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Paper-based chemical and biological sensors: Engineering aspects

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ABSTRACT

Remarkable efforts have been dedicated to paper-based chemosensors and biosensors over the last few years, mainly driven by the promise of reaching the best trade-off between performance, affordability and simplicity. Because of the low-cost and rapid prototyping of these sensors, recent research has been focused on providing affordable diagnostic devices to the developing world. The recent progress in sensitivity, multi-functionality and integration of microfluidic paper-based analytical devices (μPADs), increasingly suggests that this technology is not only attractive in resource-limited environments but it also represents a serious challenger to silicon, glass and polymer-based biosensors. This review discusses the design, chemistry and engineering aspects of these developments, with a focus on the past few years.

Contents

1. Introduction

Although the use of cellulose paper in analytical methods was known as early as the 17th century with the use of litmus paper, the first published work was reported by Hugo Schiff in 1866 describing the colorimetric detection of uric acid by a reduction of

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silver ions on a filter paper impregnated with silver carbonate (Schiff, 1866). The following years witnessed a few other applications, including the detection of cadmium (Bayley, 1878), and detection of sugar in urine (Oliver, 1883). In 1937, Yagoda reported the first hydrophobic patterning of filter paper using paraffin embossing to confine the detection zone (Yagoda, 1937). The concept was later used by Muller and Clegg to design fluidic channels (Müller and Clegg, 1949). In his seminal report, Yagoda explains the initial motivation of what we know today as paper microfluidics:

> "With the aid of a water-repellent barrier embedded in the fibers of the paper […] it becomes possible to execute the tests on filter paper of the usual porosity without loss in sensitivity owing to the spreading of the spot over a large surface. Also, as a result of the uniformity both in the area and tint of the spot, it is possible to approximate the concentration of the ion from the intensity of coloration produced by the drop of solution".

Despite these very early reports, the first significant wave of applications of paper in analytical chemistry occurred in the 1940s with the development of chromatography (Bull et al., 1949; Consden, 1948; Consden et al., 1944; Müller and Clegg, 1949). The advent of microfluidics (Whitesides 2006) and lab-on-chips (Janasek et al., 2006; Yager et al., 2006) in the 1990s generated a second boost that resulted in the current exponential growth of literature dealing with paper-based analytical devices, with the first article reviews appearing late 2009. This growing enthusiasm is a result of the unique and convergent characteristics of cellulose paper. In one side, the structural properties offer a great deal of functionality: (i) the microfiber network morphology increases the surface volume ratio and loading capacity, thereby enhancing the detection performance, (ii) the porous structure and hydrophilic character results in interesting wetting properties, giving rise to lateral and capillary-driven flows, (iii) the carbohydrate composition is a major leverage for surface functionalization following a traditional chemistry of alcohols or a simple adsorption mechanism, (iv) as a natural biocompatible polymer, cellulose paper can be used as a support for cells or microorganisms without causing adverse effects. Additionally, the high abundance of cellulose (more than 50% of the biomass), its low-cost and biodegradability, and the facile device prototyping offer very attractive commercial and environmental advantages. This review describes how recent developments take advantage of these properties to advance the sensitivity, functionality and integration of paper-based bio- and chemosensors. A remarkable number of reviews on paper-based sensors appeared last year, but they were mainly focused on diagnostics applications of μ-PADS and lateral flow systems (Hu et al., 2014; Parolo and Merkoci, 2013; Yetisen et al., 2013). In this review, we address the major design and engineering issues, from paper microfluidics, liquid handling systems, micropatterning and fabrication methods to surface activation/ functionalization strategies and the different transduction schemes. The discussion is extended to the development of biosensors using paper nanocomposites, and non-cellulose paper. We will also provide insights into the perspectives and the new challenges facing this research field.

2. Paper-based microfluidics

Cellulose paper is a permeable porous material composed of a solid matrix with average fiber diameter of 1–100 μm, and pore space with average pore size of 1–10 μm. Such geometry and the hydrophilic nature of cellulose determine the major fluidic properties of paper.

