	Dou et al. (2014)
	Synthetic dengue viral DNA	Nucleic acids	Purified DNA	Glass fiber	–	–	LFD	3000 copies	76	Choi et al. (2016a, b)
	S. agalactiae *S. pneumoniae* *S. aureus*	Intact cells	Purified DNA from clinical samples	Glass fiber	–	–	Fluorescence	SP: 4.1 × 10^2 copies	80	Seok et al. (2017)
	HIV-1	Intact cells	Spiked in saliva	FTA membrane	FTA membrane	Yes	Fluorescence	10^2 virus/mL	<40	Liu et al. (2011)
	E. coli	Intact cells	Spiked in plasma	FTA membrane	Chemical	Yes	Fluorescence	5 cells	60	Connelly et al. (2015)
	Influenza A	Intact cells	Frozen clinical nasopharyngeal swab (NPS)	PES membrane	Chemical	No	LFD	–	45	Rodriguez et al. (2015)
	Human papillomavirus (HPV)	Intact cells	Clinical cervical samples	PES membrane	Chemical	No	LFD	–	50	Rodriguez et al. (2016a, b)
	E. coli *S. pneumoniae*	Intact cells	EC: Spiked in drinking water, milk, blood and spinach SP: In clinical blood samples	Glass fibre	Chemical	Yes	LFD	EC: DW 10 CFU/mL M 10 CFU/mL S 10^3 CFU/mL B 10^2 CFU/Ml SP: NA	45	Choi et al. (2016a, b)

(continued)

Table 8.1 (continued)

Technique	Target	Amplification template	Sample	Substrate	Lysis	Purification needed?	Detection	Limit of detection	Complete assay time (min)	Reference
	P. falciparum *P. vivax* *Plasmodium* pan	Intact cells	Human whole blood samples	Glass fibre	Chemical and thermal	Yes	Fluorescence	PF: 5 parasites/mL	–	Xu et al. (2016)
	N. meningitides *H. influenzae B* *S. Pneumoniae*	Intact cells	Spiked in artificial CSF	Chromatography paper	Chemical	No	Fluorescence	–	60	Dou et al. (2017)
	Leptospira *Brucella*	Intact cells	Spiked in bull semen	Glass fibre	Chemical	Yes	Fluorescence	L: 50 cells B: 50 cells	60	Yang et al. (2018)
	E. coli *M. smegmatis*	Intact cells	Culture	Chromatography paper	Thermal	No	Fluorescence	EC: 1×10^2 CFU/mL MS: 1×10^3 CFU/mL	40	Naik et al. (2019)
	HIV-1	Intact cells	Spiked in healthy human blood samples	PES membrane	Thermal	Yes	LFD	4×10^6 virus copies/mL	90	Phillips et al. (2019)
tHDA	*M. tuberculosis*	Nucleic acids	Spiked in artificial sputum	Chromatography paper	–	–	Fluorescence	100 copies	~10	Shetty et al. (2016)
	C. trachomatis	Intact cells	Heat inactivated cells spiked in synthetic urine	Chromatography paper	Chemical	No	LFD	10^4 cells/Ml	50	Linnes et al. (2014)

(continued)

Table 8.1 (continued)

Technique	Target	Amplification template	Sample	Substrate	Lysis	Purification needed?	Detection	Limit of detection	Complete assay time (min)	Reference
	S. typhimurium	Intact cells	Spiked in wastewater, milk, juice and egg samples	Glass fiber	Chemical	Yes	LFD	WW 10^2 CFU/mL M 10^3 CFU/mL J 10^3 CFU/mL E 10^2 CFU/mL	75	Tang et al. (2017)
	N. gonorrhoeae	Intact cells	Vaginal and urethral swab samples	PES membrane	Chemical	Yes	LFD	500 genomic copies	80	Horst et al. (2018)
RPA	HIV	Nucleic acids	Purified DNA	Glass fibre	–	–	LFD	1 copy/μL	20	Rohrman et al. (2012)
	Plasmodium spp.	Nucleic acids	Purified DNA	Chromatography paper	–	–	LFD	5 copies/μL	55	Cordray et al. (2015)
	M. smegmatis *M. tuberculosis*	Nucleic acids	Purified DNA	Chromatography paper	–	–	Electrochemical	~0.040 ng/μL	–	Tsaloglou et al. (2018)
	Ebola virus	Nucleic acids	Purified DNA	Chromatography paper	–	–	Fluorescence	10^7 copies/μL	20	Magro et al. (2017)
RCA	*Circular DNA*	Nucleic acids	Purified DNA	Filter paper	–	–	Fluorescence	100 fmol/mL	140	Ali et al. (2009)
	Hepatitis C virus	Nucleic acids	Purified DNA	Nitrocellulose membrane	–	–	Fluorescence	10 pM	40	Liu et al. (2016)
iSDA	*S. aureus*	Intact cells	Nasal swab samples	Filter paper	Chemical	Yes	LFD	5000 genomic copies/sample	55	Lafleur et al. (2016)

contamination from previous experiments has been reported in the literature (Tomita et al. 2008; Naik et al. 2019). Naik and others (2019) discuss in detail a set of measures to minimize cross-contamination in paperfluidic LAMP assays.

Among the other techniques, HDA suffers from low processivity and hence fails to amplify longer targets (Vincent et al. 2004). RCA and RPA, which take place at relatively lower temperatures, increase the possibility of non-specific product formation (Sipos and Sz 2007). Absence of a thermal denaturation step in most isothermal NAATs can affect their specificity and reproducibility (Das et al. 1999). The set of primers for each isothermal amplification technique needs to be carefully designed. These considerations lead to a relatively longer period for reaction optimization when compared to PCR.

8.5.2 Choice of the Paper Substrate

As shown in Fig. 8.12b, chromatography paper and glass fibre appear to be the most preferred substrates across different amplification protocols. PES membrane and filter paper are moderately popular, while FTA cards and nitrocellulose membranes are the least used substrates of all. Despite the popularity of some substrates over others, the choice of the substrate is determined by the nature of task to be performed. There is no single paper substrate that possesses the desired characteristics for all applications.

The choice of the paper substrate also depends on its physical properties such as surface charge, and pore size. Surface charge determines the extend of sample loss by non-specific adsorption of nucleic acids and proteins on the substrates. Paper matrices with high pore sizes or porosity could lead to uncontrolled wicking of liquids (Zhong et al. 2012). Such matrices are best used as absorbent pads in paperfluidic NAATs for imbibing any excess fluid during the course of the reaction. Cellulose fibres in filter papers with larger pore sizes can swell and reduce the effective cross-section of the channel available for capillary flow. This, in turn, affects the concentration of the analyte reaching the detection area. The thickness of the paper matrix governs its tensile strength, while its bed volume determines the amount of the fluid wicked. Paper substrates that are too thin are less likely to withstand the manufacturing process. Bed volume has an inverse relationship with the intensity of the visible signal and indirectly influences the signal strength during colorimetric detections (Yetisen et al. 2013).

The burgeoning interest in DNA amplification on paper led the Klapperich group to devise experiments for comparing the amplification efficiency of LAMP, thermophilic HDA (tHDA), and PCR on five different membranes: cellulose paper (CHR), glass fibre (GF), nitrocellulose (NC), polyethersulfone (PES), and polycarbonate (PC). They characterized the membranes with respect to their surface morphology, porosity and water absorbency (Linnes et al. 2016). Taking the pore volumes of each membrane into consideration, they were cut to different sizes to hold 25 μL of reaction volume. The amplification efficiencies were compared in the

presence and absence of excess fluid to determine whether the amplification occurs inside the membrane or in the excess solution. The authors observed that CHR, PES, and PC showed statistically significant amplification by LAMP compared to the negative controls when excess liquid was present. PES and PC showed statistically significant amplification even in the absence of excess fluid. tHDA occurred in PES and PC in the presence of excess fluid, but only in PES in absence of excess fluid. The authors determined PES to be the best choice for isothermal NAATs on paper.

Paper made from wood pulp contains lignin which is a chromophoric compound (Paulsson and Simonson 2002). Lignin, even in trace amounts, significantly changes the color of paper over extended periods of time. This makes lignin-containing paper unsuitable for the colorimetric detection module of paper-based diagnostic devices. Filter and chromatography papers, on the other hand, are manufactured using high-quality cotton linters, making them preferred substrates for detection (Hepburn 1976). FTA cards are specialized substrates for use in lysis and extraction of nucleic acids. While they can be used for sample preparation from blood or gram negative bacteria, they are not suitable for extracting the DNA from gram positive bacteria.

Like surface charge, any previous chemical treatment on the paper substrate could play a role in the non-specific adsorption of biomolecules. Lack of information in the public domain about the chemical treatments performed on various commercially available paper substrates makes it difficult to explain this phenomenon. To this end, the success of NAATs on different paper substrates is still determined by simple trial and error.

8.5.3 Role of Reagent Storage, Transport and Rehydration

When discussing the development of paperfluidic POC diagnostic devices, it is necessary to consider the environmental conditions under which these tests will be performed. Most resource-poor settings do not have access to cold chain transport or storage. Therefore, the reagents on the paperfluidic devices must be stable under varying temperature and humidity conditions. The amplification reagents are dried on the paper surface for long term storage and later rehydrated by addition of the sample (Ramachandran et al. 2014; Choi et al. 2015; Shetty et al. 2016; Magro et al. 2017). Proteins are extremely sensitive to temperature and pH fluctuations. They could lose most of their functions under suboptimal storage conditions. Several groups have reported the use of stabilizing agents such as trehalose (Crowe et al. 1996), polyethylene glycol (PEG) (Chao et al. 2014), polyvinyl alcohol (PVA) (Rawat et al. 2015) and glycerol (Vagenende et al. 2009). These agents help in maintaining the structure and function of the enzymes in the dried form. Trehalose and betaine are widely used for protein stabilization (Hayashida et al. 2017; Chua et al. 2019). They can also alter the melting temperature of DNA (Weissensteiner 1996; Bezrukavnikov et al. 2014), thus facilitating preferential

amplification of certain templates. This 'dual effect' can be exploited to enhance the specificity of amplification.

Dried reagents need to be thoroughly rehydrated prior to DNA amplification. This is achieved by applying the rehydration buffer or the liquid sample on the paper substrate. On addition of a liquid, the dried reagents are transported across the paper by the fluid front. The front contains a higher concentration of the reagents compared to the rest of the fluid. The concentration gradient resulting from inhomogeneous dispersion of the assay components adversely affects the reaction efficiency. The difference in the concentration depends on the initial concentration of the analyte and the viscosity of the rehydrating liquid (Lutz et al. 2013; Ota et al. 2018). The presence of stabilising agents is known to increase the fluid viscosity, which indirectly affects the detection signal intensity (Oxaal et al. 1987; Lutz et al. 2013). A a piezoelectric ultra-low volume reagent dispenser for uniform rehydration of immunoassay reagents on a nitrocellulose membrane was reported (Fridley et al. 2012). However, it is very expensive and not suitable for POC applications.

The need for improved dispersion of the assay components upon rehydration, in turn, brings out the need for flow control in paperfluidic devices. Most reported flow control methods are yet to be extended to paper-based NAATs. Almost all DNA analysis devices require multiple manual steps, e.g. pipetting of reagents or wash buffers, folding of origami devices, sliding of a device part, etc. There are only four reports in the literature (Lafleur et al. 2016; Tang et al. 2017; Horst et al. 2018; Phillips et al. 2019) which have demonstrated a high level of integration of the different sample processing steps and automated operation of their paperfluidic analysis platform. These devices truly operate in the 'sample-in, answer-out' mode which requires only the introduction of the sample by the user.

8.6 Conclusion

There has been a widespread development in the field of paper-based nucleic acid amplification devices. While PCR remains the amplification technique of choice in conventional microfluidics, isothermal methods have replaced PCR on paper substrates. A number of commercially available paper substrates have been tested for compatibility with DNA analysis. Chromatography paper and glass fibre seem to be the most popular substrates across different techniques. LAMP appears to be the most popular amplification method due to its high sensitivity, specificity and the ability to amplify DNA from crude samples. A significant amount of research has also gone into exploring the storage of reagents (particularly enzymes) in a dried form on paper and their efficient rehydration prior to analysis. We have discussed in detail the various isothermal amplification methods used in paperfluidics. We have also discussed some sample preparation and detection techniques, while briefly touching upon some integrated devices that perform the complete analysis in an automated manner. While a large number of reports exist in the literature on paperfluidic NAAT devices, none of these platforms have been commercialized yet.

As we discussed, there are several challenges in translating these devices for use at the point of care. Fortunately, a number of research groups are actively working on addressing these challenges. We expect some of these devices soon to be available in the market.

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Chapter 9
Paper-Based Devices for Food Quality Control

Aditya Choudhary, Urmila Brighu and Kanika Saxena

Abstract Giving sheltered and nutritious food and water, both locally and universally, has for some time been an objective for improving worldwide wellbeing. Recent enactments authorized all around the world have empowered government organizations to additionally control farming and industry benchmarks, requiring the requirement for progressively deterrent methodologies with respect to food quality, safety and security. Expanding detection speed and empowering field and creation identification of point-source pollution are significant to keeping up food security just as averting hindering malady flare-ups, for example, those brought about by bacterial sullying. Besides, ongoing improvements into micro fluidic approaches for investigation have indicated incredible guarantee as stages for giving quicker disentangled techniques to recognition. So, paper-based sensors are another elective innovation for creating straightforward, minimal effort, convenient and dispensable logical gadgets for some application zones including clinical conclusion, food quality control and natural checking. This chapter centers on the advances in paper-based sensors, existing manufacture systems, logical techniques, improvement of basic, economical and expend-able stages for colorimetric and electrochemical investigation of food quality and application zones. The present status and worries of the μPAD applications in the identification of concoction perils in sustenance's from the point of view of nourishment researchers, principally for a group of people with a foundation in mechanical, food safety, environment and chemical who may have interests in investigating the capability of μPAD to address true sustenance wellbeing issues.

Keywords Food quality · Food safety · Contamination · Rapid detection · Microfluidic Paper-Based Analytical Devices (μPAD)

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9.1 Introduction

Food is an inevitable source of life and to ensure a healthy living, consuming food with impeccable nutritional quality is of paramount importance for all the human beings, and negligence to authenticate and ensure the nutritional quality of food may lead to acute deterioration of health due to short-time exposure and serious illness due to long-time exposure. The quality of food can be greatly altered to-wards a negative sphere by the presence of contaminants such as pathogens, pesticides, insecticides, herbicides, heavy metals such as lead and mercury, and other toxic materials which finds their way to the diet through agricultural and industrial processes. In a quest to enhance the flavor and visual appearance of food, food adulteration is on rise these days. Additives are being used rampantly to provide food preservation, coloring and sweetening, which can prove out to be toxic if consumed beyond specific concentrations (Sasaki et al. 2002). Foodborne illnesses are a consequence of the intake of food that contains infectious and toxic agents (WHO 2008). To combat foodborne diseases, it becomes crucial to analyse and authenticate the quality of food. Rapid and low-cost methods are in demand for timely detection and control of food quality. The problem of food quality is a significant problem in developing countries and particularly in rural areas. Therefore, diagnosis technologies need to be cost-effective, compact, and portable. Affordable technologies that can enable local communities in developing regions to improve healthcare, environmental safety, animal health, and food quality will play critical roles in achieving post-Millennium Development Goals (United Nations 2008). The WHO, which is the supreme guiding body for healthcare, has set benchmark guidelines for diagnostic technologies specifically for developing countries. These technologies must be: (i) cost-effective, (ii) sensitive for very small amount of sample, (iii) specific for testing particular contaminants, (iv) user-friendly which could be easily operated by a layman, (v) quick to show results and easy to handle, (vi) portable and compact, and (vii) delivered to target users (Urdea et al. 2006). Currently, the demand is of more sensitive and specific rapid diagnostics assays globally. The detection technology should have features such as the requirement of low-volume clinical samples; it should be lightweight and no power-requirement or battery-powered for ensuring portability. With the advances in microfluidics, microfluidic paper-based analytical device (μPAD) technology for food quality assessment has evolved considerably, thereby reducing the cost of analysis. Specifically, μPAD applications are useful for the detection of various contaminants and specific compounds that are induced in food and water during unmonitored industrial and agricultural processing. Paper substrates are increasingly being utilized for μPAD fabrication and testing of additives, pathogens or heavy metals in food.

9.2 Paper-Based Sensors in Microfluidics

Microfluidics deals with the precise flow-control and manipulation of very small quantity of fluids from 10^{-9} to 10^{-18} L (Whitesides 2006). Microfluidics has undergone rapid growth due to its advantageous ability to use minimal amount of liquid samples and specific reagents to perform channelling and detection with high resolution and sensitivity that too cost-effectively and quickly (Manz et al. 1992). Microfluidics is now being considered as the most suitable and sensitive technology for point-of-care diagnostics for assessing food quality. The rapid and cost-effective diagnosis of food quality requires cheap raw materials, easy manufacturing, compact design, and portability of the sensor, which has been facilitated by paper-based microfluidic devices. Previously glass (Ruano et al. 2000; Queste et al. 2010), silicon (Sanjoh and Tsukihara 1999; Harris et al. 2003) and polymers such as poly (dimethylsiloxane) (PDMS) (McDonald and Whitesides 2002; Leclerc et al. 2004) have been used as substrates. These have the disadvantages of being costly and are not portable. Paper has emerged as an auspicious low-cost substrate material for microfluidic device fabrication (Martinez et al. 2007; Pelton 2009; Mohammadi et al. 2015; Kumar et al. 2016a).

The impregnation or patterning of paper strips is performed with materials such as paraffin and wax because of their hydrophobicity to prevent cross-contamination for the development of paper-based sensors. It is advantageous as compared to glass and silicon as it is easy to print, coat and impregnate; it is environmentally viable technology as it offers disposal by incineration; and its accessibility has added more to its advantage and usage (Yetisen et al. 2013; Busa et al. 2016). The most important property of paper which has facilitated its use as a primary substrate for the microfluidic paper-based analytical devices (μPADs), is the power-free liquid transport by capillary action using the cellulose membrane network. Also, the detection sensitivity and chemical storage capacity within the paper itself is enhanced due to a high surface area to volume ratio for colorimetric devices (Cate et al. 2015). The only disadvantage of such systems is the low resolution and sensitivity as compared to the sophisticated conventional devices like silicon, glass or plastic devices. The application of μPADs is highly suitable for on-site monitoring that requires cost-effective analysis for regular testing especially in the developing countries where the resources are limited for sophisticated instrumentation and analytical laboratories. Hence, μPADs have proven out to be an attractive alternative to traditionally used highly advanced and costly instruments in the analytical research applications for food quality assessment.

For the application of paper-based microfluidics, the exploitation of the properties of paper is utmost necessary. The thickness of paper is in micrometers and it is produced by pressing together the cellulosic fibers. No single type of paper can be considered desirable for all the applications. Most of the studies have used chromatography papers and filter papers. Low surface tension and porosity hinders the suitability of printing paper for fabricating hydrophilic pathways for fluid flow (Zhong et al. 2012). The large pore sized filter can swell their cellulose fibers

leading to a constrained capillary flow. The surface area of the paper is a critical parameter as it accounts for the efficiency of reagent deposition, required sensitivity, and specificity to particular end-use, and reproducibility for reducing the error in analysis. Capillary flow rate relates directly to the time taken by the fluid to reach the target spot in paper-based microfluidics. It is defined as the travel speed of a liquid front moving along the flow channel. Pore size and pore size distribution are two crucial variables for the selection of materials which can be filter papers or fine membranes for the fabrication of microfluidic devices (Harvey et al. 1996). Conventionally membranes have been characterized by their pore sizes. Pore size is the diameter of the largest pore in the membrane filters. Porosity which is another independent parameter and should not be confused with pore size. It is defined as the volume of air in the three-dimensional membrane structure. It can be visualized as the volume of air in a three-dimensional membrane structure (Yetisen et al. 2013). The signal visibility or sensitivity, strength and volume of a paper are influenced by the paper thickness. Bed volume is the total useful volume of a paper or a membrane for the sample, and it depends on membrane length, thickness and porosity. An increase in thickness increases bed volume. For instance, the reagents can be spread-out easily on a thin sheet due to hindered downward penetration. The sensitivity and color intensity are low when the fluid is spread on a paper having large volume due to large thickness.

Some of the paper substrates that have been used popularly include Whatman filter paper grade 4 (Jayawardane et al. 2013; Nouanthavong et al. 2016), Whatman chromatography paper 3 MM Chr (Liu et al. 2014, 2015), Whatman RC60 regenerated cellulose membrane filter (Cuartero et al. 2015), Advantec 51B chromatography paper (Asano and Shiraishi 2015), JProLab JP 40 filter paper (Cardoso et al. 2015), Millipore MCE membrane filter (Burnham et al. 2014), Canson paper (Badawy and El-Aswad 2014), Fisherbrand P5 filter paper (Alkasir et al. 2012), and Ahlstrom 319 paper (Myers et al. 2015). The utility, advantages, and disadvantages of each of these paper substrates may be different for particular target detection and fabrication methods used. Therefore, one paper substrate may be suitable for a method and unsuitable for others. A μPAD is shown in Fig. 9.1.