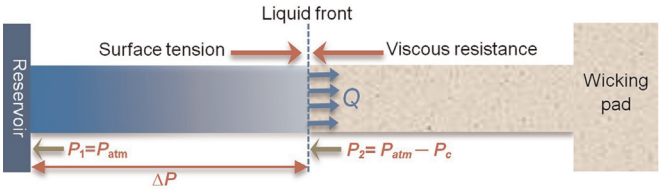

Fig. 1. Scheme of the forces involved in capillary-driven flow in paper microfluidics. P_{atm}: atmospheric pressure, P_c: pressure due to capillary forces, Q: fluid flow, ΔP: pressure drop.

First, the major flow driving mechanism is capillarity. The hydrophilic fiber surface (low contact angle) favors the adhesion and spreading of liquids, while the surface tension of the liquid tends to decrease the liquid–gas interfacial area, resulting in spontaneous wicking. A capillary-driven flow is then generated through the porous medium and sustained by the difference in pressure between the wetted area and the relatively dry area at the liquid front where the pressure is lower (Fig. 1). Wicking in cellulose paper is usually predicted and analyzed using two different models: the Lucas–Washburn equation that was derived from the Hagen–Poiseuille law by considering the pores in the medium as isotropic capillary tubes, and Darcy's law that was phenomenologically developed. Both of these models and their limitations will be introduced.

Secondly, the micrometric pore size of cellulose paper results in Reynolds numbers lower than 1:

$$Re_p = \frac{\rho V \delta}{\mu} < 1 \quad (1)$$

Where ρ is the fluid density (kg/m³), V is the fluid velocity (m s^{-1}), δ is the characteristic pore size (m) and μ is the fluid dynamic viscosity (Pa s=N s/m²=kg/(m s)).

This is a major parameter that determines the domain where paper microfluidics can be studied and modeled. The first consequence of $Re_p < 1$ is the laminar flow where the viscous forces are dominant. As a result, the flow velocity mostly depends on the equilibrium between the surface tension and viscous resistance.

$Re_p < 1$ will also enable a reliable approximation of the fluid flow through a cellulose filter paper by Darcy's law, which was first experimentally established to characterize fluid flow in hydrogeological settings. Darcy's law is only applicable for incompressible and Newtonian fluids (constant density and viscosity). It describes the volumetric flow rate in porous permeable media such as sand filled pipe as a function of the elevation, fluid pressure and proportionality constant:

$$Q = -\,\mathrm{AK}\frac{\Delta h}{L} = -\,AK\frac{\Delta[(P/\rho g) + z]}{L} \quad (2)$$

Where Q is volumetric flow rate (m^3/s), A is the flow area perpendicular to L (m^2), K is the hydraulic conductivity (m/s), h is the hydraulic head or height (m), which is the sum of the pressure head (P) and the elevation (z); L is the flow path length, which is affected by the tortuosity of the medium (m); g is the acceleration of gravity (m/s^2); ρ is the water density (kg/m^3). The negative signs in the equation indicate that the hydraulic head decreases in the direction of the flow. In other words, the fluid moves from high pressure towards low pressure zones. The hydraulic conductivity (K) is the proportionality coefficient and is a function of both the permeability of the medium and the fluid properties. It can be expressed as:

$$K = \frac{\kappa \rho g}{\mu} \quad (3)$$

Where κ is the intrinsic permeability of the paper (in m^2), ρ is the

fluid density, μ is the dynamic viscosity of the fluid, and g is the acceleration of gravity (m/s^2).

For one dimensional lateral flow on paper strips, where the elevation is constant ($\Delta h = 0$) and $Re_p < 1$ (gravity and inertial effects are neglected), Darcy's law reduces to a simple linear relation between the volumetric flow rate Q and gradient of pressure ΔP occurring over the length L of the paper strip (Osborn et al., 2010):

$$Q = -\frac{\kappa A}{\mu L}\Delta P \tag{4}$$

where κ is the permeability of the paper (in m^2), A is the hydrophilic paper cross-sectional area perpendicular to flow, μ is the dynamic viscosity (Pa s), and ΔP is the pressure drop across the length L, which is the combination of the atmospheric, hydrostatic and capillary pressures.

For a constant cross-sectional area or channel width, the time needed for the liquid front to travel a certain distance L_f can be expressed as:

$$t = \frac{V}{Q} = \frac{V\mu L}{\kappa A \Delta P} \tag{5}$$

Where V is the volume of the liquid at time t.

During the wicking process, the distance moved by the liquid front (L_f) in the paper strip can also be estimated using the Washburn Equation, by assuming $Re_p < 1$ and a Hagen–Poiseuille flow through rigid and isotropic cylindrical pores: (Bell and Cameron, 1905; Washburn (1921))

$$L_f^2 = \frac{\gamma D t}{2\mu} = \frac{\gamma D \cos(\theta)}{4\mu} t \tag{6}$$

where L is distance traveled by the liquid front within a the porous material (paper) whose pore diameter is D, t is time, γ and μ are respectively the surface tension and viscosity of the liquid and θ is the contact angle.

According to this equation, the distance traveled by the liquid can be increased by increasing the surface tension of the liquid. It also shows that the liquid front velocity decreases with time due to viscous resistance.

It is important to note that even at $Re_p < 1$, deviations can be observed from Darcy's law and Lucas–Washburn equation. This is due to the multiple approximations (pore size, geometry and distribution, constant porosity and permeability) and assumptions (perfectly wetting liquid, non-limiting source, negligible swelling effect) used to calculate the parameters of interest. One of the major sources of digression is that the model assumes that flow takes place across the entire cross-section of the paper strip, while actually it only occurs through interconnected and inhomogeneous pore channels. Despite the complex geometry of cellulose paper, Darcy's law provides an accurate description of paper microfluidics and has been experimentally and theoretically confirmed. Also, it provides more flexibility for modeling two or three-dimensional flows as compared to the Washburn equation, which is limited to a single one-dimensional flow.

Most of the research and modeling performed today on paper microfluidics use the two models or modified versions of Darcy's law and Washburn equation. A number of modifications to the equations have been introduced to address cases where one of the initial assumptions is violated. One example is that the Washburn equation assumes constant pore geometry over time and space and thus does not account for any fiber swelling effect that normally occurs during paper wicking. Schuchardt and Berg proposed a modified equation based on the assumption that the pore radius in the wetted area of a porous medium such as cellulose paper will decrease linearly with time as a result of wicking and fiber swelling. This assumption led to the development of a relation for wicking rate in swelling media (Schuchard and Berg, 1991). Schuchardt and Berg assumed that the pore radius continually decreases, which is not the case in fully wetted paper strips where the swelling rapidly reaches its maximum. Consequently, their model is only applicable during the wetting process.

The Washburn equation was also modified to include the gravity term and extend its application to cases where the inertia of the liquid column cannot be neglected (Masoodi and Pillai, 2012; Szekely et al., 1971). This is the case in vertical wicking where the liquid ultimately ceases to rise due to the balance of surface tension and gravity. Such consideration is particularly important when making three-dimensional microfluidic paper devices. More recent work focused on the study of fluid flow in different channel geometries in paper strips as a way to enhance sample pretreatment and detection abilities. Yager et al. investigated the relationship between flow transport time and the channel width. Based on this study, they proposed new designs to control the delivery time of reagents to a detection region by varying the widths of the input strips (Fu et al., 2011b). In another interesting work, Osborn et al. showed that many classic laminar-flow-based devices can be recreated in simple and low-cost paper networks including diffusion-based separation, hydrodynamic focusing, mixing and dilution (Osborn et al., 2010). A number of other designs for liquid flow control are described in Section 3.1.2. For further details on the analytical development of the different wicking equations and predictive models of liquid flow in porous media, the readers are directed to a recent dedicated book by Masoodi and Pillai (Masoodi and Pillai, 2012).

3. Design and fabrication methods

As in any biosensor development, the fabrication of paper-based biosensors requires the integration of three important components: a fluid handling systems, a transducer capable of converting one form of energy to another, and surface functionalization for sample pretreatment or specific receptor-ligand interaction.

3.1. Fluid handling systems

3.1.1. Microchannel patterning

To direct liquid flow on paper devices, the paper needs to be patterned into hydrophilic channels separated by hydrophobic walls. The patterns are used to prevent the spreading of liquid into undesired areas, concentrate chemical reactions in specific detection zones, and allow parallelization of multiple reactions in a single paper strip. One of the first methods used for the fabrication of microchannels for paper-based biosensors is photolithography. Photolithography is a conventional fabrication technique in microtechnology based on UV exposure of a photoresist through a transparent mask containing the desired patterns (Davaji and Lee, 2014; Gu et al., 2014; Hu et al., 2014). Commercial photoresists such as SU-8 can provide excellent patterning features with sizes below 1 μm. However, photolithography has two major drawbacks: (i) the hardened, easy cracking of photoresist barrier is likely to influence bending and folding damages, (ii) the procedure usually requires expensive instrumentation and needs multiple steps (Carrilho et al., 2009; Dou et al., 2015), although home-made photolithography systems can be developed and used for low resolution patterns. In order to reduce the cost of fabrication, cheap epoxy-based photoresist was used to replace the commercial SU-8. The procedure for photolithography fabrication of microfluidic devices on paper is illustrated in Fig. 2A (Martinez et al., 2010, 2008b).