One challenging feature in food analysis using paper-based devices is the method of handling and pre-treatment of the samples before the detection. It is easier to test liquid samples such as drinking water samples or beverages because they do not require any pre-treatment of the sample before detection (Li et al. 2011, 2015; Alkasir et al. 2012; Shi et al. 2012; Jayawardane et al. 2013, 2014; Wang et al. 2013; Kuek Lawrence et al. 2014; Asano and Shiraishi 2015; Zhu et al. 2015; Cuartero et al. 2015). However, food specimens are necessarily in solid or semi-solid form, and therefore, a suitable pre-treatment step is required for increasing the mobility of the target entity before introducing into the μPAD for detection. For pre-treating fruits, vegetables and meat samples, various methods have been used (Mani et al. 2013; Nouanthavong et al. 2016). Processes such as elution (Liu et al. 2014, 2015), or boiling (Cardoso et al. 2015), to dissolve the

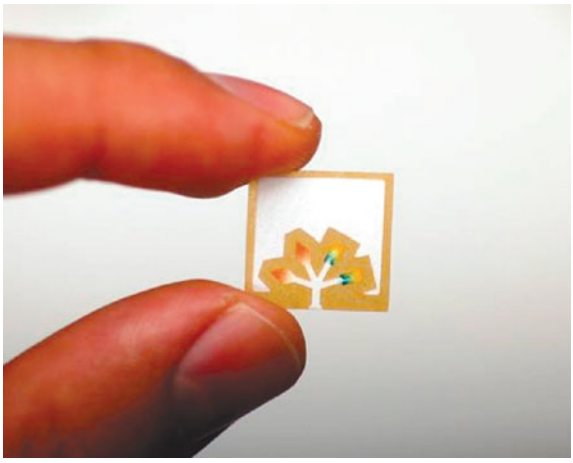

Fig. 9.1 A paper-based analytical device (Martinez et al. 2010). Copyright © 2010, American Chemical Society

constituents of the semi-solid pulp in water, followed by filtering the solids and then testing the liquid containing the target for μPAD detection. For pathogen detection, the swab sampling technique is popularly used which is less time-consuming and are based on colorimetric detection (Jokerst et al. 2012).

9.3 Fabrication Techniques

Complex diagnostic applications can now be achieved by fabrication of paper-based microfluidics in two or three dimensions for channeling the fluidics in both the horizontal and vertical dimensions. The manufacture of paper-based microfluidic devices involves the creation of barriers by filling the pores and shaping and cutting to facilitate the movement of fluid under capillary action through channels. Fabrication of μPADs is based on forming patterns on sheets of paper to create hydrophilic channels (where the fluid flowas) bounded by hydrophobic barriers (Fig. 9.2) (Martinez et al. 2010).

A number of methods of fabrication have been experimented in the past. These methods include photolithography (Martinez et al. 2008a), silane and UV/O_3 patterning (He et al. 2013), poly(dimethylsiloxane) (PDMS) printing (Bruzewicz et al. 2008), inkjet etching (Abe et al. 2008, 2010), plasma treatment (Li et al. 2008), printed circuit technology (Zhang and Zha 2012) and subtractive (dehydrophobization) laser treatment (Chitnis et al. 2011). These techniques were synthesized on lab-scale but could not be scaled up for large-scale commercial manufacturing due to their high cost and complex design. The following are some popularly used methods for fabrication of paper-based sensors.

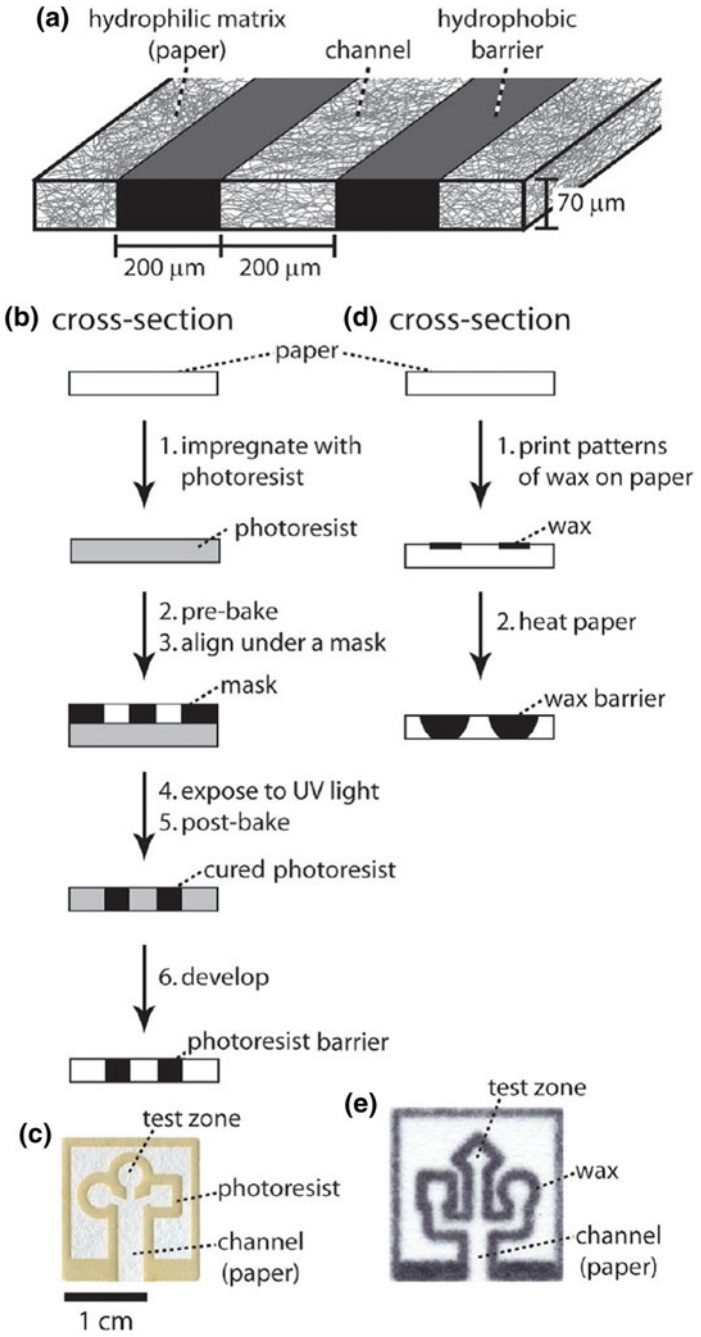

Fig. 9.2 **a** Schematic representation of a paper-based microfluidic device. **b** Formation of hydrophobic barrier using photolithography **c** A device fabricated by photolithography three independent test zones. **d** Hydrophobic barriers by wax printing **e** A device fabricated by wax printing with a central channel that wicks fluids into three independent test zones (Martinez et al. 2010). Copyright © 2010, American Chemical Society

9.3.1 Two-Dimensional Cutting

There are two ways of constructing 2-dimensional paper based sensors: (i) computer-controlled cutters (Fenton et al. 2009) and (ii) CO_2 laser cutting (for nitrocellulose) (Fu et al. 2010; Kumar et al. 2016b). For the 2-dimensional patterning of paper or nitrocellulose using a computer-operated 2-dimentional knife plotter paper channels are attached to suitable plastic chips for fabricating 2-dimensional sensors. The device includes a cutter that is programmed to move and allow the cutting of desired shapes and features. CO_2 laser cutting of nitrocellulose has also been performed, which produces lateral dimensions down to 1.5 mm (Fu et al. 2010). These instruments are not robust when compared to wax-printing and therefore require sealing and backing by using vinyl or polyester plastic films. The packaging is required, which controls intrusion of humidity through the air and protects the assay from contamination.

9.3.2 Wax Patterning

Wax-based fabrication or patterning techniques at lab-scale of the paper-based microfluidic devices have proved amenable since they are cost-effective methods in which no harmful or toxic reagents are used. Printing, dipping and screen printing are the primarily used methods for wax patterning. Wax screen printing involves putting solid wax using a screen onto a paper sheet (Dungchai et al. 2011) and the paper is placed on a hot plate or oven so that the wax melts and diffuse into the paper such that hydrophobic barriers are formed. Another method which eliminates the necessity for complicated instrumentation is wax dipping (Songjaroen et al. 2011). In this method of fabrication technique, an iron mold of desired shape is formed by laser cutting. The assembled mold is then dipped into molten wax and the pattern is transferred onto a piece of paper. Although, both wax dipping and screen printing have the advantage of cost-effective manufacturing as they do not require high-cost fabrication machines, they have certain disadvantages too as they can show defects in patterning and dissimilarity between batches.

9.3.3 Flexographic Printing

This technique is particularly used for commercial manufacturing of μPADs. Flexographic printing of polystyrene has enabled production of huge quantity of paper rolls specifically to be used in microfluidics (Olkkonen et al. 2010). First, an impression roll is fixed with a substrate paper. The requisite ink is filled into the ink slot followed by directing the polystyrene solution onto a cylindrical roller having already embossed microfluidic patterns. The polystyrene solution is evenly

distributed by the cylindrical roller onto the printing plate, having embossed patterns. The system then prints the pattern on to the substrate. Flexographic printing uses contemporary manufacturing techniques and therefore it is preferred method for large-scale production (Songjaroen et al. 2011).

9.3.4 Alkyl Ketene Dimer (AKD) Printing

Forming μPADs by using alkyl ketene dimer printing has shown the potential for commercial-scale production. Cellulose reactive hydrophobization agents can be used for the selective hydrophobization of paper for fabricating paper-based microfluidic channels (Li et al. 2008, 2010). The cellulose reactive agents to paper are applied to chemically modify the fiber surface. These reagents react with the OH groups of cellulose so as to impart hydrophobicity. This technique is particularly useful for printing fine and intricate hydrophobic patterns invisible to the naked eye.

9.3.5 Three-Dimensional (3D) Paper-Based Microfluidics

The recent developments in the μPAD application have introduced 3D paper-based devices. These devices have an edge over 2D devices and are currently dominating the market. 3D devices offer certain advantages over 2D devices because they have lesser footprint than a 2D device and enables three-dimensional fluid movement. These are manufactured using multiple layers of paper, which results in compactness and higher sensitivity of device. The configuration of a 3D fabricated paper-based device and its functions for fluid mobility are shown in Fig. 9.3 (Han et al. 2016). The use of 3D fabricated paper-based sensors minimises the sample volume that is lost in swelling the paper and increases the distribution time. The key fabrication challenge is maintaining the fluid flow through layers by keeping in contact the hydrophilic portion of layers. This contact is maintained by fixing one layer of paper over the other and clamping all the layers by using tapes, clamps, adhesives, or protective covers to hold the layers in position (Martinez et al. 2008b). Fluidic channels in a 3D device can be further divided into multiple channels to offer a multi-tasking feature so that different presence/absence tests can occur simultaneously. Each layer in a three-dimensional paper-based microfluidic device can be so assembled to perform a specific function to facilitate the overall assay.

9.3.6 Additional Functional Elements

The control of fluid flow and timing is an important aspect of μPADs, which requires many additional functional elements after the fabrication of these sensors.

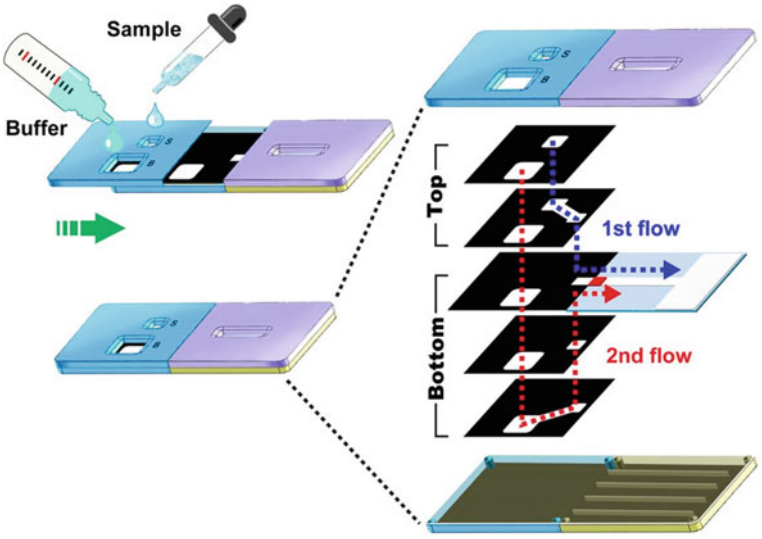

Fig. 9.3 A 3D μPAD and its functional features. Black and white regions represent the hydrophobic wax barrier and hydrophilic flow channel, respectively (Han et al. 2016)

Analytical assays having paper as a base device require several functions to inject sample, control and direct fluid flow, display of the response, power source, etc. Further, some detection tests may require a fixed amount of sample to be induced in the system, a fixed travel time, a power supply for the timer or display unit (particularly for powering absorbance, fluorescence or electrochemical assays), and in situ reagents for selective analysis. A 3D device may require much more functional units than a 2D device. The complexity of analysis increases the requirement of functional elements. These elements should confirm with the prime characteristics of a μPAD which are low-cost, lightweight, requirement of low sample volumes and easy to be used in source-limited settings.

a. **Valves and Switches**

These capabilities are crucial for multiple-step procedures involving signal amplification and also for facilitating the allowance of controlled fluid flow in the requisite channels. The presence of valves and switches is essential where the fluid needs to be transferred from one channel to the other. This is achieved by assembling thin gaps between fluid-flow channels which can be removed by applying small pressure at that particular point by using a pin or a ball-point pen. Removing the gap will allow the fluid to wick to the other connected channels. Programmable valves are also being used to construct 2D microfluidic networks using miniature circuits (Lutz et al. 2011).

b. **Mixing**

In μPADs, mixing the sample fluids with reagents is an essential requirement. Capillary-driven passive mixing has been used, but it has certain disadvantages, which includes non-uniformities and inefficient fluid mixing. Threads have also been used to build mixing capabilities (Li et al. 2008; Reches et al. 2010). Threads are sewn into the system to create short-circuits or pathways to transfer fluid from one channel to the other by capillary action. Another advanced method has been developed which uses miniature battery circuits to form surface acoustic waves to induce mixing (Rezk et al. 2012).

c. **Fluidic timers and metering**

The substances present in the device itself can be used as timers such as the amount of wax present in the paper or dyes. A previous calibration by relating the amount of wax and time taken for the fluid to travel is done. Similar concept is used in case of dyes to adjust the wicking rates.

d. **Power**

As these μPADs are portable, the requirement of an inbuilt source of power also becomes essential for specific electrochemical analysis. Fluidic batteries can be installed in paper-based microfluidic devices to generate electricity when a sample is introduced to the device. Galvanic cells can be integrated with the fluid pathways (Thom et al. 2012).

e. **Displays**

The display of result is very critical to the fabrication of μPADs. Generally, the color change is used as an indication of presence/absence of the required entity. Thermochromic displays have been used traditionally (Siegel et al. 2010). The thermochromic inks are heated using electric current to obtain a color change.

9.4 Applications to Food Quality Testing

Contamination and deterioration of food quality can occur by excessive use of additives such as artificial sweeteners and colors, pathogenic contamination, presence of heavy metals, pesticides, insecticides, or herbicides. The methods of detection of such contaminants using μPADs is summarized below:

9.4.1 Control of Food Adulteration

Artificially synthesised food supplements are being widely used in the food and beverage industries. Sweeteners such as glucose, fructose and sucrose, and other

food additives, are being used for enhancement of the flavor or color. Although most of these food additives may be nontoxic but, excessive consumption may deteriorate the nutritional quality of the food to which it is added and may become toxic if consumed above a certain concentration. This has pushed a strong demand for quick, sensitive, and cost-effective methods of analyses. Adkins and Henry (2015) developed an electrochemical μPAD that utilized microwire electrodes instead of screen-printed electrodes for detecting glucose, fructose, and sucrose in beverages. Colletes et al. (2016) developed a paraffin-based PAD for detecting glucose in the hydrolysis of liquors by using paper spray mass spectrometry (PS-MS). Nitrites are also popularly used as food additives to hinder the synthesis of disease-causing microorganisms and also to prevent rancidity caused by lipid oxidation (Honikel 2008). Nitrite can react with secondary and tertiary amines to form carcinogenic nitrosamine compounds, therefore there is a need to control the presence of nitrite in food (Zhang et al. 2014). Nitrite detection can be performed by colorimetric methods such as Griess-colour reaction method for visual detection of the presence of nitrite in food. Two pH indicators, phenol red and chlorophenol red are used. The image of the μPAD can be captured and processed to ascertain hue (H) and saturation (S) in the HSV color model. Similarly, Griess-color method was also used by Cardoso et al. for nitrite detection in ham and sausage with a detection limit as low as 5.6 μM (micromoles per litre). The image of the μPAD was taken for the colorimetric analysis and CMYK (cyan, magenta, yellow, key) model was used. Food colorants can also be detected by μPAD Zhu et al. (2015). The image analysis of colored compounds has facilitated the rapid detection of contamination.

9.4.2 Pathogen Detection in Food

μPADs for pathogen detection in food are primarily based on low-cost optical methods. In such arrangements, the results are either analyzed or by using image analysis software. A swab sampling technique is used to collect pathogens from foods, and then they are cultured in media before introducing to a μPAD and the detection is achieved by a color change. Park et al. (Lim et al. 2009) developed an optical technique known as Mie scattering strategy for detection of Salmonella Typhimurium. A novel method was developed by Jokerst et al. for the detection of pathogenic bacteria in ready-to-eat meat samples to concentrations as low as 10^1 CFU mL^{-1} within 12 h or even lesser (Jokerst et al. 2012). Zuo et al. (2013) developed a fluorescence (FL) method pathogen detection using a μPAD, which was a hybrid of PDMS and glass.

9.4.3 Pesticides and Herbicides Detection in Food

Combinations of paper chromatography and chemiluminescence in μPAD have been used for the determination of pesticides and herbicides in fruits and vegetables. Pesticides like organophosphate and carbamate has been detected by using colorimetric method. Organophosphates like paraoxon and malathion can be detected by using colorimetric techniques. Pesticides such as pentachlorophenol (PCP) which is a xenobiotic are also finding their way into the crops we eat (Dai and Copley 2004; Fuentes et al. 2013; Nesakumar et al. 2016). Long-time exposure to such pesticides can lead to bioaccumulation and cancer. A μPAD device was developed by Sicard et al. (Sicard et al. 2015) for the on-site colorimetric detection of organophosphate pesticides using smartphone application. This detection was based on the inhibition of immobilized acetylcholinesterase (AChE) by the pesticides. The inhibition of AChE occurs with increasing pesticide concentration and reduces the color intensity. (Badawy and El-Aswad 2014) developed a method based on similar concept for the detection of pesticides such as methomyl and carbamate. The toxicity of the pesticides is indicated by the degree of inhibition of the AChE (Pundir and Chauhan 2012). Paper-based methods have also been used for detecting herbicides like methyl viologen by using fluorescence (Su et al. 2015).

9.4.4 Heavy Metals in Food

Several methods have also been developed for heavy metal detection in food and water contaminants using μPAD technology. However, electrochemical techniques are particularly popular for heavy metal detection. An electrochemical method was developed by Nie et al. (2010) for ethanol detection in water for potential food quality control purposes. Halide detection in food supplement has also been accomplished by using an electrochemical μPAD via cyclic voltammetry developed by Cuartero et al. (2015). Colorimetric method of detection has been used for detecting copper, chromium, nickel and mercury (Li et al. 2015). Arsenic has also been detected using gold nano-particles (Nath et al. 2014).

9.5 Summary

The conventional analytical measurement systems have presented a disadvantage of being costly for the rapid and cost-effective analysis of food quality in resource-limited settings particularly in rural areas in developing countries. Countering this problem, the emergence of μPADs for food quality analysis is a rapidly proliferating technology which is quick, economically viable, user-friendly and very appropriate for on-site testing. This technology is still on its journey to be

realised as commercial-scale products to cater to the needs of a mature platform. The advancement and evolution of structural properties of paper, enhancements in the development of new hydrophobization materials using various contemporary fabrication methods will play an exciting role in the realization of new and improved paper-based microfluidic devices. The understanding of fundamentals of capillary flow has allowed the construction of paper-based assays with improved control capabilities. The fabrication of paper-based devices both in 2D and 3D along with various control units has increased its suitability and has established the ability of μPADs to detect specific contaminants such as heavy metals (lead, mercury, copper etc.), disease-causing pathogens, food colorants and additives, and other targets of interest. The detection of these contaminants has been demonstrated by many researchers in real food and water samples using colorimetric, fluorescence and electrochemical techniques. This has resulted in a significant improvement in the productivity of the governmental agencies responsible for food safety and quality. The potential of μPADs for food safety monitoring has also ensured the monitoring of food crops by rapid analysis of pesticides, herbicides and insecticides thus keeping a check on potential biomagnification and bioaccumulation. Exponential development in the field of nano-electronics and artificial intelligence has resulted in the development of image analysis software, digital cameras, improvements in battery life and compact battery designs. The revolutionary development in the field of smartphones has proved out to be an icing on the cake offering quick access to knowledge and tools for the rapid assessment of food quality and hence ensuring healthy living standards.

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Chapter 10
Paper Based Sensors for Environmental Monitoring

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Abstract Environmental pollution has become the major concern for the environmentalists in the recent times. Continuously increasing population and development of human race demands abundant amount of resources such as electricity and water for domestic as well as industrial work. At the same time, the by-products and remains of industrial processes are being discharged to the environment in form of harmful gases and effluents. Hence, the need of the hour is to monitor and control these contaminants, especially in air and water. The sensors required for this type of monitoring should have stability, cost effectiveness, disposability, and flexibility. Paper based sensing devices have become a promising mean to detect the pollutants and monitor the air and water quality. Paper based sensing devices possess some unique properties such as liquid transport through capillary action, ease in mass production, flexibility of shape/size, and disposability. Paper based sensors also offer high sensitivity, cost effective process, fast response, and flexibility as per the end user requirement. Due to above mentioned characteristics, the paper based sensors have become promising devices for the present environmental monitoring scenario. This chapter explains the fabrication methods, mechanisms, and applications of the paper based sensors for environmental monitoring.

Keywords Sensors · Pollutants · Microfluidics · Adsorption · Chemiresistive

10.1 Introduction

The global development in industrial and transport sector has posed a catastrophic threat to the environment. Rising population demands facilities like clothes, transport vehicles, healthcare, education, and safety in abundance. These demands

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have revolutionized the respective industries with majorly focusing on fulfilling the demands. These industries discharge a huge quantity of solid, gaseous, and water wastage per day.

These waste products are sometimes discharged directly to the environment such as dumping grounds, open environment, rivers, and ponds (Rai et al. 2018), etc. The phenomenon of 'Global warming' has attracted a lot of attention of world leaders in recent years which relates directly to the air and water pollution. Many countries have now started to consider pollution as a major threat to mankind and are taking various critical steps to reduce it. To maintain the air quality various gas sensors are used to identify and quantify the different harmful gases. These harmful gases are: CO, CO_2, H_2, NO, NO_2, SO_2, H_2S, CH_4, C_2H_2, and O_3, etc. (Chauhan and Bhattacharya 2017; Chauhan et al. 2019a; Kumar et al. 2019). Similarly, various pollutants present in water are identified by using various water sensors. Heavy metals such as: As, Pb, Fe, Cu, Cr, and Al etc. mixes with water during various industrial processes such as steel making, heat treatment, textile colouring, and tanning (Chauhan et al. 2017). At the same time harmful dyes and other chemical compounds are also mixed with water during these operations. These compounds present in the water are the root of various water borne diseases such as cholera, diarrhoea, abdominal pain, headache, and fever, etc. (Chauhan et al. 2019b). The polluted water discharged into rivers and ponds is also harmful for the animals and humans which use this water.

Hence, it becomes a necessity to monitor and control these environmental pollutants. The monitoring system of air and water pollutants demands certain characteristics such as fast detection, accuracy, cost effectiveness, simplicity, and ease of fabrication. At the same time the detection system should be able to detect the presence of contaminants as quickly as possible to take appropriate actions. The traditional methods for the contaminant detection such as: inductively coupled plasma spectroscopy (ICPMS), gas/liquid chromatography, and mass spectroscopy are very accurate and effective but require complex processes to achieve the result (Liana et al. 2012). These methods have the drawbacks of exhaustive sample preparation requirement and complex quantitative analysis. Also, these methods require very expensive machinery, costly reagents, and expert operator to conduct the tests. These drawbacks make conventional detection methods less useful for online/onsite monitoring of the environmental pollutants.

Recent advances in paper based sensing technology have revolutionized the area of environmental monitoring. Now paper is not just a writing, packaging, drawing, and printing material but also serves the purpose of flexible substrate for the sensing material. Paper possesses certain properties such as: liquid flow through capillary action, flexibility, low cost, availability, and lightweight which makes it to be extremely useful for certain type of sensing applications. The cellulose fibre is the main constituent of paper and has hydrophilic nature which allows the liquids to flow through capillary action. Apart from that the properties of the paper can be improved as per requirement by chemical functionalization and treatments. The

paper based sensors are widely used for clinical and analytical chemistry due to their portability flexibility, and disposability (Park et al. 2018).

The first paper based sensor was developed in 1956, to monitor the glucose level in urine (Comer 1956). The later developments resulted into the development of paper based 'dipsticks' and commonly used pregnancy test kit (Von Lode 2005). These type of detection kits are based on immunoassay detection method which commonly consists a sample pad, a reagent pad, and a test line. The sample pad receives the sample to be tested, the reagent pad contains antibodies conjugated to target antigen specific signal indicator, and the test line captures the antibodies which are immobilized on the surface. The signal given in these type of devices is generally a color which is only a qualitative analysis (Yes/No type) of the sample. The example of commonly used paper based detection in chemistry is pH level measuring strips also known as 'Litmus paper'. The color of the litmus paper when dipped in a solution indicates its pH level (acidic or basic) as shown in Fig. 10.1. With further advancements in the paper based sensing technology, the quantitative analysis has also been possible up to some extent. The intensity of the color of the indicator corresponds to the concentration level of a particular gas or analyte (Huang et al. 2014). In the current paper based detection scenario, there are several sensing techniques such as: colorimetry (Li et al. 2018), electrochemistry (Sicard et al. 2015), chemiluminescence (Wang et al. 2018a), electrochemiluminescence (Delaney et al. 2011), and electrical (Mirica et al. 2013) are being used. The application of these techniques is based on the requirement of the particular system such as environmental conditions, type of analyte, and the accuracy level required. These techniques have their advantages and disadvantages which are to be considered before selecting it in a sensing system.

Hence, the paper based detection systems have emerged as an effective sensing lab-on-chip devices due to their fast analysis, portability, disposability, and onsite real time detection for the medical, environmental and industrial applications. Low cost of paper based devices can be easily mass produced and widely used specially in healthcare where a fast analysis is required. This chapter reviews the development/fabrication methods, analysis techniques, application areas, and challenges of the paper based environmental sensing devices.

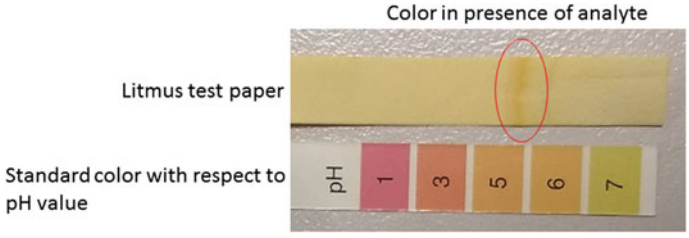

Fig. 10.1 Litmus test paper for the detection of pH level of analyte

10.2 Development of Paper Based Sensor

The choice of paper in paper based sensors becomes essential because it governs the sensing performance of the device. It adheres the sensing material onto its surface which further interacts with the entity to be sensed. Sometimes, the choice of paper depends upon the fabrication steps involved and applications with specific requirements. In recent times, filter paper has become the most favorite choice for the paper based sensing devices (Li et al. 2010). The filter papers are available with different grades which is basically describes the porosity, flow rate, and retention property of the paper (Liana et al. 2012). Few studies suggested the modification of the paper properties by adding the hydrophobic/hydrophilic films to optimize the fluid flow property. The filter paper is made of cellulose fibers which due to capillary action gets swelled by absorption of liquid. Hence the grade number should be clearly chosen so that the porosity can be maintained throughout the detection process.

For detection of biomolecules such as proteins, enzymes, and DNA nitrocellulose membrane is used due to its specific binding properties (Scida et al. 2013). The coating of wax on the paper substrate is used to create hydrophobicity and microchannels. Sometimes, apart from the conventional paper the glossy paper is also used. The glossy paper is fabricated by using inorganic filler material with cellulose fiber. Glossy paper is non-degradable and has smooth surface which is useful in surface based applications.

After selecting the most suitable paper the fabrication and pattering of the paper based sensing device is carried out. In some of the detection methods there is a requirement of fabrication of microchannels, electrodes, or specific layers of some other materials. There are several techniques and methods available for the fabrication of these features by using chemical or physical means. Some of the methods reported earlier by the researchers are photolithography (Shaker et al. 2011), screen printing (Sarfraz et al. 2013), plasma treatment (Li et al. 2010), wax treatment (Sicard et al. 2015), and inkjet printing (Lee et al. 2011) etc. The selection of these methods depends upon the quality of paper, application, cost, and other requirements of the end user. Gosselin et al. proposed a low cost fabrication protocol for a microfluidic device on an embossed paper (Gosselin et al. 2017). The embossing of the paper are used to create hollow microchannels for the fluid flow in colorimetric glucose detection. In this study two layers were coated on the paper substrate. The first layer of Styrene Butadiene Rubber (SBR) provides the waterproofing so that the analyte liquid could not penetrate the paper matrix. While, the second layer of Poly (Vinyl Alcohol)/copolymer Styrene Acrylique (SA) provides the hydrophilicity for allowing the fluid flow on the surface of the paper. The microfluidic channel was created on this coated paper by embossing method. In brief, the paper was pressed against a master (with negative carvings) of the desired shape inside a heated chamber as shown in Fig. 10.2. This device successfully demonstrated the flow of glucose assay through the microchannels (shown in Fig. 10.3).

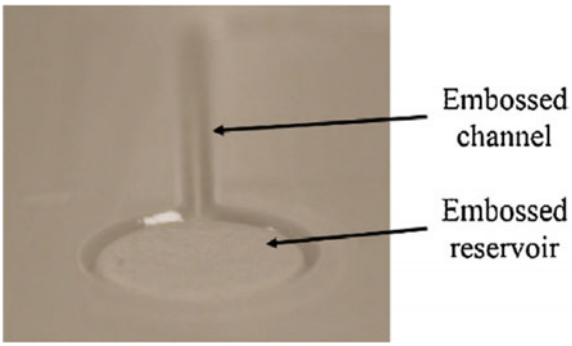

Fig. 10.2 The image of microchannel and reservoir on the embossed paper. Reprinted with permission from Gosselin et al. (2017). Copyright (2017) Elsevier, Sensors and Actuators B: Chemical

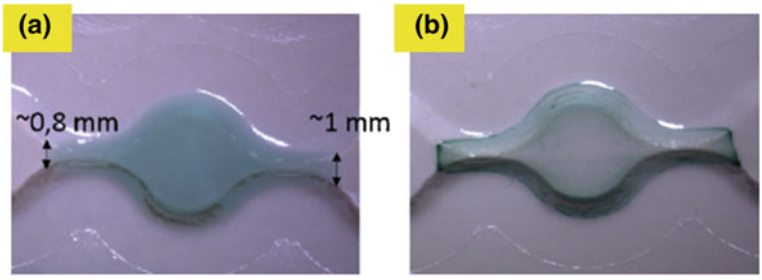

Fig. 10.3 The central well of the microchannels fabricated on an embossed paper with fluid before (**a**) and after evaporation (**b**). Reprinted with permission from Gosselin et al. (2017). Copyright (2017) Elsevier, Sensors and Actuators B: Chemical

Such types of system can be easily used in environmental monitoring such as heavy metals detection from the wastewater etc.

10.3 Detection Techniques

The paper based sensors have different detection techniques for identifying the analyte. These detection techniques are explained in the following section.

10.3.1 Calorimetry Detection

This test is based on the ability to give a semi quantitative detection based on the reaction between target substance and chemical reagents. This reaction involve

indicator which results in the color change with the completion of reaction. The detection results can be directly visualized or with the help of some computer software. Generally computer software is used only when the direct visualized is not possible.

This detection techniques is widely used as it provides accurate results at lower cost. Both enzymatic techniques and gold, silver nanoparticles are used in the calorimetry detection. In enzymatic technique, enzyme-substrate complex is formed due to the reaction between enzyme and specific substrate. This complex produces a visible color change. The enzyme is immobilized on the paper with the help of some reagents. Many innovations are made to decrease the limit of detection (LOD) and improve the detection sensitivity (Kumar et al. 2016, 2017, 2018; Almeida et al. 2018). In a study, paper is modified by multi-layering for uniformed color intensity detection of uric acid and glucose (Wang et al. 2018b). Horseradish peroxidase (HRP) combined with specific oxidase (uricase and glucose oxidase), chitosan and different kinds of chromogenic reagents were coated on the microfluidic paper. Produced Hydrogen peroxide reacts with substrates and also oxidizes the co-coated chromogenic reagents which produces color change. Here HRP is used as catalyst and to make the color uniform. The device is then placed under LED lamp which will suppress the external light. Mobile phone and image J software is used for the further analysis of the recorded images. The schematic of the colorimetric detection technique is given in Fig. 10.4.

10.3.2 Surface-Enhanced Raman Spectroscopy (SERS) Based Detection

Another method of detection is using gold (AuNPs) and silver nanoparticles (AgNPs) due to their change in absorption spectrum with change in the size. They are immobilized in the paper and are incorporated to label the secondary antibodies

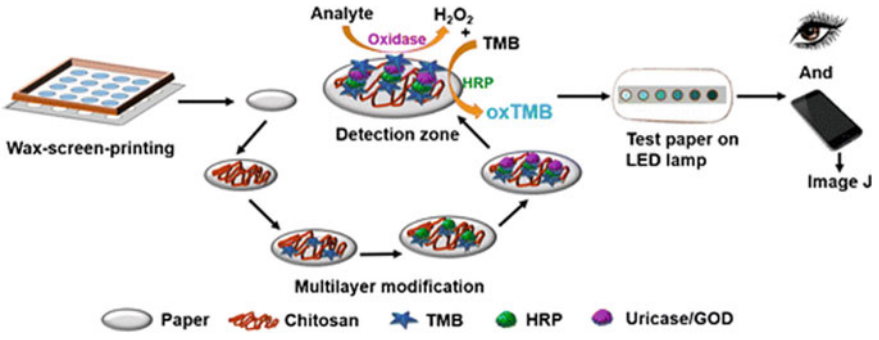

Fig. 10.4 Schematic of screen printed colorimetric assay based detection. Reprinted with permission from Wang et al. (2018b). Copyright (2018) Springer, Nature).

as detecting components. The color change is observed because of the aggregation of nanoparticles with the antibody-analyte (antigen) interaction. This change in the property of the nanoparticles is quantified with the surface plasmon resonance (SPR) effect which utilizes the plasmons. The disturbance of electrons from their mean position is the reason for the generation of these quantum oscillations known as plasmons. This also increases the electromagnetic field near the surface of the nanoparticles which can become the basis for the surface-enhanced Raman spectroscopy (SERS) (Wei et al. 2015). The Raman spectrum is different for different molecules depending upon the vibrational modes present. The SERS effect enhances the Raman cross section after the interaction of a molecule with AuNPs (Li et al. 2018) and AgNPs (Wei et al. 2015). The electromagnetic field of near the nanoparticle gets enhanced due to this type of interaction which can be detected from the spectroscopy analysis.

However, these nanoparticles leaks from the paper upon washing due to which leaching indicator and binders are incorporated in the device. Apart from the binder, many other reagents are also used to increase the specificity and enhance the detection. One of the successful attempt were made to detect the contamination of mercury ion in water with the help of gold nanoparticles (Chen et al. 2014). Citrate capped gold nanoparticles with the average size of 13 nm were synthesized and is mixed with ssDNA in the colloidal form to make the ssDNA-attached gold nanoparticle colloid. This colloid is printed on the paper to make the working device. As Hg^{2+} ion comes in contact with the printed colloid, it reacts with the present thymine to form the thymine- Hg^{2+}-thymine coordination bond and this aggregated the attached gold nanoparticles to show the color change. Such type of studies confirm that SERS based sensors can be used for the detection of biomolecules and metallic ions from different type of test samples.

10.3.3 Electrochemical Detection

By the virtue of its maturity and well-understood working principle, electrochemical detection is widely used in sensing applications. Integrating it with paper based devices gives us low cost, portability, high selectivity, sensitivity, low power consumption as its benefits to name a few (Määttänen et al. 2013). When an electric current is passed through the electrolyte, electrodes functions in a specific manner which leads to the detection of some entity on further analysis. A typical electrochemical sensor consists of three different electrodes: working electrode, counter electrode and reference electrode as shown in Fig. 10.5. Current is passed between the working and counter electrode which can be easily fabricated on the paper using conducting inks. In the electrolytic cell arrangement, the analyte is detected at the working electrode and the counter electrode is used to maintain the electrical circuit. It collects the current flowing in the circuit and also limits to the current flowing in the system and reference electrode. The reference electrode is generally made of

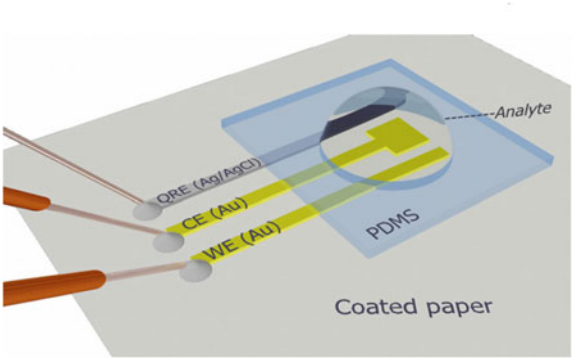

Fig. 10.5 Schematic of paper based electrochemical sensor with illustration of electrodes. Reprinted with permission from Määttänen et al. (2013). Copyright (2013) Elsevier, Sensors and Actuators B: Chemical

Ag/AgCl and is placed away to avoid any contact with the reaction to maintain the known constant potential (Määttänen et al. 2013).

Many experiment directly uses this setup while constant modifications are made frequently to enhance the detection on paper based device. For detection of trace amount of heavy metals (Cd^{2+} and Pb^{2+}) in the water using electrochemical detection (square-wave anodic stripping voltammetry), miniaturized carbon based sensor was fabricated (Shen et al. 2017). The contaminated water being the analyte here is flown along the microfluidic channel and both the electrodes are made facing each other at the ends of the channel. This device detects the trace amount of heavy metal, and is very robust with results highly reproducible.

10.3.4 Luminescence Based Detection

This detection technique includes fluorescence, chemiluminescence and electrochemiluminescence, and is widely used with the microfluidic devices. It is more sensitive compared to the photometric measurement because of the signal strength dependence on intensity of incident radiation and analyte concentration (Almeida et al. 2018). In this method, direct emission of the light as well as fluorescence quenching of analyte is used for the detection. Xin-Ran et al. fabricated a polymer grafted paper based fluorescent sensor by using CdTe quantum dots (Wang et al. 2015). Paper based device was fabricated to detect Cu^{2+} ions using fluorescence inhibition. The schematic of sensing mechanism and fluorescence spectra is shown in Fig. 10.6. Chemiluminescence and electrochemiluminescence are generally more sensitive than fluorescence measurement owing to the low background light signal. This low signal is due to the absence of the excitation light source. Here also, detection can be done by emission or inhibition. However, electrochemiluminescence measurements are considered to be better due to the electrochemical control of timing and location of excitation on the paper based device which is not possible in chemiluminescence.

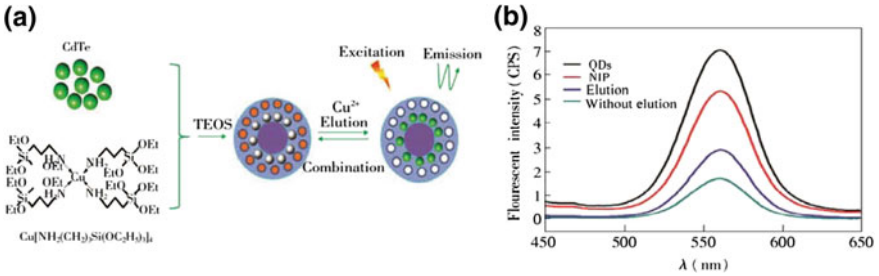

Fig. 10.6 Schematic diagram representing the sensing mechanism involved in the detection of Cu +2 (**a**), and the result of fluorescence intensity variation during detection (**b**). Reprinted with permission from Wang et al. (2015). Copyright (2015) Elsevier, Chinese Journal of Analytical Chemistry

These techniques are widely used and integrated with the microfluidic device for the low cost and robust detection of the analyte. In similar approach. a microfluidic detection chip was fabricated for detection of formaldehyde in various food sample, which consist of micro paper analytical device (Liu et al. 2018). This μ-PAD is fabricated by the wax printing and implanted with acetoacetanilide reagent. Basic Hantzsch reaction process is used for the detection in which, formaldehyde-acetoacetanilide complex (dihydropyridine) is produced. The concentration of formaldehyde is detected from the UV light – induced fluorescence intensity of the formed complex.

10.4 Paper Based Sensors for Environmental Monitoring

Paper based sensors are widely used in environmental pollution monitoring. The application of these devices for water and air quality monitoring is described in following sections:

10.4.1 Paper Based Sensor for Water Quality Monitoring

Paper based sensors are widely used for water quality monitoring. Water based contaminants such as harmful heavy metals, chemicals, dyes, and bacteria are very injurious to the health of flora and fauna. These contaminants should be detected and removed from the usable water as early as possible to eliminate the spreading of the various waterborne deceases. The onsite and fast detection system is required for these contaminants to improve the water quality. The fast detection system cannot be based on the conventional methods such as liquid chromatography and mass spectroscopy. The advantage of fast detection by using paper based sensors

provides a feasible solution to the water health quality monitoring system. Hossain et al. developed a paper based sensor for the detection of organophosphate pesticides in food and beverages (Hossain et al. 2009). In this method, the paper substrate is used to detect pesticides by means of change of colour from yellow to blue due to enzyme catalysed hydrolysis of the substrate in presence of the pesticide acetylcholinesterase and indophenyl acetate (IPA). The paper strips were successfully tested for milk and apple juice samples. As shown in Fig. 10.7, the reagentless lateral flow paper based sensor detects pesticides effectively. Polyvinyl Amine (PVAm) is used as a cationic capture agent on the paper which captures anionic products of the reaction and preserves the response. This type of sensor can be used for the detection of pesticides from water samples.

Sicard et al. followed the similar approach and developed a paper based analytical device (μPAD) to detect organophosphate pesticides (Sicard et al. 2015). The mechanism for the detection was based on the inhibition of coated

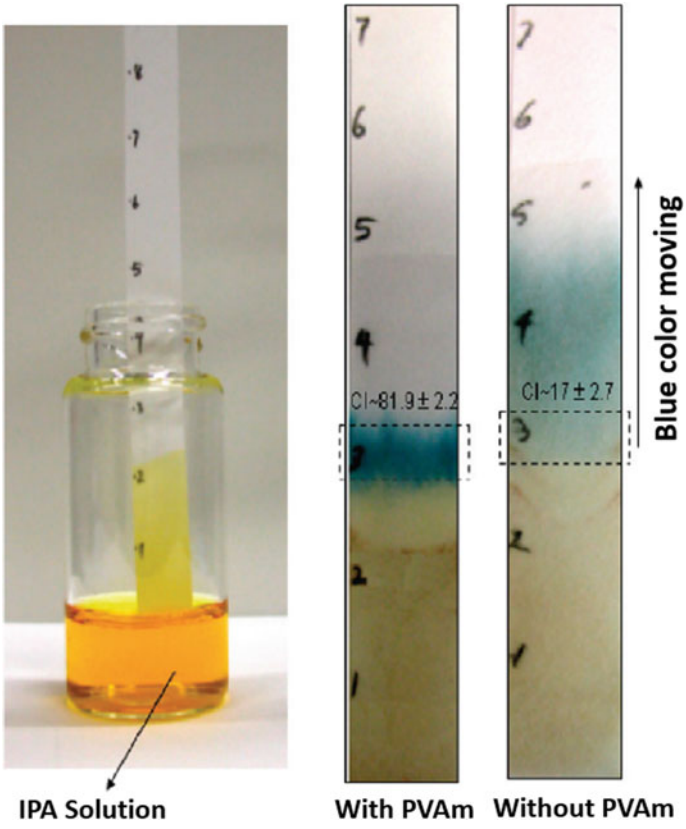

Fig. 10.7 Paper strip for the detection of pesticides from the food products in a lateral flow detection system (The dashed boxes show the coated regions). Reprinted with permission from Hossain et al. (2009). Copyright (2009) American Chemical Society, Analytical Chemistry

acetylcholinesterase by the contaminants present in the water sample. Also, the monitoring system was linked to the smartphone and centralized web portal to collect the data on large scale as shown in Fig. 10.8.

The steps involved in the detection are explained schematically in Fig. 10.9. The detection mechanism is based on the change of color of indoxyl acetate from

Fig. 10.8 Schematic of detection system by using cell phone and web portal. Reprinted with permission from Sicard et al. (2015). Copyright (2015) Elsevier, Water Research

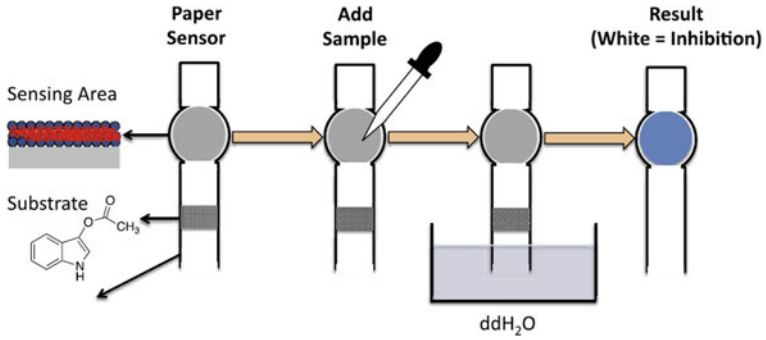

Fig. 10.9 Steps for pesticides sensing from water samples. Reprinted with permission from Sicard et al. (2015). Copyright (2015) Elsevier, Water Research

colorless to blue in absence of pesticides and white in presence of pesticides. The pesticides present in the water sample inhibit the acetylcholinesterase (AChE). The AChE was sandwiched between the silica gel layers over the treated paper substrate.

10.4.2 Paper Based Sensor for Air Quality Monitoring

Paper based sensors are also very useful in detection of harmful gases present in environment. Various toxic gases generated from industries, automobiles, and chemical industries etc. are released in the atmosphere (Chauhan and Bhattacharya 2018). These gases are harmful to human and animal life because of their toxic and explosive nature. Paper based sensors are now widely used for the rapid detection of these harmful gases which is helpful in monitoring, regulating, and alarming the nearby controlling and safeguarding authorities.

Wang et al. developed a metal-organic framework (MOF) with amino functionalization on a paper substrate and utilized it for the colorimetric and luminescence detection of toxic sulfur dioxide SO_2 gas (Wang et al. 2018b). SO_2 gas is widely used in making of pesticides, dyes, and pulp making. The long-term exposure to the SO_2 gas causes respiratory and nervous system damage for human beings. The MOF's are crystalline porous materials with π-conjugated structure which facilitates the luminescence property. Wang et al. used Zinc and benzenedicarboxylate (BDC) as metal and organic counterparts, respectively (Wang et al. 2018b).

In this work, amino group acts as an electron donating group and transfers electrons through the metal ions during the formation of MOF. The interaction with SO_2 gas hinders this electron transfer phenomenon and results into luminescence turn on effect. From Fig. 10.10, it is evident that the luminescence intensity of the MOF-NH_2 is maximum for SO_3^{2-} ions with respect to the other interfering ions present in the solution. Also, the luminescence response of MOF- NH_2 coated paper for different anions is shown in Fig. 10.10c. The similar paper based sensor coated with MOF- NH_2 was exposed to the different harmful gases and the maximum luminescence can be observed for SO_2 gas in Fig. 10.11.

Following a similar approach, Maity and Ghosh went one step ahead and developed a visual color changing paper based sensor for ammonia gas detection (Maity and Ghosh 2018). This method doesn't require any special instrument but visual analysis is sufficient to detect the presence of ammonia gas. In this study, the disposable paper was coated with perovskite halide $CH_3NH_3PbI_3$ (MAPI) which has black color. In presence of toxic ammonia gas the color change from black to yellow due to the conversion of MAPI into PbI_2. Figure 10.12 shows the change of color of the test paper from black to yellow after exposure of toxic NH_3 gas (30 ppm). The X-ray diffraction analysis (Fig. 10.12b) confirms the formation of PbI_2 from MAPI in presence of NH_3 gas. These studies indicate that paper based gas sensing devices are being explored and widely used for environmentally toxic and polluting gases.

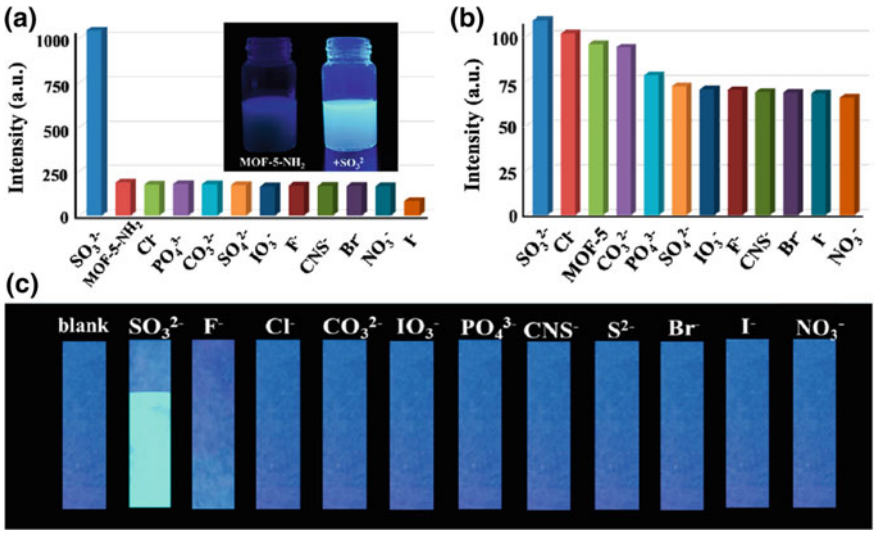

Fig. 10.10 **a** Variation of luminescent intensities of MOF-5-NH_2 and **b** MOF-5 with other anions. **c** Image of test strips in presence of different anions under UV light (365 nm). Reprinted with permission from Wang et al. (2018b). Copyright (2018) American Chemical Society, Analytical Chemistry

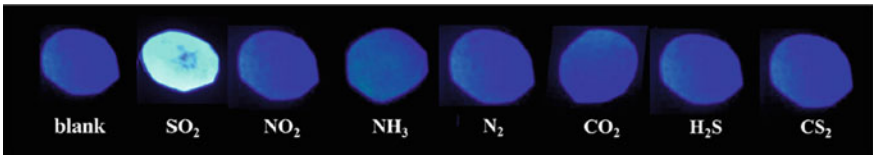

Fig. 10.11 Luminescent response of test strips in presence of different gases under UV light (365 nm). Reprinted with permission from Wang et al. (2018b). Copyright (2018) American Chemical Society, Analytical Chemistry

10.5 Challenges

Although paper based environmental sensors possess large number of advantages over the conventional detection methods but there are certain challenges which are required to be addressed for the future of paper based sensing devices. One such issue is the admission of the sample to the test paper. The sample to be tested is sometimes at a considerable distance from the testing region of the paper which alters the concentration to be analyzed. Also there are chances of evaporation of the sample. There is also the requirement of improving the immobilization efficiency of biomolecules, enzymes, and other nanomaterials over the porous paper substrate. The nanoparticles can be attached to the fibres during fabrication of paper. Also the treatment of the paper can be done with functionalization of different groups

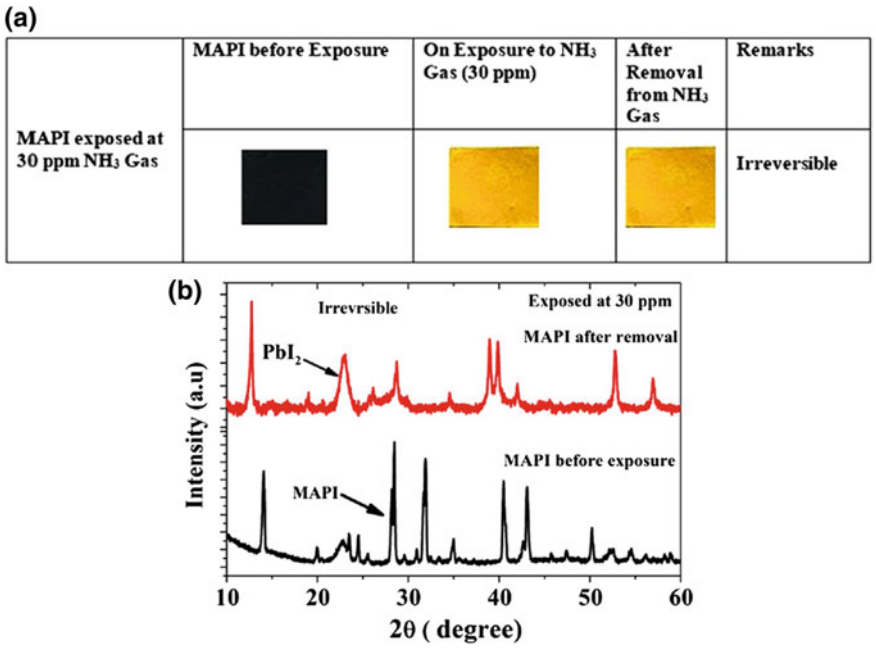

Fig. 10.12 **a** The colour response of test paper in presence of NH_3 gas (30 ppm) and **b** XRD analysis of the test paper before and after exposure of NH_3 gas. Reprinted with permission from Maity et al. (2018). Copyright (2018) Nature, Scientific reports

(carboxylic, hydroxyl, and amino etc.) to facilitate the attachment of nanoparticles and create hydrophobicity/hydrophilicity. There is also the need of different type of papers depending upon the application. The electrode printing for electronics require smooth hydrophobic type paper. The analysis of colorimetric detection method sometimes becomes subjective to the user. Now a day's smartphones are being used to minimize the issue of relative color change for the small variation of analyte properties.

10.6 Conclusion

In this book chapter paper based environmental sensing devices have been discussed. Paper based sensors have advantage of improved microfluidic activity due to high porosity, capillary action of cellulose fibres, flexibility of the paper, and easy functionalization/treatment of the paper. The paper based substrate provides easy, fast, and cheap point of care detection of environmental as well as bio-entities. The paper based sensors are also efficient in immobilization of enzymes, bio-molecules, and chemical compounds due to their high porosity and adsorption capability. In this chapter, the environmental aspect of the paper based sensors have

been studied. Paper based devices are widely used in water quality monitoring by the rapid and simple detection of pesticides, heavy metals, and other chemical pollutants present in the water samples. At the same time, paper based gas sensors are also trending for the effective and simple detection of polluting gases such as NH_3, SO_2 etc. The main advantage of paper based sensors working on colorimetric detection is the visual analysis without involving any sophisticated instruments. The paper based sensors are now being used in environmental as well as other field of detection technology.

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Chapter 11
Paper-Based Energy Storage Devices

Poonam Sundriyal and Shantanu Bhattacharya

Abstract The increasing focus on the environment-friendly electronics and requirement of easily disposable nonhazardous waste has introduced the cellulose paper as an attractive choice for energy storage devices. The disposal of commercially used energy storage devices is a major problem, and it has a great threat to a sustainable environment. Paper-based flexible and low-cost energy storage devices are recently gaining much research attention and are highly needed in various industrial applications. This field is undergoing exciting innovations and holds enormous opportunities for further developments. In this chapter, we review the current developments of the paper-based energy storage devices, their fabrication methods, design strategies, application areas, and challenges for their further growth. An industrial/technological roadmap is also provided to help the readers to understand the future directions in this field.

Keywords Paper · Energy storage devices · Flexible electronics · Supercapacitors · Batteries · Scalable manufacturing

11.1 Introduction

With the rising problem of electronic waste generation and adverse climate change, recently, much research focus has been paid to develop environment friendly and sustainable electronics. Out of the different components of an electronics device, the choice of a suitable substrate is an important criterion which directly affects the environmental compatibility of the electronics. In recent years, cellulose paper has emerged as an effective replacement for conventional rigid metal substrates for

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low-cost and green electronics. Paper based electronic devices are gaining much attention due to several advantages of paper such as; low-cost, easy availability, recyclability, biocompatibility, biodegradability, combustible nature, lightweight etc. Paper also provides high surface area and absorption properties which enable regents to be stored in it. Furthermore, the wicking capability of the paper makes it an attractive choice for different microfluidic applications. Paper based devices are especially suitable for the flexible electronics which holds enormous potential for future electronics with flexible and wearable capabilities. Till date, a variety of paper based flexible electronic devices (like supercapacitors, batteries, transistors, generators, sensors, radio frequency identification tags, solar cell arrays, electronic displays, health monitoring devices, etc.) have been widely studied by researchers and some of them are well commercialized in the market.

Among all the electronic devices, the energy storage unit (mainly supercapacitors and batteries) is an important part of all electronics. Although some of the flexible electronics are now commercialized, the slow developments of flexible energy storage devices are still limiting the further development and wide adoptability of these electronics. In order to full utilization of the flexible electronic devices, further efforts are required to improve the flexibility and performance of the energy storage devices. Paper, which is an ancient flexible substrate since last 2000 years is a smart replacement for the existing flexible substrates. Paper-based energy storage devices are suitable choice for flexible electronic devices due to their high flexibility, easy fabrication and ease of integration with other electronic parts irrespective of their shape and sizes. The rough and porous surface of the paper provides fast and easy ion transport pathways across the entire structure and therefore improves power performance of an energy storage device (Hu and Cui 2012). Facilitated by the power of 'printed electronics', the paper based energy storage devices have huge potential for the thin and scalable devices ranging from small printed letter size to the large area scale requirements.

To date, various paper based energy storage devices have been reported for different applications, such as; paper based supercapacitors for self-powered nanosystems (Yuan et al. 2012), paper-based battery for microfluidic applications (Hamedi et al. 2016), paper-based supercapacitor for portable electronics (Guo et al. 2017; Sundriyal and Bhattacharya 2017, 2019), urine-based paper battery for healthcare sensor (Liu and Crooks 2012), etc. As the existing paper based energy storage devices needs much improvements to fulfill the flexibility and performance criterion of the present and next-generation flexible electronic devices, a lot of research focus is required to improve the existing devices for real life applications. This chapter will cover the recent advances in the synthesis, device design, fabrication methods and application areas of the paper based energy storage devices. The challenges for the practical application of these devices and the future perspective of this exiting field will be discussed in detail.

11.2 Fabrication Methods

The suitability of a paper based energy storage device directly depends on the fabrication method. Paper based electronic devices emerged due to the low-cost, availability and environment friendliness of the paper substrate. Therefore, the fabrication method should also be chosen in a way that can provide high performance of the device without adding much cost and chemical waste to the surrounding. The simple and low-cost manufacturing methods should be used for the device fabrication of any paper based electronics. The vacuum evaporation has been used as the first method for making paper-based electronic device in the late 1960s. However at present; it is not a suitable method due to its high cost, complexity and problems with the large scale manufacturing. In the recent years, the solution based methods such as inkjet printing and screen printing have gained much popularity as the smart manufacturing methods for the fabrication of the paper based electronic devices. The other methods such as; pencil drawing, vacuum filtration, chemical and physical deposition, and dip coating have also utilized for developing the paper based energy storage devices. The details of the existing fabrication methods are as following.

11.2.1 Printing

Printing on the paper is a simple, rapid and a highly scalable method to fabricate the paper based energy storage devices. Paper is a compatible choice for all printing methods and is widely used to print the new-papers and various office letters. The use of suitable functional materials inks in the printing machines instead of the commercial inks can develop the printed electronic devices with the required shapes and sizes without much effort. The fabrication of the printed paper-based energy storage devices have realized the development of devices ranging from small printed letters to the large area printing. A major challenge for the development of the paper based electronics is the extremely rough surface of the paper. However, the uneven and rough surface of the paper with enormous pores is advantageous for the energy storage devices as it facilitates the easy ion transport and provides high rate performance of the device. The printing route is a smart method which has recently emerged as a potential method for the development of the thin film and flexible electronics. Despite its high suitability for the paper electronics, the preparation of the required ink is a great challenge which hinders its wide adoption. Preparation of the printable ink with the required rheological properties is a critical and difficult task which governs the print quality and the performance of the developed device. The rheological properties such as viscosity and surface tension of the inks must be optimized to get the required inks.

The widely used printing methods are inkjet printing, screen printing, gravure printing and meyer rod coating. The inkjet printing and meyer rod coating are scalable methods which can be well utilized for the next generation of the large area electronics. Specially, the inkjet printing method is currently extending from the traditional office printing to the development of the high performance paper based supercapacitors/batteries. A lot of research focus has been devoted for the development of the printable inks from the energy storage materials. These materials include conducting materials (graphene, carbon nanotubes, silver nanoparticles, gold nanoparticles etc.), energy storage materials (MnO_2, Bi_2O_3, NiO, Activated Carbon, MXene, PANI, polypyrrole (PPy), and polythiophene etc.), and electrolytes (ionic gel electrolyte and PVA-KOH gel electrolyte). The paper substrate is generally converted to the energy storage device by coating/printing with a conducting material to develop a conducting matrix followed by the deposition of the positive/negative electrode materials and applying electrolytes over it. Figure 11.1 shows the schematic of the fabrication procedure of the paper-based energy storage device using inkjet printing method. While constructing a printed paper-based electronic device the wettability of the paper substrate should be properly optimized to get the proper patterns. Recently Choi et al. (2016) have suggested to add a primer layer over the paper substrate which can effectively reduce the ink spreading and maintains proper wettability of the paper based substrates for printed applications (Fig. 11.2). Other than the substrate wettability, the post-treatment temperature should be carefully selected which will not adversely affect the print quality or strength of the paper substrate. Recently, some other methods such as ultra violet curing have been reported for the proper post-treatment of the printed paper based supercapacitors (Choi et al. 2016).

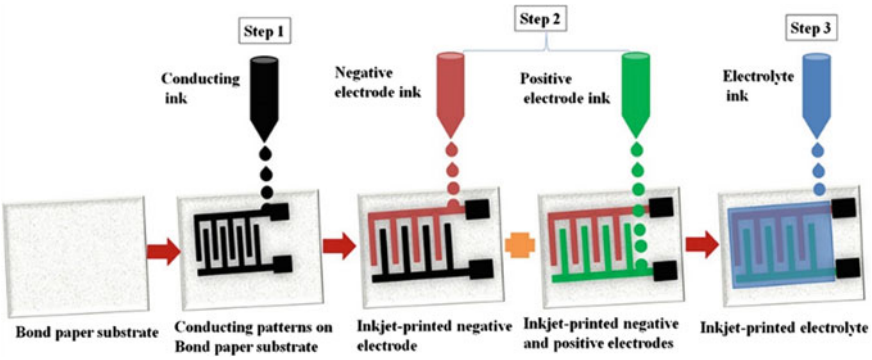

Fig. 11.1 Schematic illustration of the stepwise fabrication of the paper-based energy storage device by inkjet printing method (Sundriyal and Bhattacharya 2019)

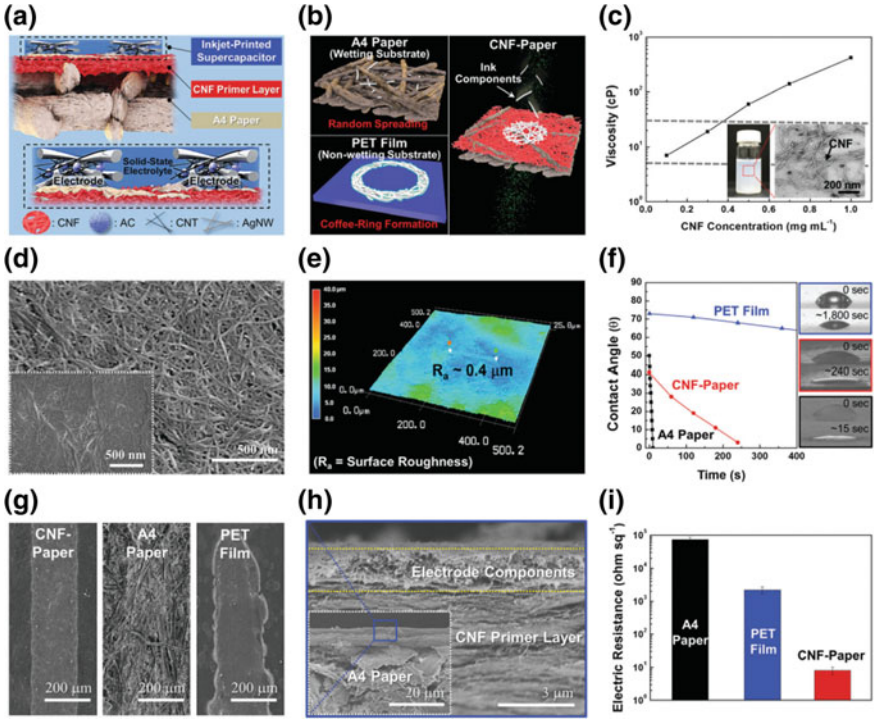

Fig. 11.2 Deposition of cellulose nanofiber (CNF) layer on paper substrate: **a** schematic illustration of the device structure and components with printed layers, **b** effect of substrate on the print quality, **c** viscosity versus CNF concentration graph; FESEM image of the **d** CNF printed paper, **g** print quality on different substrates, **h** cross-sectional SEM image of the printed layers; **e** surface profilometry of the CNF printed paper, **f** time-dependent contact angles of different substrates, and **i** electrical resistance of the printed electrodes on different substrates. Reprinted with permission from Choi et al. (2016), © Royal Society of Chemistry 2016

11.2.2 Pencil Drawing

Pencil is a commonly used writing tool which is composed of graphite and SiO_2. The conducting tracks comprising of graphite flakes can be easily drawn on the paper substrates without much effort. The various scales of the pencil leads ranging from 9B to 9H indicate hardness or the relative content of graphite on them. The required conductivity can be obtained by selecting the suitable pencil lead or by multiple writing of the same pattern on the paper substrate. The papers with a surface roughness of 1–5 mm are most suitable for making electronic devices with pencil drawing. Yu et al. have developed high performance supercapacitors with the help of 4B and HB pencil drawings (Yu and Zhang 2017). They have applied multi walled carbon nanotubes (MWCNT) along with the graphite flakes of the pencils, which have formed the strong π-π bonds between these two allotropes of the carbon

(Fig. 11.3a, b). They have observed that the graphite structure in the HB pencil was compact and ordered while the structure was loose and disordered (Fig. 11.3c, f). There was no adverse effect of MWCNT on the surface wettability of the drawn patterns on the paper substrate (Fig. 11.3g, h). The developed paper based device exhibited extraordinary power performance with a power density of 15.1 W cm^{-3} and stable device performance up to a scan rate of 150 V s^{-1}. Some other studies are also available for the fabrication of paper based supercapacitor and battery device using this method (Wang and Zhou 2011; Yu and Zhang 2017; Zheng et al. 2011). Regardless of the simplicity of this method, it is not much effective due to its inability to make the large area electronics and for developing the non-carbon based energy storage devices. Most of the high performance energy storage devices require transition metal oxides or their combinations with the carbon materials. This method has no provision to choose the energy storage materials based on the user convenience.

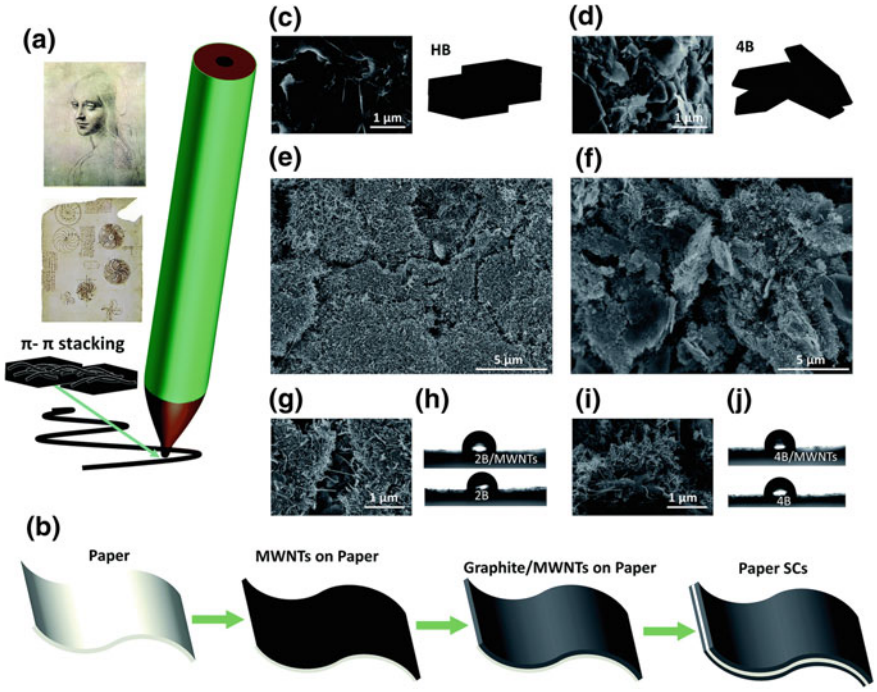

Fig. 11.3 **a** Graphite-MWCNT drawing on paper using a pencil, **b** schematic illustration of the fabrication steps paper SCs, **c**, **d** FESEM images of the HB and 4B graphite, **e**, **f** FESEM image of the graphite-MWCNT structures, and **g**, **j** contact angle variations in different graphite-MWCNT structures (Yu and Zhang 2017)

11.2.3 Chemical and Physical Deposition

The chemical and physical deposition methods are the most commonly used techniques to from a thin layer of the metals over the paper substrates. Although the most efficient deposition methods such as chemical vapor deposition and the sputtering are very complex and expensive, they are popular due to their reliability, precision and ability to form the very thin and highly crystalline metallic layers. Zhang et al. have developed a filter paper based supercapacitor device using electroplating and electrodeposition methods (Zhang et al. 2015). Figure 11.4 shows the schematic illustration that how a filter paper can be converted to a Ni/MnO_2 based electrode which can serve as an efficient positive electrode for the supercapacitor device. Much literature is not available for the fabrication of paper-based energy storage devices (Kim et al. 2010; Zhang et al. 2015; Wang et al. 2013). The most common drawback of these methods is their complexity, sensitive instrumentation and high-cost, which suppress their use for the low-cost electronics.

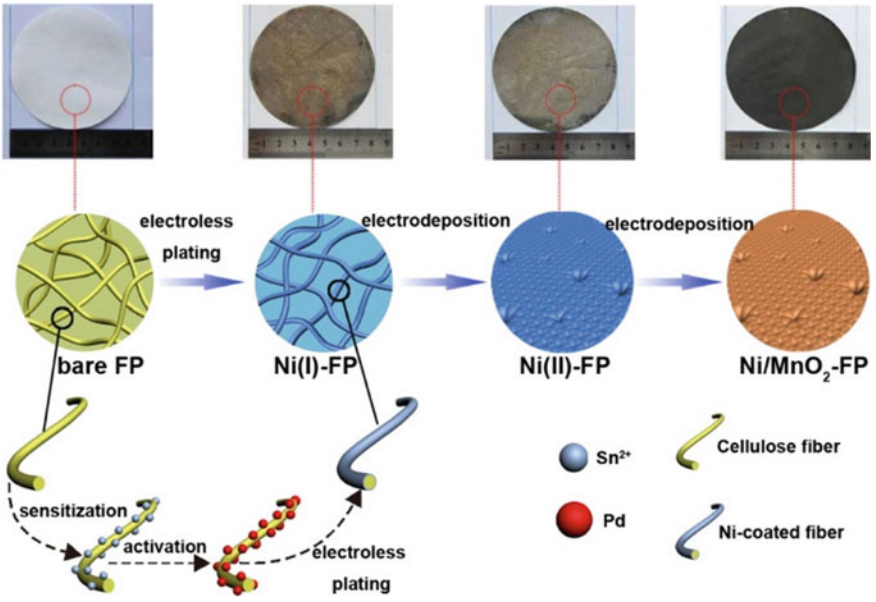

Fig. 11.4 Schematic illustration for converting filter paper (FP) to the high performance electrode for supercapacitor application using electroless plating and electrodeposition technique (Zhang et al. 2015)

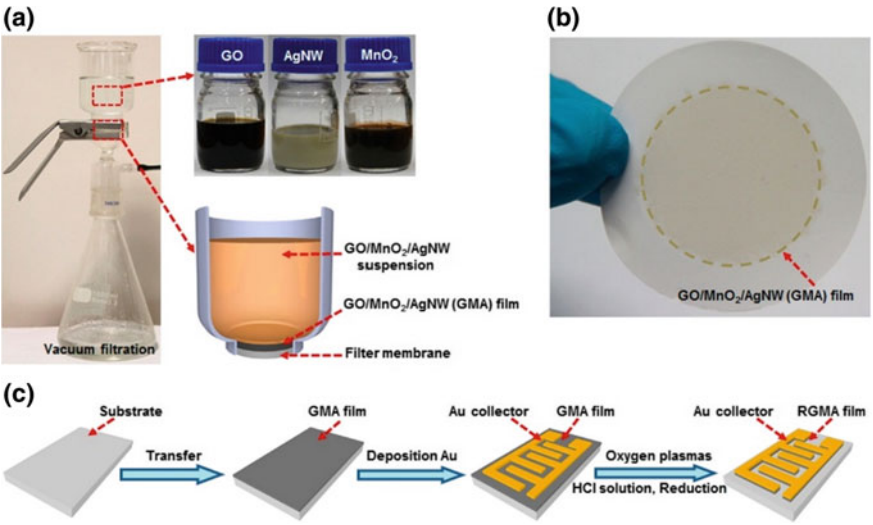

Fig. 11.5 **a** Fabrication of GO/MnO_2/Ag electrode on paper substrate using vacuum filtration method, **b** photograph of the GO/MnO_2/Ag paper electrode, and **c** schematic illustration of the fabrication for micro-supercapacitor (Liu et al. 2015)

11.2.4 Vacuum Filtration and Dip Coating

Vacuum filtration and dip coating are solution based methods which can easily and rapidly fabricate the paper based supercapacitors and batteries. A lot of studies reports the manufacturing of paper based electronics using these methods. Recently Liu et al. (2015) have developed paper-supported high performance electrodes for micro-supercapacitors using vacuum filtration method (Fig. 11.5). The fabricated devices exhibited extraordinary high stability of the device with an excellent rate capability up to a scan rate of 50,000 mV/s and good cycle life with 90.3% capacitance retention after 6000 cycles.

11.3 Conclusion

The paper based energy-storage devices have a great potential for the flexible electronics area. For development of a paper-based energy storage device, selection of a proper manufacturing method is essential. Despite several lab-scale demonstration for paper based supercapacitor/batteries, their commercialization is limited mainly due to their poor cycle life performance and low mechanical strength. Therefore some strong and durable matrix such as; fibers, graphene or carbon nanotubes should be added in the paper pulp while processing the paper substrates. The rapid degradation of paper in the wetting environment is the other problem that

must be addressed for actual realization of the paper based electronic devices. The paper-based device should be encapsulated in an environment friendly casing to avoid any moisture content in it. In conclusion, paper is a smart choice for the low-cost flexible electronics devices which is promising for a green and sustainable electronics. However, further focus is required to improve the existing paper based energy storage devices to make them industry/consumer ready electronics.

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Chapter 12
Paper-Based Devices for Wearable Diagnostic Applications

Mohit Pandey, Krutika Shahare, Mahima Srivastava and Shantanu Bhattacharya

Abstract Paper-based devices has gained widespread applications with its unique properties like affordability, rapid detection, user-friendliness, sensitivity, specificity etc. These low costs devices are considered as powerful tool for point of care testing because of the inherent microfluidic nature, disposability, flexibility, and ability to store and analyze the sample. With the advancement in fabrication techniques and the requirement of real-time diagnosis and health monitoring, paper-based devices also emerged in the field of wearable diagnostic applications. Depending upon the biocompatibility, paper-based devices are integrated with different materials to be used as in vitro and in vivo wearable devices. In this chapter, we will cover the principles of the paper-based devices along with the evolution of the wearable devices and also briefly review the recent development in the wearable diagnostic devices.

12.1 Introduction

Despite the constantly occurring global urbanization, more than 55% of the population still lives in rural areas. These inhabitants live in a resource-limited and technology-deprived developing world with lack of human resources. In such arenas, it becomes quite significant for advances like wearable diagnostics to improve the current healthcare situation and environmental safety. Various techniques are

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currently used for the detection of biological markers in the body fluids like urine, blood and serum which yield consistent and reliable results. Hence, there is an unsatisfied need for the development of easy-to-use, reliable and inexpensive detection systems to evaluate results in a resource-limited environment of the developing world. Rapid diagnostics include various realms like environmental monitoring (e.g. detection of contamination levels in water), immunoassays (e.g. detection of infectious diseases, respiratory problems, etc.) (Kumar et al. 2018), urinalysis (pregnancy detection, glucose level detection, etc.) (Kumar et al. 2016, 2017), food safety (e.g. detection of food allergens, drug residues etc.) and bioterrorism (e.g. plague, orthopox, etc.) (Yetisen et al. 2013).

These diagnostic tests are less robust than their laboratory bench counterparts but provide simple yes/no type answers when the response time is critical to the user. The simple microfluidic paper-based technology uses hydrophilic paper as the substrate to test a biological substance like blood or urine, or an environmental reagent for the hydrophobic analyte of interest by creating microfluidic channels (Warren et al. 2014; Grau 2017). The reagent containing the required analyte reaches the detection zone by capillary action and is detected owing to chemical reactions which change the color or electrochemical properties. Calorimetric changes, being the most frequently used method for detection depends on color changes formed from ligand-analyte binding. This chapter describes the various detection techniques, fabrication methods, applications and challenges of paper-based devices for wearable diagnostic applications.

12.2 Fabrication Techniques of Microfluidic Paper-Based Analytical Devices

12.2.1 Photolithography

A photoresist (light sensitive liquid to make a film) is used to fabricate the paper-based device. The exposure system may include a UV lamp (Martinez et al. 2008). This is used to transfer geometric patterns from the photomask to the photoresist. Light is permitted on the parts of the photoresist where the photomask is transparent to light. Chemical reactions occur when the photoresist is exposed to light. It may become either soft or hard depending on the nature of the photoresist i.e. positive or negative. The photoresist is then put into a developing solution and then etched. Although photolithography is a widely used technique for fabrication, it requires access to clean rooms (Kumar and Whitesides 1993) and is relatively an expensive technique.

The general procedure for photolithography is mentioned below:

Surface coating: This includes coating the wafer with a chemical (HDMS or Hexamethyldisalizane) that enhances adhesion of it to the photoresist. It provides a clean surface for the photoresist to stick onto the wafer. The wafer is baked to

remove any water molecules present after which HDMS is applied to create a hydrophobic surface. The wafer is then cooled at room temperature.

Spin coating: The photoresist is spin-coated onto the wafer where the thickness of the photoresist is largely dependent on the viscosity of the resist and the spin velocity.

Softbake: Post the application stage until the desired thickness, the wafer and the photoresist are soft-baked to remove any residual solvents from the resist after which they are cooled to room temperature.

Mask alignment: Alignment of 1 micron can hugely affect the quality of the resultant wafer. Hence, this is a crucial step where each layer must be aligned within specifications to the previous layers and the subsequent layers.

Exposure: The resist is exposed to light. Chemical reactions occur on the parts exposed to light and the photoresist may become either more or less acidic. The resist that becomes more acidic on exposure are called positive photoresists and the ones that become less acidic are called negative photoresists.

Development: A developer solution is used to dissolve parts of the photoresist. In a positive photoresist, the parts of the photoresist exposed are dissolved while the unexposed parts stay on the wafer. In case of negative photoresists, the unexposed photoresist parts are dissolved and the exposed parts stay on the wafer.

Hard-baking: The temperature of hard-bake is higher than soft bake. This process hardens the photoresist for the subsequent process. Post this stage, the photoresist is cooled to room temperature.

Post process cleaning and Inspection: This process involves cleaning of the resultant wafer and looking for alignment mismatches and defects.

Figure 12.1 shows the procedure for FLASH fabrication in paper using photolithography.

12.2.2 Inkjet Printing

This method is utilized for device fabrication for detection of proteins, pH and glucose simultaneously (Abe et al. 2008). This technique is advantageous for large scale production of paper-based sensors as polymers used for photoresists are expensive for large scale fabrication (Li et al. 2010). This process uses polystyrene and other organic solvents as consumables and works on the simple principle of propelling drops of it on filter paper substrate. Some of the advantages of inkjet printing include higher efficiency, reduced cross-contamination, rapid fabrication and high resolution. On the other hand, this process requires a customized expensive bio-ink printer and an extra heating step for curing purposes. Figure 12.2 represents the procedure of inkjet printing followed by Abe K. and team.

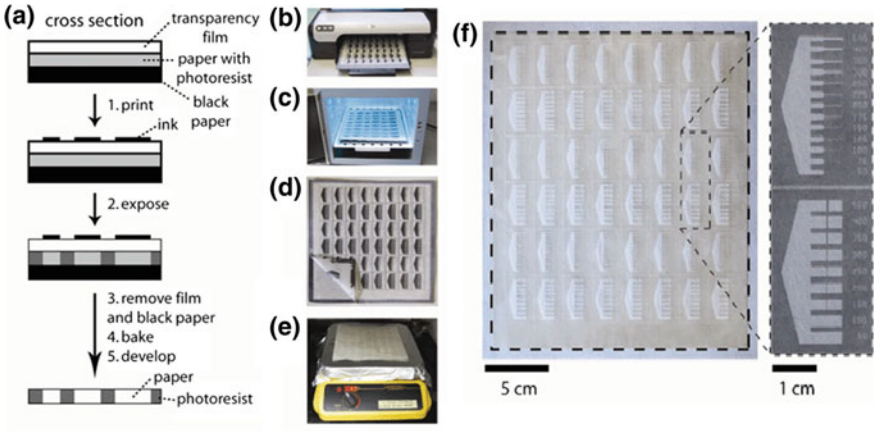

Fig. 12.1 Procedure for the FLASH fabrication of devices in paper. **a** Schematic of the method used. **b** Printing of the design of microfluidic channels. **c** Exposure to UV light. **d** Removal of photoresist-impregnated paper from black construction paper and transparency film. **e** Baking of paper on hotplate. **f** Microfluidic devices ready to use after been developed in acetone and isopropyl alcohol solution. Reproduced from Martinez et al. (2008), with permission from Royal Society of Chemistry

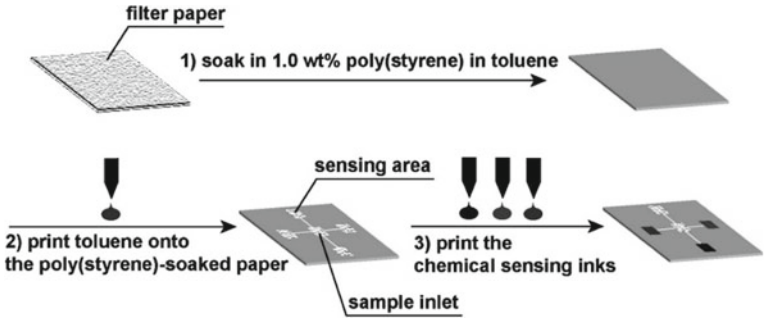

Fig. 12.2 Fabrication method of inkjet-printed microfluidic device for multi-analyte sensing. Central sample inlet area is connected to different sensing area and reference area with microfluidic channels. Step 2 indicates patterning and 3 indicates printing of chemical reagents for sensing. (The pen symbol indicates the use of inkjet printer). Reproduced from Abe et al. (2008), with permission from Royal Society of Chemistry

12.2.3 Laser Cutting

Laser cutting makes use of CO_2 to cut or make patterns on the paper and double sided adhesive (DSA) to fabricate the device (Chitnis et al. 2011). The hydrophilic patterns are then remodeled to have hydrophobic coating. Recently, laser cutting has had its applications in the detection of tuberculosis due to its simple manufacturing

method which is relatively inexpensive, yet robust (Mani et al. 2016). However, one disadvantage of laser cutting is that it requires specialized and customized equipment such as a laser engraver, a 2D graphics software and double sided adhesive for assembly purposes. Laser cutting works on the detection method of chemiluminescence where the recognizing element is the catalytic action of iron in hemoglobin.

12.2.4 Wax Printing

This process uses wax either from a pen or a printer to create microfluidic channeling patterns on the paper to control the flow of the fluid consisting of the analytes and the reagent (Lu et al. 2009). This method has several advantages including lower cost non-toxicity and simplicity as compared to other techniques. It uses filter and chromatography papers as substrates and electrochemical or colorimetric detection techniques to detect analytes like iron and glucose. Drawbacks of this method include the low resolution and the technique being unstable at higher temperatures.

12.2.5 Polydimethyl-Siloxane (PDMS) Printing

A solution of PDMS is poured over a stencil to form patterns on paper. Due to it being hydrophobic, it increases the flexibility of the sensors. Hence, the sensor can be bended without rupturing the microfluidic channels. The advantages of this method over photolithography are numerous including lower cost increased flexibility, no requirement of UV exposure and no post printing steps (Bruzewicz et al. 2008). However, the resolution obtained by this method is less and sophisticated equipment is required for fabrication of molds. This technique uses colorimetric detection method to detect analytes like proteins, glucose and pH. Figure 12.3 shows the PDMS printing technique used by Bruzewicz D. A. et al. Some of the other fabrication techniques include hot embossing, wax dipping, wax screen printing, hydrophobic silanization, origami and many more.

12.3 Detection Techniques

12.3.1 Colorimetric Detection

Colorimetric detection has its roots in as early as the 1st century. Plinius Secundus, a philosopher and Roman Empire commander used gallnut to detect the presence of iron in hydrated copper acetate. Certain analytes contain a group of atoms/molecules which absorb certain wavelengths of light. Such groups of atoms/

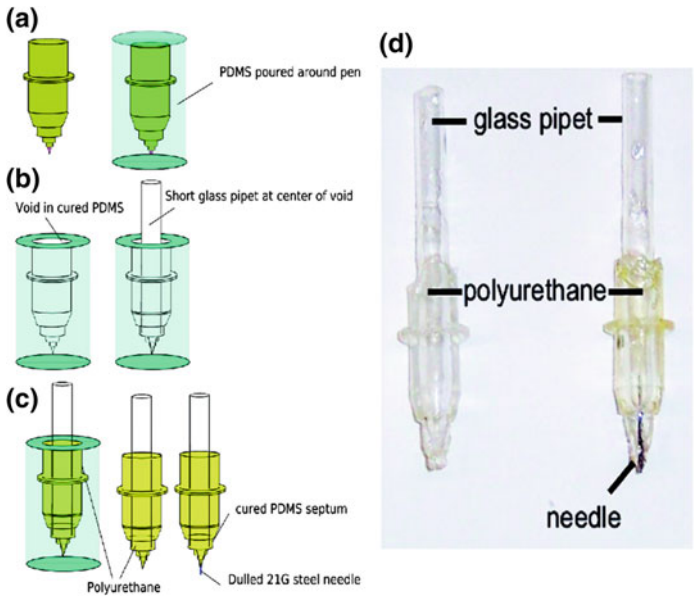

Fig. 12.3 Schematic illustration of the fabrication process to produce alternative pens for the plotter. **a** Left side have the original pen while right side have pen embedded in PDMS. **b** Cutting the original pen from the PDMS and thus creating a void which can hold a glass pipet. **c** Polyurethane is used to fill the void and curing under UV light. Piercing the PDMS septum with a blunt 20G needle forms the tip of the pen (D) Photograph of a replica-molded plotter pen. Reproduced from Bruzewicz et al. (2008), with permission from Royal Society of Chemistry

molecules are called chromophores and it is due to this absorption of certain wavelengths of light, that the substances are perceived to possess color. Certain wavelengths are absorbed in an atom because of a certain kind of electron density distribution of that atom. The principle of colorimetric detection depends on making these target atoms react with chemical reagents to alter the electron density distribution for the analyte to be detected using light.

Colorimetric detection technique is quite useful in microfluidic paper-based analytic detection because it gives quick yes/no type of results with decent reliability and good efficiency (Liana et al. 2012). The technique includes chemical reactions between chemical reagents (acid-base indicators, dyes or enzymes) and target substances. Sometimes, due to the presence of interferents, the there is a possibility of getting a 'false positive'. These interferents react with the sensor to give an appearance that the actual analyte is present. Therefore, careful study of the presence of interferents need to be done before using a certain kind of sensor. Another drawback of this technique includes the kind of paper used for sensing (Evans et al. 2014). It is experimentally found that thicker sensor papers have higher resistance for the fluid flow and hence give poor color readings. The intensity hence, depends upon the thickness of the paper and the volume of the reagent used.

The results of colorimetric detection can be either done manually for instance litmus test detection for acids and bases or done using software. The latter method is preferred owing to reliability and lack of human error. The method, in totality, is widely celebrated as it is inexpensive, easy, efficient and disposable.

12.3.2 Electrochemical Detection

Electrochemical techniques are largely employed for non UV-absorbing analytes. These analytes do not respond to colorimetric detection due to the absence of chromophores hence, electrochemical techniques need to be used. Advantages of electrochemical detection include cost, portability, high selectivity, low electrical power consumption, and minimal instrumentation. An added advantage of this method is that the sensitivity of the method does not depend on the size of the instrument. Electrochemical sensors use three electrodes: working electrode, counter electrode and reference electrode. When current is passed through these electrodes, chemical reactions occur that enable the simultaneous detection of various analytes. The sensors are made by depositing conductive ink on a paper matrix. In the detection unit, the analyte is detected on the working electrode. The counter electrode is used to collect the current flowing through the cell and control the current passing through the reference electrode. The reference electrode on the other hand, is situated away from the reaction place and is responsible for maintaining a constant potential. This technique is attractive to the microfluidic paper based analytical device industry as it does not require a light source and is a label free method.

12.3.3 Chemiluminescence Detection

Chemiluminescence is the emission of light by chemical reactions. Certain compounds attain higher energy states as a result of chemical reactions. That is, the electron jumps to a higher energy state. After a while, the electron jumps back to the lower states, thus, emitting light of certain wavelength and an energy corresponding to the energy difference of the two energy states. Hence, light of a certain color is emitted during the transition of the electron to the ground state.

In the process, chemical energy of the electrons is converted into light. For instance, luminol reacts with hydrogen peroxide to produce light. A detection device was manufactured for an enzymatic reaction where uric acid produced hydrogen peroxide during decomposition of the substrate. Hydrogen peroxide, on the other hand, reacted with rhodanine derivative to produce chemiluminescence.

This technique is one of the simplest techniques because it does not require heavy instrumentation methods. It is efficient, reliable, precise and the most sensitive technique. One drawback of this method is the requirement of high concentration of the enzyme which may run the risk of clogging up the paper.

12.3.4 Fluorescence

Fluorescence phenomenon includes an atom absorbing a photon of certain wavelength and emitting a photon of a longer wavelength. The most common example of fluorescence is the household tube light. Electric current causes the mercury atoms to collide which raises its energy. The excited mercury atoms then emit ultraviolet light. Fluorescence detection technique involves fluorescence occurring due to the interaction between target molecules and the fluorescent dyes called fluorophores. There exists a light source which causes the excitation. The source of light is of a certain wavelength and induces fluorescence in the fluorophore. The excited phase has a lifetime of its own after which fluorescence occurs. The emitted photos are separated from the exciting photons and the emitting photons are finally detected. To quantify the luminescence, a fluorimeter device is commonly used. The fluorescent dyes used are bio-compatible but must be checked regularly for photo bleaching which would in turn, reduce the efficiency and reliability of the process. The future and the feasibility of this process will be heavily dependent on the cost reduction and miniaturization of the fluorescent devices.

12.3.5 Electrochemiluminescence

This technique is the mixture of chemiluminescence and electrochemical techniques. The sensing mechanism depends upon the luminescence generated by electrochemical reactions taking place. This technique has the advantages of both chemiluminescence and electrochemical detection techniques. Due to reactions occurring, the molecules attain an electronically higher energy states. Light is emitted when the electron returns to the ground state. A photodetector reads the emitted electron in a dark room. The applications of electrochemiluminescence includes the detection of tumor markers, analytes and ions. This technique possesses advantages like better sensitivity, smaller sample volumes and simple instrumentation.

Some other detection techniques used in microfluidic paper-based analytical device include detection using gold nanoparticles, hybrid sensors and ELISA based immunoassays.

12.4 Applications

Owing to the ease of detection of yes/no type of results, the simple instrumentation, the time of response and robustness of the process, paper based device finds their application in variety of wearable diagnostic applications. Sweat and blood serum are commonly used for the detection of many biological parameters like blood sugar level, Cl ion concentration, etc. These parameters are needed to be monitored frequently to detect any abnormalities as they are responsible for many biological activities like maintenance of osmotic pressure, muscular activity, etc. Recently S. Cinti reported the detection of chloride ion in blood serum as well as in sweat and compared it with gold standard method i.e. ion selective electrode which is adopted in the routine clinical analyses. The design of the electrode is printed first with wax printing to make the selective hydrophobic zone and screen printing is used to print the conductive ink containing silver. The testing area is then loaded with the required chemical and blood serum or sweat sample is used for the electrochemical detection of Cl ion. The response time of the sensor is 60 s in which the three electrode assembly becomes homogeneously wet and the shelf life of the device is 1 month. This reported device is very robust and can sustain bending, twisting, shows very satisfactory linear relation with the clinical analyses data and thus, can be used as the wearable device for Cl ion monitoring (Cinti et al. 2018). Figure 12.4 shows the robustness test of the paper based device.

Similarly, Castro L. F. and team demonstrate the implementation of paper based microfluidic wearable sensor for the salivary monitoring of glucose and nitrite. The μPAD is designed on silhouette studio software having one sampling zone and two detection zone and cut using craft cutter printer. For its integration with 3D printed silicone mouth guard, sampling zone is replaced by short microfluidic channel. Colorimetric detection technique is used in the μPAD, in which glucose is detected through glucose oxidase and horseradish peroxidase, while nitrile is detected through griess reaction. Chitosan solution is used to enhance the detection of the glucose. The volume of the saliva sample is optimized to prevent the coffee ring effect on the μPAD. A liner regression plot is plotted with concentration and color intensity. Also, exponential decay is observed with time when wearable sensor is subjected to monitor the glucose concentration after ingestion of chocolate (de Castro et al. 2019). Figure 12.5 shows the integration of the paper based device with mouth guard holder.

Blood being the most important biological fluid is diagnosed for various diseases, infections and to monitor functions and activity of various organs. Vella and team demonstrated the use of paper based microfluidic device for the detection of two markers which is responsible for the function of liver. They designed a vertical flow device in which blood sample is used to detect alkaline phosphate, aspartate aminotransferase (both liver function markers) and total serum protein. Wax based printing is used to create the selective hydrophobic vertical zones. This configuration gives them benefit of smaller size requirement, rapid assay development, light weight device and easy construction (Vella et al. 2012). Due to complex

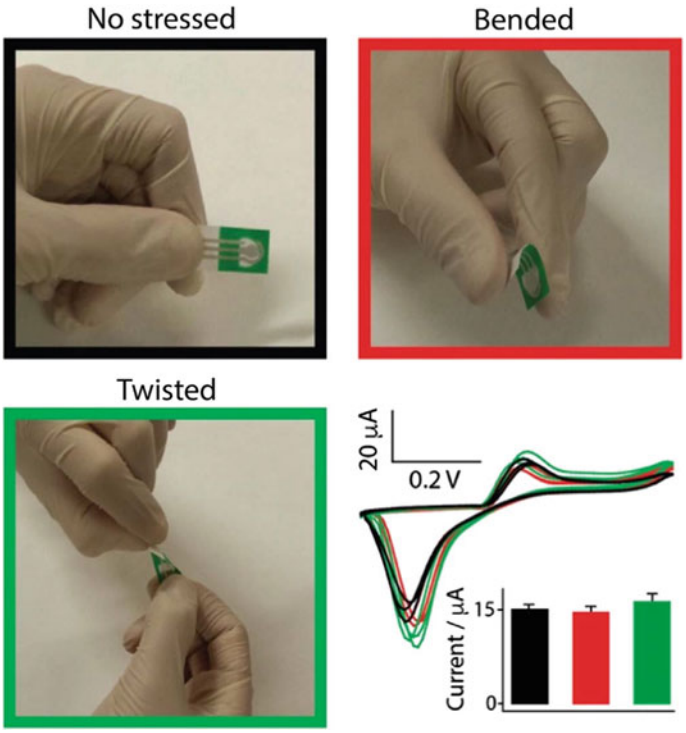

Fig. 12.4 Robustness of the paper-based device after 100 repeated bending test (red) and twisting test (green) and compared the cyclic voltammetry response without mechanical stress test. Reproduced from Cinti et al. (2018), with permission from Elsevier

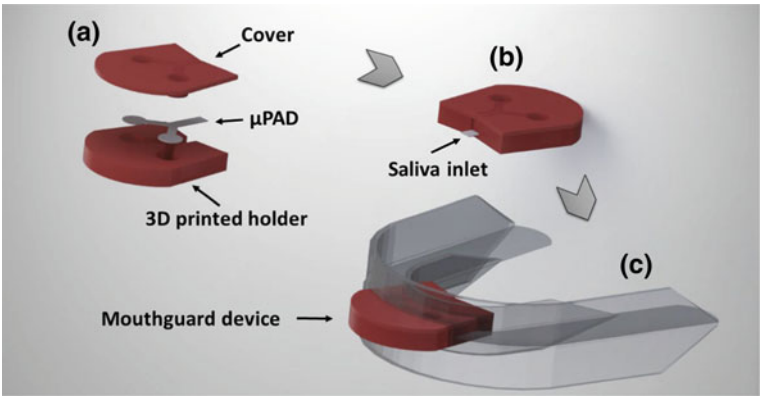

Fig. 12.5 Integration of the µPAD with the 3D printed mouth guard holder for wearable paper-based device. Reproduced from de Castro et al. (2019), with permission from Springer

composition of the blood, many diagnostics require plasma separation from the blood. This problem is solved by Xiaoxi Yang and team by fabricating the paper based device for the separation of plasma from the blood. They agglutinated the red blood cells and separate them on the device for plasma analysis by wax printing for ensuring selective hydrophobic area. The blood sample was collected at the central zone and plasma is separated on the perimeter to react with present reagent to show the detection of analyte (Yang et al. 2012). Another Colorimetric detection is reported by Chin-Chung Tseng and team, where they detected creatinine concentration from raw blood to ensure the proper functioning of the kidney. They used Jaffe reaction and integrated it with paper based microfluidic technology. They analyzed color output with in house fabricated detection box having camera and light source. This prevents environmental conditions to affect the color analysis (Tseng et al. 2018). Apart from blood, urine is also being used as the sample for the diagnostics and monitoring with paper based microfluidic devices. E. L. Rossini and team detected two important biomarkers; creatinine and uric acid in urine. They detected these biomarkers for the renal function with colorimetric detection technique; creatinine by Jaffe reaction and uric acid by reduction of Fe^{3+} to Fe^{2+}. The sampling zone is divided into three zones for the detection. The analysis shows a good correlation with the clinical chromatographic data and thus, can be used for the wearable and monitoring applications (Rossini et al. 2018).

Colorimetric test sometime becomes a little difficult for real time monitoring thus, integration of electrochemical sensors with the paper based diagnostic device have attracted huge attention over the recent development. Such recent attempt has been reported by Qingpeng Cao and team where they fabricated a 3D paper based device integrated with electrochemical sensor for real time monitoring of sweat metabolites. This 3D wearable device is fabricated by wax screen printing patterns on paper and folding it to form the five stacked layers. These different layers have patterns for sample collector, vertical channel, electrode assembly and sample sink. Three electrode system is printed with graphite ink and Ag/AgCl and in experiment, 0.01 M PBS solution is used for in vitro amperometric measurements. The sensor is used to detect and monitor the glucose concentration from the sweat sample with as low detection limit as 5 mM due to low glucose content in sweat (Cao et al. 2019). Figure 12.6 represents different layers fabricated in the device and the working of the device.

Similarly, Sadri et al. fabricated a low cost epidermal paper based electronic device (EPEDs) by the combination of highly conductive nanoparticles, spray deposition of silanizing agents, encapsulating polymers, and laser micromachining. This EPED is used as electrophysiological sensor for recording electrocardiogram signals, electromyograms signal and is showed working underwater also. One side of the paper is silanized with solution of long chain fluorinated organosilane in isopropanol over a hot plate to create uniform a very thin layer and other sided of the paper is coated with the suspension of Ag nanoflakes (AgNFs) in toluene. After drying for 10 min this AgNFs layer acts as the conductive layer with low resistivity of 60 nΩ m. The EPED is cut with laser in stretchable form which here is open-mesh serpentine design and is transferred to the skin with the help of medical

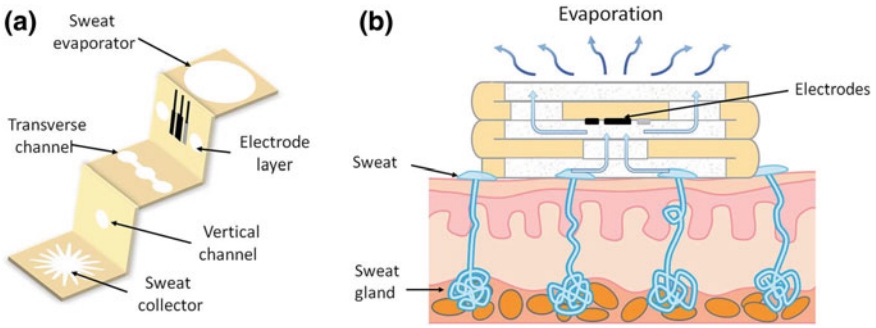

Fig. 12.6 Schematic of 3D fabricated device. **a** The layered structure of 3D device showing different layers fabricated. **b** Shows the working of 3D stacked assembly with sweat flow. Reproduced from Cao et al. (2019), with the permission under creative commons (Open Access)

glue and water-soluble tape. Figure 12.7 represents the three electrode assembly, disposability of the device and comparison of different electrophysiological signals obtained from this device and conventional device.

These EPEDs are simple, inexpensive, and compatible with mass-printing technologies, washable, mechanically stable, and easy to apply/dispose. The fibrous cellulose structure makes them breathable and provides stability to the soldered electronic components. This EPED can be powered wirelessly via inductive coupling and can be used as implants for stimulation, therapeutics and localized heat treatment by Joule heating for treatment of cancer (Sadri et al. 2018).

12.5 Challenges and Future of Microfluidic Paper-Based Analytical Devices

In the developing world, it is the need of the hour to come up with low cost, easy, reliable and portable diagnostic systems. Lateral flow tests are hence the need of the hour, which have an added advantage that it does not require the used to be skilled in any manner. Use of paper as substrates in such tests, reduces the cost of goods, is easily available and quick to use. The cost of manufacturing and developing paper based microfluidic channels for reactions to occur is far cheaper than plastic-based microfluidic system (Martinez et al. 2010). The silk-based platform (Fab-chips) can also be a cheaper alternative. A major portion of the cost of production of Fab-chips goes in antibodies. However, even if the cost of antibodies is removed, paper-based systems are three-fold cheaper than Fab-chips. The advantage of the latter comes from the fact that Fab-chips would put the Indian artisans to good use and improve the country's economy.

Another area of development in this sector is the area of separation. The blood diagnosis process requires the separation of plasma from the blood for sample

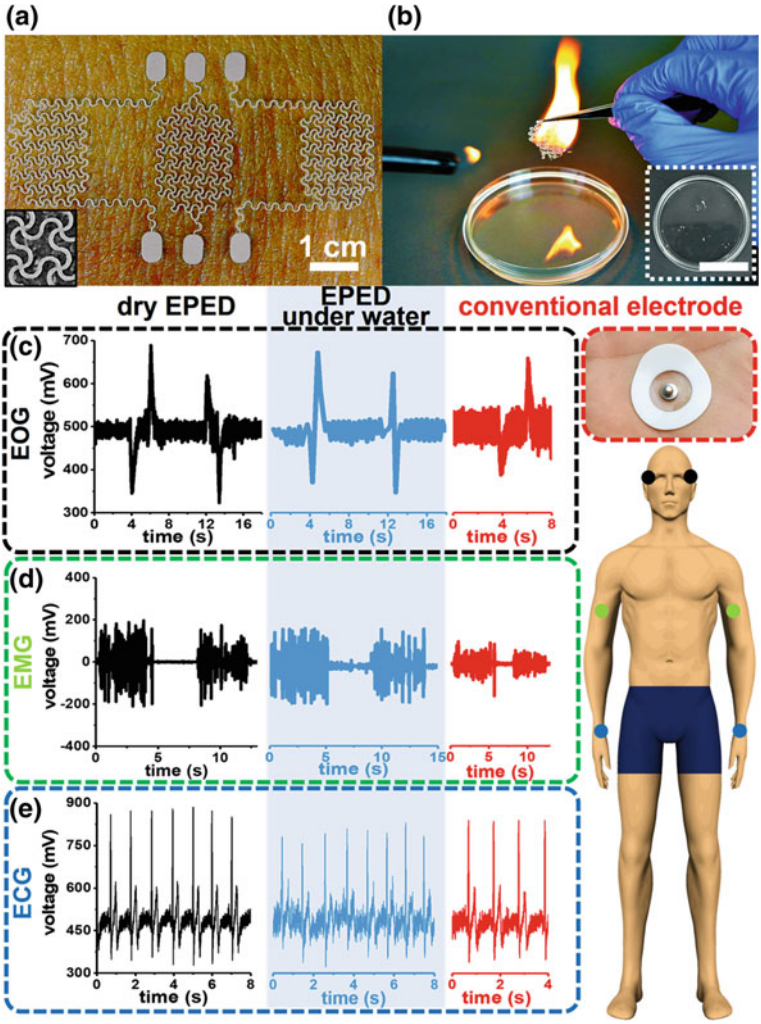

Fig. 12.7 EPED to monitor the electrophysiological signals. **a** The three electrode assembly to for measuring, ground and reference mounted on skin. **b** Disposability of the device by incineration. **c–e** EOG, EMG and ECG signals of the human body measured with ambient condition and underwater and compared with the conventional electrode. Reproduced from Sadri et al. (2018), with permission from Royal Society of Chemistry

analysis. This process is quite expensive. Hence, there is a need to develop a cheaper alternative for the sampling process for the easy and cheap separation. A sophisticated level of research is hence required in this area of separation for further analysis.

Apart from this, commercialization of paper-based diagnostics is an issue. It is difficult for a diagnostic company, or a research group in diagnostics to incur profits

by using paper-based products. Although the manufacturing costs are low, it is difficult for the market behavior to shift if the area is mature in a certain other therapeutic method or an existing assembly line. Hence, expecting a shift from the existing methods of diagnostics, without the paper-based technology having extraordinary advantages, is a little ambitious, but not impossible.

The future of a paper-based microfluidic diagnostic company depends on how well it bridges the gap between the needs of the people and development of new technology instead of replacing the existing one. One such area of development is theranostics (a field of testing employed for selecting targeted therapy), done to monitor levels of drug intake during the patient's clinical trials. Also, a diagnostic company needs to smartly analyse the persistent diseases in the vicinity of its location. Further development of paper-based technology to target those diseases would prove beneficial in this case.

According to the current market analysis, the United States holds the largest segment of in vitro diagnostics while the fastest growing market is China. After a detailed study of the world market in diagnostics, it is found that the fastest growing sector in the field is the integration of molecular diagnostics with point-of-care testing.

12.6 Summary

Due to a plethora of advantages of paper based microfluidic devices, it is an emerging approach in the current market and promises to grow manifold in the next five years. These devices are equipment free, easy to use and dispose, portable, require smaller volumes of analytes and can detect multiple analytes at the same time, making it a desirable option in clinical applications. Apart from these, the technology strives to become easier, more user-friendly and much more efficient according to the guidelines of WHO (Mabey et al. 2004).

Understanding the dynamics of capillary flow will surely help in enhancing the performance of the currently existing micro-fluidic technology used for wearable applications. Sensing and detection technologies also strive to evolve with evolving research in the field. Another area of improvement and research is the quality of paper used for substrate. Some of the performance criterion that can be taken into consideration are the long term storage and the absorption levels of the paper used. For analyzing purpose, quantitative equipment reducing human intervention to give more fool-proof, reliable results, will play a significant role in future diagnostics. One are of research is the use of mobile phones to quantitate and analyze the results of diagnosis. Advances in regions like better camera resolution, smarter phones and image quality would definitely accelerate the research on telemedicine.

Paper-based microfluidic technology has seen various applications including disease detection, monitoring of forensic samples and detection of environmental agents. In addition to this, the technology can also be used in agricultural industries for monitoring food quality or toxins. Although the area is largely advancing, there

exists limitation of it. The quality of the paper used, the sensitivity and the reliability of the results of such easy diagnostics are some of the leading issues of the industry. Some of the manufacturing methods are complicated and need to be simplified before commercialization (Dungchai et al. 2010), the response time and the cost of manufacturing must also be lowered simultaneously.

There is still room for improvement in the area, although various detection and fabrication techniques are well utilized. Owing to the sensitivity, specificity, reliability, ease and the cost of the approach, paper-based microfluidic devices see huge applications in the clinical industries.

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Chapter 13
Paper Microfluidic-Based Devices for Infectious Disease Diagnostics

Mohit Pandey, Mahima Srivastava, Krutika Shahare and Shantanu Bhattacharya

Abstract Infectious diseases are still a burden in the global healthcare sector. As per the WHO report, millions of people get affected annually at a worldwide level. An early diagnostic is a critical step in the prevention of their spread. The demand for point-of-care diagnostics of deadly diseases has increased significantly. From the past decades, several technologies have been developed for the diagnosis of these diseases. Among these, paper-based microfluidic devices present a unique opportunity for point-of-care diagnostic of various infectious diseases due to their simplicity, affordability, ease of fabrication, rapid detection, robustness, sensitivity and low limits of detection. As compared to laboratory-based diagnostics, the paper-based platform provides a cost-effective solution which plays a significant role in developing countries with limited resource settings. This chapter presents a brief overview of recent developments in paper microfluidic-based technology for the detection of various infectious diseases.

Keywords Infectious diseases · Paper-based device · Colorimetric detection

13.1 Introduction

In areas of scarce resources, cleanliness, and facilities, several infectious diseases are prevalent, despite the continuous struggle by the World Health Organization (WHO). Thus, timely diagnosis and monitoring of these diseases are essential for

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minimal treatment and low mortality. Modern day technological advancements in the field of public healthcare have caused a shift in the detection of viruses in clinics to kit-based detection. A need for portable, low-cost, disposable, and user-friendly medical diagnostics system has prompted the development of paper-based microfluidic devices, capable of detecting pathogenic microorganisms and chemical contaminants.

Microfluidics refers to the science of behavior, precise control, and manipulation of fluids that are geometrically constrained to a sub-millimeter scale. It is advantageous as low volumes of sample and reagent are required, and rapid detection of infectious diseases is attainable, even for the most dangerous pathogens like HIV, HBV, ZIKV, with high accuracy in identification. Paper is a reasonably intricate material, and it finds utilization in developing sensors and devices in analytical chemistry, owing to its physical properties, its availability, and easily convertible characteristics. These devices typically consist of a series of hydrophilic cellulose that steers fluids from inlet to the desired outlet. Amongst the variety of papers available, the choice of paper to use largely depends upon the fabrication steps involved in the development of a device and their areas of application. Microfluidic chips integrate preparation of samples, different reactions, separations, and identification on a microchip, which can be efficiently used as a substitute for conventional laboratories and clinical detection.

The characteristics and behavior of different microorganisms like bacteria, fungi, and viruses can be studied to examine infectious diseases caused by them and for the development of new, competent diagnostic methods, and devices. In this chapter, we focus on the dominant infectious disease-causing pathogens including *Escherichia coli* (*E. coli*) bacteria, Plasmodium parasite, Mycobacterium tuberculosis (TB) bacteria, *Vibrio cholerae* bacteria, Dengue virus (DENV), Human Immunodeficiency virus (HIV), Hepatitis B virus (HBV) and Zika Virus (ZIKV). We discuss the need and processes of detection of these microorganisms through microfluidics and then focus on various methods of health diagnostics. Finally, we discuss the commercialization of these microfluidic techniques.

13.2 Pathogen Detection

Detection of pathogens is one of the essential applications of paper-based microfluidic sensors in medicine since the antibiotics chosen depends upon the agent causing the disease. Here we discuss the detection of pathogens very commonly present.

13.2.1 Escherichia Coli

Foodborne diseases have been widespread across the world for decades, and they lead to extreme health, economic, and social problems. The most common

pathogen, *Escherichia Coli*, also known as *E. coli* bacteria, is mostly found in the intestines of humans and animals. The initial symptoms of being infected by these bacteria are severe abdominal cramps, fever, and vomiting, whereas, hemolytic uremic syndrome (HUS) can occur in the later stages and lead to death. It presents a challenge based on the life-threatening nature and microscopic concentration of *E. Coli* O157: H7, sufficient to cause disease. Adulterated food including ground beef, and unpasteurized milk, unclean water or close contact with the infected cause spreading of the disease. The traditional methods of detection of bacteria like counting colonies on a plate, and immunology-based methods are, although susceptible, yet time-inefficient and have complicated operation techniques. Therefore a fast, selective, and reliable technology to detect deficient concentrations of bacteria has been developed using nanomaterial-based biosensors.

Several methods of detection have been employed for qualitative and quantitative detection of *E. coli* O157: H7, such as colorimetric technique, electrochemical technique, and optical-based detection. A colorimetric biosensor uses *E. coli* proteases. The proteolytic activity these proteases is acts as a biomarker. *E. coli* O157: H7-specific peptide substrate is labeled with magnetic nanoparticles and further immobilized on a gold sensing platform. This gives a hand-held pep-tide probe in strip format. On being exposed to *E. coli* O157:H7 proteases, the magnetic nanoparticle peptide moiety is attracted to the sensor stripe, mounded with permanent magnets at the back, and as a consequence, a color change is observed (Suaifan et al. 2017). Detection by electrochemical chip provides better sensitivity and a faster response. A microfluidic chip was developed, combining dielectrophoretic and impedance analysis systems. It realized the real-time monitoring of bacteria accumulation in the cross-electrode chamber. The system automated the whole concentrating operation by remote computer control, recording the change of electrical impedance to detect *E. coli* (Zhou et al. 2019). Optical-based detection method uses a combination of fluorescence detectors and microfluidic chips. Nuchtavorn et al. introduced separation and detection using chip-based capillary electrophoresis platform with laser-induced fluorescence detection. The sensitivity of this method depends upon the capability of the fluorescent labeling reagent. A combination of aminated nanomagnetic beads and carboxyl quantum dots is used to quantify the DNA of *E. coli* O157:H7. Thus the development of fluorescent nanoprobes and quantum dots combined with micro-fluidics can cause rapid detection of this bacteria with high sensitivity (Zhou et al. 2019; Nuchtavorn et al. 2012).

13.2.2 Plasmodium

Plasmodium is a protozoan group which is a parasite of vertebrates and insects. These cause malaria in humans and commonly transmitted by the bite of an infected female Anopheles mosquito, called malaria vectors. Plasmodium falciparum poses the most significant threat as it causes severe illness and can lead to death. The existing tests have proven to be unreliable. It has, therefore, become a necessity to

have low-cost and rapid devices for diagnostics wherein no previous training or laboratory setup is required and can detect deficient concentrations of parasites. The difference in the deformability of healthy and infected red blood cells has been used to develop methods for malaria diagnosis. As parasites in the blood mature, the red blood cells progressively lose deformability and become stiffer. This deformability is used as a biomarker to estimate the infection stage of malaria. It is found that more stiff infected RBCs are more likely to displace to the walls of the microfluidic channels. More than 80% of the iRBCs (in trophozoites/schizont stages) could be isolated by splitting the primary microchannel into side channels. The specificity yet needs to be improved as other infections like sickle cell anemia also cause changes in the deformability of RBCs (Chen et al. 2019). In another paper-based sensors for malaria diagnostics, there are different wells containing buffer gold enhancement reagents and sample pads are kept adjacent in the design for color development by promoting lateral flow of reagents. A sample test is conducted where, on the test line, a murine antibody specific to *PfHRP-II* is coated as a capture reagent for the antigens in the sample. To visualize the binding reaction, a secondary antibody specific to *PfHRP-II* tagged with gold nanoparticles, is used as an optical tag. A gold enhancement reagent is used to improve the intensity of the signal, which bind with the GNPs. A high-intensity blue color appears, as a result of increased size of gold nanoparticles. The amplification of signals take a longer time for detection than unamplified immunoassay but the limit of detection is largely improved by this amplification, up to 2.9 ng/mL (Ragavan et al. 2018).

13.2.3 HIV

The Human Immunodeficiency Virus (HIV), belonging to the Retroviridae family is a single-stranded enveloped RNA. It is a virus that impairs the function of the immune system of the body by infecting the protective cells as it enters the body. As the infection increases, it deteriorates the immune system progressively, and the individual becomes prone to life-threatening "opportunistic infections" and cancers and leads to the Acquired Immunodeficiency Syndrome (AIDS) dis-ease. HIV is easily transmitted through blood transfusion from the infected and re-using contaminated surgical equipment, syringes and needles, unprotected sexual intercourse, and oral sex with an infected person. During pregnancy, childbirth, or breastfeeding, HIV can also be transmitted from the mother to the infant. In the initial days of infection, no visible symptoms appear. As it progresses, symptoms like fever, diarrhea, swollen lymph nodes, and cough appear. Although the cure for HIV infection doesn't exist, yet early diagnosis and continued usage of antiretroviral drugs prevents or reduces the reproduction of the virus in the body, and thus help in maintaining health for more extended periods.

Conventional techniques used for the detection of the virus in the host are based on quantitative PCR. But these methods are expensive, require longer times and insensitive to lower concentrations of virus. Paper-based detection can be used to

determine whether the target is present in the blood or serum. The earliest biomarker after the infection is HIV p24, a capsid protein of HIV. The peroxidase-mimicking porous platinum core-shell nanocatalysts (PtNCs) is integrated into a Lateral flow immunoassay device (LIFA) to account for the sensitivity of detection. To determine the presence of the target analyte in the sample, a colorimetric detection is carried out using LFIA. In an LFIA, the core process is initiated when the sample hydrates a detection label (typically an intensely colored nanoparticle), and by capillary action, it is drawn along the paper membrane and thus have the detection label bound to the test line through a target analyte. The test line and the nanoparticles need to be decorated with antibodies (affinity ligands), which could bind different regions of the target analyte. The high sensitivity of LFIA requires faster binding reaction kinetics towards solid phase. While the sample flows past, the particle labels must bind to the test line. Through the catalytic activity of the nanoparticle, a powerful amplification mechanism is incorporated in the PtNC amplified LFIA, which increases the intensity of the signal through locally depositing dyes at the test line. In a vessel containing orthogonally biotinylated camelid antibody fragments (nanobody-biotin), and lyophilized antibody modified PtNCs, a plasma (or serum) sample is introduced. These antibody segments can bind to discrete epitope regions of the p24 capsid protein. The solution is drawn up the strip towards the streptavidin bearing test line by capillary action, using a nitrocellulose reaction membrane lateral flow strip and an absorbent pad. Higher concentrations of the target (100–10,000 pg mL^{-1} p24) show a positive test result indicated by a clear black line, which occurs when particles bound at the test line are intrinsically absorbed. Catalytic amplification of the PtNCs is required for the detection of lower concentrations of the target. The oxidation of chromogenic substrate is enabled by the disproportionation of H_2O_2, achieved by this amplification. The oxidation of substrate by PtNC generates an insoluble colored product captured on the test line. The assay can thus be easily read by eye because of the presence of the unambiguous colored product (Loynachan et al. 2018).

13.2.4 HBV

Hepatitis B Virus, of the hepadnavirus family, affects the liver in humans and causes an infectious disease known as Hepatitis B. This disease causes health problems globally, and both acute and chronic infections occur. Usually, there are visible symptoms at the initial stage. The symptoms that appear include dark urine, vomiting, and abdominal pain, nausea, and yellowing of the skin and eyes. Eventually, liver cancer and cirrhosis may develop and sometimes lead to death. Exposure to infected blood or body fluids transmits this virus. The transmission also occurs through vaginal, seminal and menstrual fluids, reuse of syringes and needles in a medical setup, and dental and surgical procedures. As recommended by the World Health Organization, vaccination for HBV is a necessity and must be done for all infants.

Simple paper-based sensors have been developed to detect HBV from blood or serum samples. Simple paper-based sensors have been designed to detect HBV from blood or serum samples. This is achieved by observing changes in the optical properties of polythiophene (PT) impregnated polyvinylidene fluoride (PVDF) membrane, when the target nucleic acid, HBV-DNA, forms a complex with PT. Orange to purple color change occurs when the complex is formed by electrostatic interactions between the nucleic acid and polythiophene (Ammanath et al. 2019).

13.2.5 ZIKA Virus

Zika Virus belongs to the family Flaviviridae, transmitted mainly by the bite of Aedes mosquito, which is active during the daytime. It can also be transferred to the fetus from mother during pregnancy, organ transplant, blood transfusion, and sexual contact. Zika virus was successfully isolated in the Zika Forest of Uganda, where it derives its name from. The symptoms include fever, rashes, muscle and joint pain, conjunctivitis, and headache and lasts for 2–7 days. These symptoms appear only after about 3–14 days after being exposed to the virus. Zika virus causes massive complications, especially during pregnancy, wherein it causes microcephaly and congenital anomalies in the fetus or could result in stillbirth and preterm birth. Zika virus also triggers Guillain-Barré syndrome, myelitis, and neuropathy, more commonly in adults.

A system for the rapid diagnosis of Zika infection has been developed as a response to the Zika virus outbreak. An abiotic and sterile, substrate based microfluidic system that can be used outside laboratory setup has been established, by freeze-drying cell-free transcription and translation systems onto paper with genetic sensors (Pardee et al. 2016). The RNA extracted is isothermally amplified, and freeze-dried paper sensors can be rehydrated using these. In the substrate disc, the color shift from yellow to purple indicates the detection of a suitable trigger RNA. At room temperature, these biomolecular components remain stable, and thus their storage and distribution becomes easier (Nasseri et al. 2018).

13.3 Health Diagnostics

The development in technology is carried out with the common though; to achieve better health and living condition for the human being. Paper-based microfluidic helped a lot in gaining the lower limit of detection of different analytes with the benefits of low cost, ease of manufacturing, and flexible point of care diagnostics devices. Paper-based microfluidic devices are being constantly developed and improved for the detection of various pathogens in the water, food and biological fluids like blood, serum, urine, etc. and also for the monitoring of the glucose, uric acid and physiological parameters to monitor the good health.

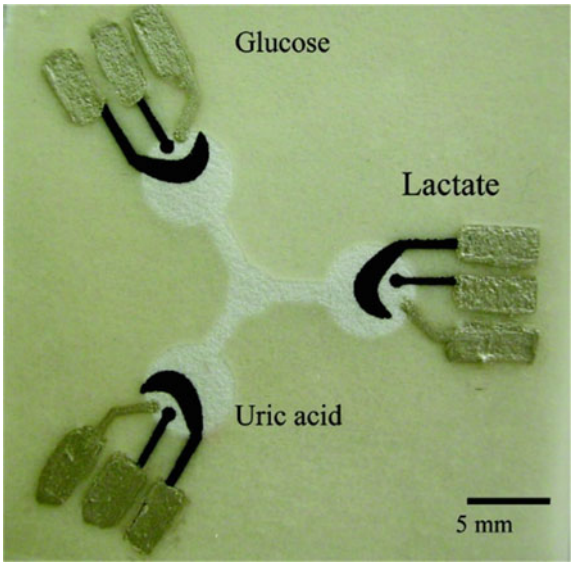


Fig. 13.1 Three electrode assembly for all the three analytes. The sample is divided into three separate test zones with the help of hydrophilic channel. Reproduced from Henry et al. (2009), with permission from American Chemical Society

Henry et al. (2009) demonstrated the detection of glucose, lactate, and uric acid using the paper-based microfluidic device. They fabricated the electrochemical detection technique with microfluidics on a paper and characterization of the analytes were done with cyclic voltammetry. Screen printing technology was used to fabricate the electrodes, and photolithography was used to make the microfluidic channels on the filter paper. Glucose oxidase, lactate oxidase, and uricase enzymes are used to react with glucose, lactate, and uric acid, respectively, and the determination of analytes was done using chronoamperometry. This device shows the integration of electrochemical detection with the paper microfluidic as a robust alternative for the point of care monitoring. Figure 13.1 shows the paper-based device fabricated by Henry et al. consisting of hydrophilic channels and three separate testing zones.

Takahashi and team (Takahashi et al. 2018) fabricated a synthetic platform for the analysis of microbiome sample. They address the need for a simple and affordable paper-based platform to analyze microbiome sample which is responsible for many biological functions of the body. mRNA from 10 different bacteria which affect human health and four clinically relevant host biomarkers were detected using RNA toe-hold switch sensors and compared with the RT-qPCR. Further detection of toxin mRNA in the diagnosis of Clostridium difficile infections was highlighted. Yang et al. (2018) worked for the health and wellbeing of cattle by detecting sexually transmitted infection (STI) pathogens using paper-origami DNA microfluidics. They tested the semen samples from elite bulls and detected three bovine infectious reproductive diseases through fluorescently. The paper device was fabricated by hot wax print-ing five channels on the paper, which includes positive and negative controls and three pathogen targets. There were three

components in each device; wax printed filter paper, single-sided adhesive acetate film sealed plastic plate with five paper spots and glass fiber disk to adsorb nucleic acid from the sample in the case of high concentration of the chaotropic agent, guanidine thiocyanate. Pathogen DNA from one viral pathogen and two bacteria (bovine herpes virus-1, Brucella, and leptospira) was extracted, amplified, and detected. This data was either collected visually or digitally with a mobile phone camera. The limit of detection with this device was also evaluated using pathogen-inoculated semen sample and found to be as low as 50 leptospira organisms, 50 CFU Brucella, and 1 TCID50 BoHV-1. Figure 13.2 represents different parts of the device and five separate reaction chambers, one each for the detection of three analytes, positive and negative control.

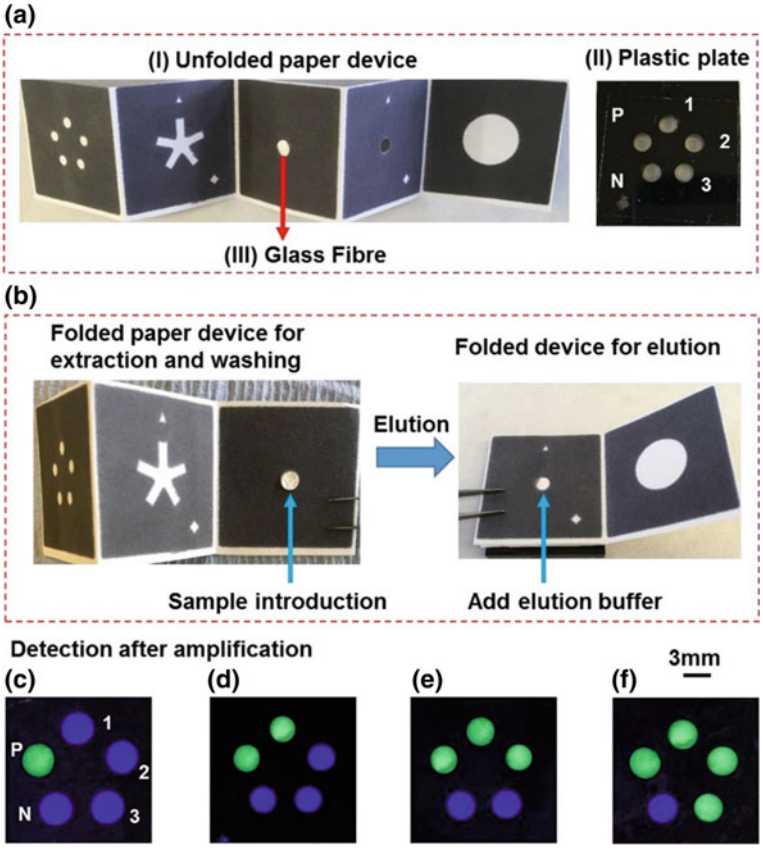


Fig. 13.2 Design of the paper device having five different zones for positive, negative control and for detection of three targets. **a** Shows the unfolded filter paper, plastic plate having five reaction chambers and glass fiber. **b** Sample introduction into glass fiber followed by washing and elution (**c**–**f**) detection of different analytes. Reproduced from Yang et al. (2018), with permission from American Chemical Society

Kumar et al. (2018) reported a novel lateral flow immunoassay for the detection of dengue NS1. They used the tapered flow design for paper microfluidics simultaneously leveraging on the benefits of Au-rGO as the detection label. rGO used provides a high surface to volume ratio inhibits the gold nanoparticle aggregation, which eventually increases the number of active binding site for the anti-dengue antibodies. Due to this modification, the concentration gradient of the antigen bound nanoparticles at the test line increases, which increases the color intensity. This enhancement of the signal enables them to reach the extremely low detection limit of 4.9 ng/ml. The taper angle of the device has been optimized to achieve the high sensitive limits and rapid detection (10 min) of NS1. The method has been demonstrated with spiked in human serum sample and resulted in the 11 fold improvement over the previously reported values. This lateral flow immunoassay can be used for the rapid detection of several target diseases at an early stage.

Suaifan et al. (2017) developed a novel biosensor for the detection of *Escherichia coli* O157:H7 through a peptide probe. This device is based on the ability of *E. coli* proteases to change in the optical response. The gradual increase in the golden color, which is visually observed is due to the surface modified, magnetic nanoparticles. The color intensity and concentration of the *E. coli*. are correlated and is quantified using image analysis software. The detection is with magnetic nanoparticle-specific (MNP-specific) peptide probe, with a limit of detection as low as 12 CFU/ml in both samples and 30–300 CFU/ml in a spiked food sample. This sensor is rapid, easy to use, low cost and easy to mass produce and thus, can be used with the regulatory authorities and by the food manufacturer for better control of the food quality and health. Figure 13.3 represents the schematic of the fabrication technique of the colorimetric biosensor to detect *E. coli* O157:H7.

Similar such attempt has been reported by Bo Pang and team where they developed a paper based ELISA test for the detection of *E. coli* O157:H7. This enzyme-linked immunosorbent assay (ELISA) device operates with shorter operation time, higher sensitivity, lower cost and lower limit of detection (10,000 CFU/ml). The filter paper was pretreated with chitosan and glutaraldehyde to increase the bacteria immobilization and then incubated with anti-*E. coli* primary antibody. Horse radish peroxidase conjugated anti-goat secondary antibodies are also incorporated in the paper. With the tetramethylbenzidine-hydrogen peroxide (TMB-H_2O_2) solution, the color signal was obtained and is analyzed by imageJ software (Pang et al. 2018). Figure 13.4 illustrate various steps involved in the fabrication of p-ELISA based biosensor for the detection of *E. coli*.

Rengaraj et al. (2018) developed a rapid, low cost, on site screening method for the pathogenic microorganism in the drinking water. They fabricated carbon electrodes onto hydrophobic paper and used carboxyl group to functionalize electrodes. Concanavaln A (Con A) is used as the biorecognition element and device is tested as impedimetric sensor for bacteria in water. This device can detect bacteria up to lower level as 1900 CFU/ml. This portable paper based device shows linear

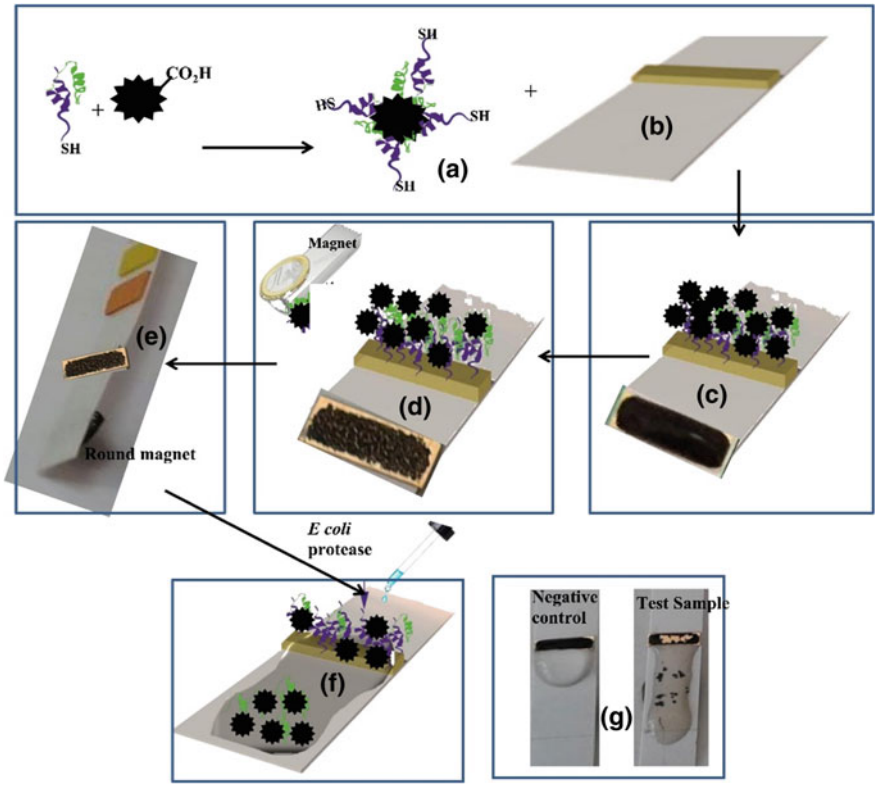


Fig. 13.3 Schematic representation on the actual image of the colorimetric biosensor to detect *E. coli* O157:H7. **a** *E. coli* O157:H7 specific peptide substrate functionalized with magnetic-nanoparticles (MNPs). **b** Gold sensing platform. **c** Immobilization of the sensing monolayer. **d** Removal of unattached MNPs by external magnet. **e** Side view showing magnet attachment. **f** Sample addition. **g** Detection of *E. coli* and negative blank control on paper based biosensor. Reproduced from Suaifan et al. (2017), with permission from Elsevier

increase in the probe charge transfer resistance with bacterial concentration ranging from 10^3 to 10^6 CFU/ml. Sun and team (2018) fabricated a paper based analytical device with combining PVC pas and filter paper for the detection of cronobacter spp. Pathogen in food and water. This device consist of a micro spot which changes color on the bacteria detection from colorless to indigo due to species-specific enzyme association. The testing time of this device is 10 h or less and is capable of detecting live bacteria with concentration as low as 10 CFU/cm^2.

For the early diagnosis of plasmodium falciparum malaria, Santos et al. developed a paper based platform with minimal amount of reagents for detection of histidine-rich protein. The device consist of different regions for sample addition, detection zone and absorbent pad, all connected with microfluidic channels for

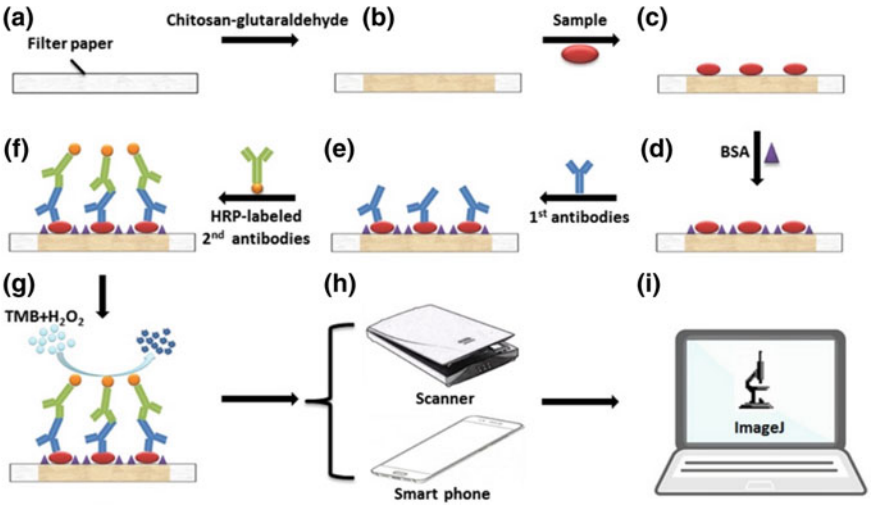


Fig. 13.4 Schematic representation of paper-ELISA. **a** Selection of Whatman #1 filter paper; **b** pretreatment of paper with chitosan-glutaraldehyde; **c** attaching the sample on the paper; **d** blocking with the help of BSA; **e** Application of primary antibody; **f** incubation of secondary antibody labelled with HRP; **g** color development with TMB; **h** data collection as an image using scanner or printer; **i** analyzing acquired image. Reproduced from Pang et al. (2018), with permission from Elsevier

performing lateral flow assay. This whole assembly is fabricated through CO_2 laser cutter. This modified shape causes delay in flow at the detection zone as compared to the conventional lateral flow systems which provides adequate time for reaction. With the captured antibody reactions, this device shows good sensitivity and detectability visual and calculated limit to be 5.0 and 4.5 ng/ml that is sufficient to identify the disease at early stage and thus, cutting down the requirement of nanostructures or other amplification techniques (dos Santos et al. 2018). Figure 13.5 represents the overall layout and schematic for the fabrication of paper based device for detection of malaria.

Colleen N. Loynachan and team developed a paper based lateral flow immunoassay for the detection of p24, one of the earliest and most conserved biomarker of HIV. This novel device is serum stable and consist of porous platinum core-shell nanocatalysts (PtNCs) which surpasses the current commercial and published sensitivities of paper based detections. The specific antibody has been modified with platinum nanocatalyst to have high specificity and affinity towards p24. This catalytic activity enable the naked eye detection of p24 biomarker in the spiked serum sample even in the low femtomolar range and reduced the detection time to less than 20 min (Loynachan et al. 2018).

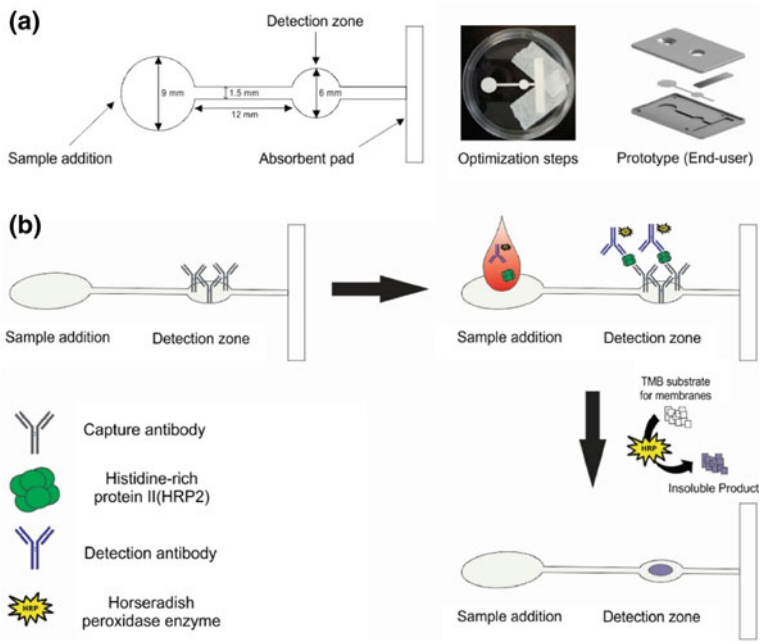


Fig. 13.5 **a** Overall layout of the device; designing, optimization and prototyping for end user. **b** Schematic for the preparation of paper based platform for diagnosis of *Plasmodium falciparum* malaria. Reproduced from Sun et al. (2018), with permission from Elsevier

13.4 Commercialization and Challenges

With the development of newer technologies for the point of care diagnosis, commercialization plays a very critical role in providing access to this technology to in need community. Most of these commercial technologies available are based on either antibody or DNA primer although various other means are available for diagnostics purpose. The devices having these analytes are much easier to modify to cater to different variety of diseases and thus covering a broader spectrum of diseases (Su et al. 2015). Table 13.1 shows the commercially available microfluidic-based system for point of care infectious diseases diagnostics.

However, implementation of this ideation on a commercialize stage is full of various challenges. These innovative platforms are designed with the thought to cater to the resource scare part of the world, and thus, pricing of the device should be low. Due to this low pricing, the profit associated with the product is also small, which is not appealing to many profit-seeking venture capitalists. This is one of the biggest hurdles in the way of commercialization. Luring other financial support from the non-government organization and private, not-for-profit organization working in the health domain becomes the only option for many groups.

Table 13.1 Commercially available microfluidic-based system for POC infectious disease diagnostics

Pathogens[a]	Analytes	Technical features	Company[b]	Website
Bacterial and viral	Primer	Disposable cards with benchtop analyzer, on-card sample processing	Cepheid	cepheid.com
Influenza, Bacterial	Primer	Portable detector (3 M™ Integrated Cycler), discs with on-board extraction	Focus Dx (Quest)	focusdx.com
Bacterial	Primer	Disposable cards with integrated heating, detection, sample processing in a portable instrument	HandyLab (BD)	bd-world.com
Viral	Primer	Automated analysis on FilmArray™ instrument	Idaho Technologies	BioFireDx.com
Bacterial, HIV/AIDS	Primer	Lab-in-a-tube platform for automated analysis	IQuum	iquum.com
Bacterial	Primer	Compact tabletop analyzer utilizing membrane-based quantum dot detection	LabNow	labnow.com
HIV/AIDS, MRSA	Primer or Antibody	Compact, benchtop analyzer, manipulation of nano- and micro-droplets	Advanced Liquid Logic	liquid-logic.com
Viral	Primer or Antibody	Disposable plastic discs (spinit®); label-free, portable SPR analyzer	Biosurfit	biosurfit.com
HIV/AIDS, hepatitis, flu	Primer or Antibody	Integrated fluidic cartridge and low-cost, low-power fluorescence imaging using planar waveguides	Mbio Diagnostics	mbiodx.com
Malaria	Primer or Antibody	Disposable cartridges composed of thin-film laminates and injection-molding	Micronics (Sony)	micronics.net
HIV/AIDS	Antibody	Disposable cartridge, portable analyzer, automated image based immune hematology test	Alere	alere.com
Bacterial, HIV/AIDS	Antibody	Portable analyzer, injection-molded plastic cassette, low cost optical detection of silver films	Claros Diagnostics	opko.com
Bacterial, HIV/AIDS	Antibody	Compact tabletop fluoro cytometry instruments with disposable cards	LeukoDx	leukodx.com

[a]MRSA, Methicillin-Resistant *S. aureus*; HIV/AIDS, Human immunodeficiency virus infection
[b]Acquisitions of POC microfluidics companies. Reproduced from Su et al. (2015), with permission from Elsevier

Government and philanthropic foundation can play a very crucial rule in research, development and finally in rolling out the product in the market (Tay et al. 2016).

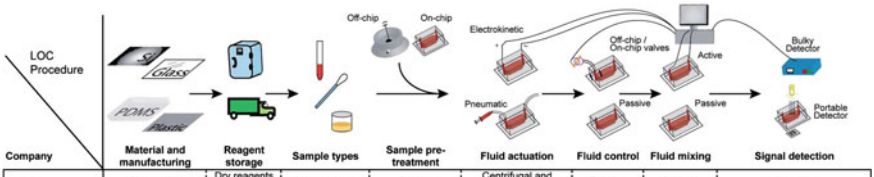
Apart from finances, another major issue for the commercialization run is the integration of different technologies on a single platform. Commercialization for point of care diagnostics needs a robust product which is easy to use by the patient itself. This "easy to use" is different from working in the lab and working on the field. In the lab, there are many resources available which might not be readily available on the field and thus, make the use of the product little uneasy. This is one of the major problems which is not catered by many research groups working in this field and formalizing a startup for diagnostics. Also, various technologies like fluid delivery, on-chip storage of sample and results, heating/cooling, pre-treatment of sample like dilution, mixing, etc. has to be combined with the detection technology to fabricate a fully functional product (Chin et al. 2007).

Other challenges that become very important for commercialization are approval from the regulatory authorities. The criteria for these approvals are different in different countries which are based on the socioeconomic status of the country, market availability for the product, and existing competition for the new product. Although some criteria that are generally followed by various authorities for point of care diagnostics applications are fully automated test, use of unprocessed specimen, no specialized training required to operate, results easy to interpret and unaffected by environmental conditions. Apart from fulfilling these criteria, the pathway of commercialization in many developing countries is very slow due to which the newer technology becomes old when it gets into the commercialize stage. Although this problem can be tackled by coordinating between different NGOs, government agencies and authorities (Chin et al. 2012). Figure 13.6 represents a technology map of design choices made by selected microfluidic companies.

In many countries, government bodies are taking many initiatives to help various startups and small companies to get them closely connected to government functioning and also to help them gel well among themselves and with different manufacturers, raw material suppliers, etc. These schemes help the government to monitor all the groups associated with the commercialization of point of care diagnostics to provide help or intervene whenever required.

13.5 Conclusion and Future Perspectives

The WHO is aiming at the complete elimination of infectious diseases through affordable and sensitive diagnostics. The limitations of existing conventional methods used for infection diagnosis, such as higher costs, technical complexities, longer processing time and poor sensitivities can be overcome by "smart" paper-based sensors (Tay et al. 2016; Liana et al. 2012). Paper-based microfluidic techniques can be successfully implemented as an economical and reliable manner. In developing countries, the number of trained personnel and equipment are deficient, and hence, the development of paper-based portable devices can potentially



Company	Material and manufacturing	Reagent storage	Sample types	Sample pre-treatment	Fluid actuation	Fluid control	Fluid mixing	Signal detection
Abaxis	Plastic disc	Dry reagents on disc	Whole blood	On disc	Centrifugal and capillary	Passive	Passive	Absorbance
Advanced Liquid Logic	Glass, insulated electrodes	On- and off-chip reservoirs	Whole blood	On chip	Electrokinetic (electrowetting)	Electrokinetic	Electrokinetic	Fluorescence, chemiluminescence
Alere (formerly Inverness Medical)	Plastic and elastically deformable materials	On cartridge	Whole blood (capillary)	On cartridge	Mechanical (peristaltic), capillary	Passive	Passive	Fluorescence
Biosite (Alere)	Strip with textured microstructures	On test strip	Whole blood (venous), plasma	Centrifugation	Capillary	Passive	Passive	Fluroescence
Cepheid	Disposable plastic cartridge	On test cartridge	Whole blood, sputum	Sample dilution buffer, shaking	Pneumatic (syringe plunger)	Rotary valves	Passive, manual	Fluorescence (with molecular beacons)
Claros Diagnostics (Opko)	Plastic cassette	On cassette	Whole blood, urine	On cassette	Pneumatic	Passive	Passive	Absorbance
Daktari Diagnostics	Plastic cartridge	On cartridge blister packs	Whole blood	On cartridge	Pneumatic	Passive	Passive	Electrochemical (impedance spectroscopy)
Diagnostics For All	Paper	Dry reagents on paper	Whole blood, urine	On stamp	Capillary	Passive	Passive	Colorimetric
Epocal (Alere)	Film, epoxy laminates	Dry reagents	Whole blood	On chip	Capillary, pneumatic, electrokinetic	Electrokinetic	Electrokinetic	Electrochemical, chemiluminescence
Focus Dx (Quest)	Plastic (polypropylene)	PCR reagent prep needed	Nasal & pharyngeal swabs	Nucleic acid extraction	Centrifugal	Passive	Passive	Fluroescence
HandyLab (BD)	Disposable cartridges	Dry reagents	Vaginal, rectal, nasal swabs	Broth enrichment, cell lysis (MRSA)	Electrokinetic, thermal pneumatics	Valves	Passive	Fluorescence (with molecular beacons)
i-STAT Corp (Abbott)	Plastic cartridge with silicon microchip	On cartridge	Whole blood, urine	On cartridge	Pneumatic (air bladder), capillary	Passive	Passive	Electrochemical (potentiometry, amperometry, conductivity)
Micronics (Sony)	Plastic, laminates, paper	On cartridge	Whole blood, stool	On cartridge	Pneumatic, capillary	Passive, valves	Passive	Absorbance, colorimetry
MBio Diagnostics, Inc.	Plastic, transparent planar components	On chip	Whole blood	On chip	Pneumatic	Passive	Passive	Fluorescence (with planar waveguides)
Philips	Plastic cartridge	Dry reagents on cartridge	Whole blood, saliva	On cartridge	Pneumatic	Passive	Passive	Optical (frustrated total internal reflectance)
TearLab	Polycarbonate	None	Tear	On chip	Capillary	Passive	Passive	Electrochemical
Zyomyx	Glass, plastic	Dry reagents	Whole blood	Bead-conjugated Ab incubation	Gravitational	Passive	Passive	Colorimetric (cell stacking)

Fig. 13.6 Technology map of design choices made for a set of common procedures central for production of integrated point of care diagnostics device by selected microfluidics companies. Reproduced from Chin et al. (2012) and Chin et al. (2007), with permission from Royal Society of Chemistry

help patients. These devices use high-tech biomarkers, and bioassays, which are cheap and thousands of reactions required for linking reagents to specific microorganism is implemented in minutes. Extensive research work shows that the detection of various disease-causing pathogens can be done with great precision and accuracy using only small volumes of sample and high sensitivity and integrability can be achieved with minimum limits of detection. However, these can be improved to achieve direct detection of complex diseases and viruses and their quantitative estimation (Lim et al. 2017). It is also required to remove the pretreatment steps for several detections to increase the rapidity of the test. Related clinical symptoms are present for several infectious diseases; a device with multiplex functionality is also desirable. For a specific contagious disease, characterization of a set of biomarker signatures may be a significant advancement in future biomarker screening (Chen et al. 2019). With advancing techniques, new areas of application come into the picture. It is expected that microfluidic detection and analysis will help in optimization of drug doses and help personalize medicine for more than one disease. The challenges, including fabrication techniques and incorporating different materials and markers for different detection techniques onto paper, further research needs to be done. Multidisciplinary collaboration, including industry sciences, researchers, managers, and clinicians can accelerate developments in this field.

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