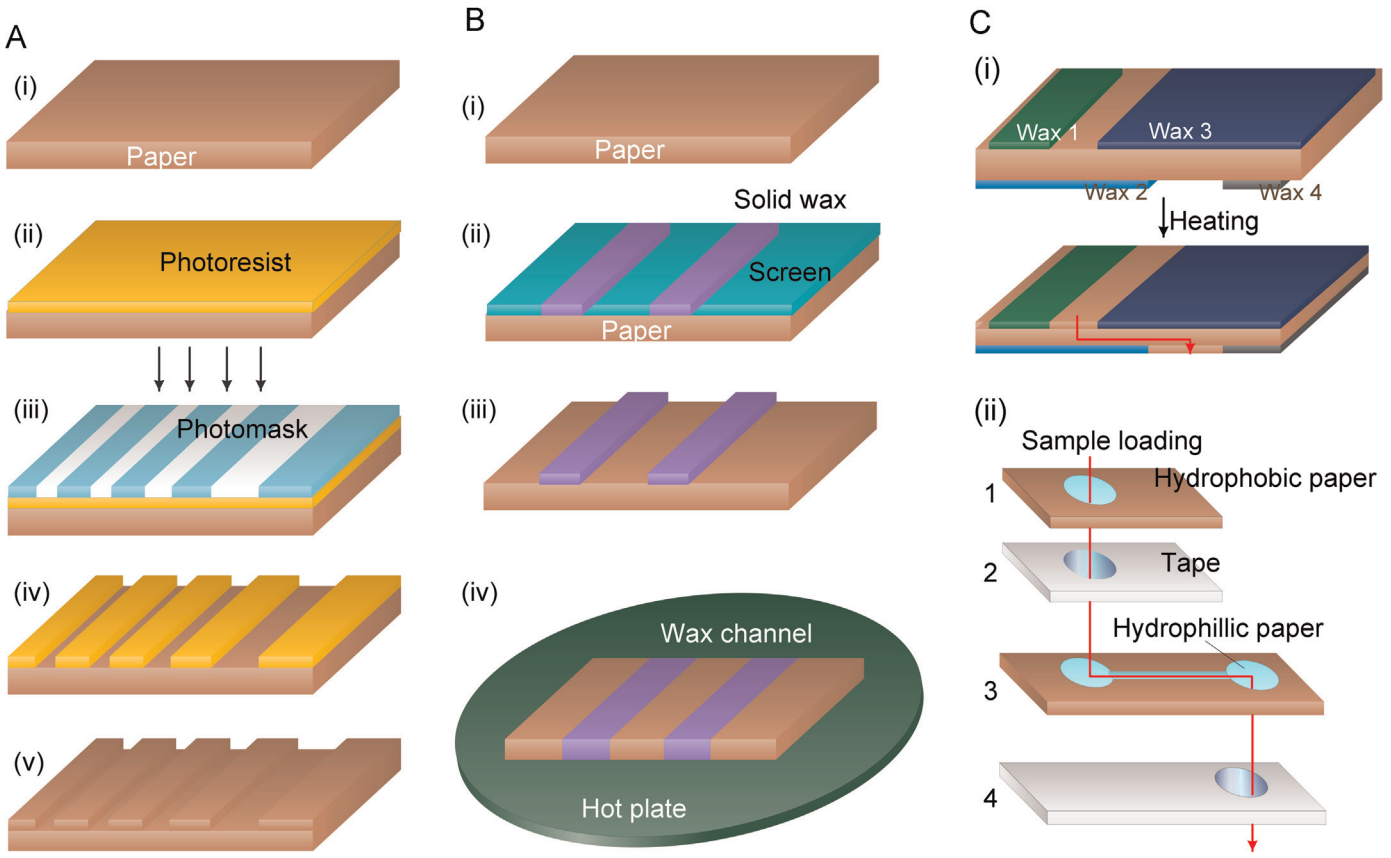

Fig. 2. Microchannel patterning process on paper-based biosensor. (A) 2D patterning on paper using photolithography with photomask and photoresist: (i) cleaning paper; (ii) photoresist coating, (iii) UV exposure, (iv) developing process, (v) etching process (Martinez et al., 2008b). (B) Printing technique using wax, polymer or organic solvent, (i) cleaning paper, (ii) printing with solid wax, (iii) mask removal, (iv) melting wax into paper (Dungchai et al., 2011). (C) 3D microfluidic fabrication on paper. (i) Multilayer microchannel fabricated inside a single sheet of chromatography paper by wax printing technique (Lei et al., 2014). (ii) Multilayer paper-based microfluidic fabrication using layer-by-layer of paper and adhesive tape (Thom et al., 2014).

The most recent and popular approach used in the fabrication of paper-based microfluidic devices involves printing. Different materials were used to print on paper for microchannel fabrication, leading to different techniques including wax printing (Ge et al., 2012a; Lewis et al., 2012), ink-jet printing of polymer such as alkyl ketene dimers (Barona and Amirfazli 2011; Yoon et al., 2011), and screen printing of silver and carbon ink for electrode fabrication (Lei et al., 2012; Yafia et al., 2015). Although wax printing has a lower resolution than photolithography (about 700 μm minimal barrier line width), the hydrophilic areas are not exposed to a photoresist, polymers or solvents. Hence, it does not require a processing step to create hydrophilic areas (Dungchai et al., 2011). As mentioned earlier, Yagoda introduced wax patterning on filter paper in 1937 (Yagoda, 1937). Today, a large number of research groups adopted the concept for portable paper-based bioassays (Jin et al., 2015; Lu et al., 2009). A commercial wax printer was introduced in 2009 and was used to print patterns of solid wax on the surface of filter paper. Wax printed paper was put on top of a hot plate at temperature above 60 °C for few seconds, allowing wax to melt and penetrate fully into the patterned paper (Dungchai et al. 2011). This process creates complete hydrophobic barriers that define hydrophilic channels, fluid reservoirs, and reaction zones from a simple computer design (Fig. 2B). Another technique that can eliminate the necessity of complicated instrument in wax printing is replication (Songjaroen et al., 2011). Patterns can be transferred onto paper by dipping an assembled iron mold into a wax solution at 120–130 °C. Using this technique, the patterned paper can be obtained in less than one minute as compared to 15 min needed in printing techniques. This method is claimed to produce up to 90 pieces per hour with inexpensive equipment, but it is limited by the required fabrication of different molds using laser cutting. Table 1 summarizes and compares different methods used for the fabrication of paper-based microfluidic channels. Depending on the purpose and specific application, other techniques were also used. Hydrophilic patterns can be created on a hydrophobic paper such as parchment paper, wax paper, palette paper, by using oxygen plasma treatment or laser treatment (Chitnis et al., 2011; Gao et al., 2014; Lei and Huang, 2014). Other mask-free patterning techniques have been used to generate microchannels by simply cutting filter paper with a craft-cutting tool (Abbas et al., 2013a; Glavan et al., 2013). Yi et al. also reported a mask-free patterning method of two-dimensional (2D) and three-dimensional (3D) microchannels on paper by laser beam cutting (Yi et al., 2010). However this method requires glass slides or polymer-based plates to sandwich the paper sheet, which leads to a multilayer structure. Despite their unique advantages, plasma treatment and laser fabrication techniques failed to prove amenable for mass production due to their multistep nature and relatively high cost instruments (Chitnis et al. 2011; Li et al. 2008).

The fabrication and patterning techniques discussed above can be extended to more complex 3D microfluidic systems. The use of 3D paper-based microfluidic devices offers a wide range of capabilities over lateral flow in 2D systems. The advantages includes the ability to (i) conduct a large number of assays in a miniaturized device, (ii) rapidly distribute sample through the *z*-direction and laterally through the *x*, *y* planes, and (iii) perform multiple assay steps in parallel by taking advantage of wicking through paper layers (Ma et al., 2015; Martinez et al., 2008a; Mosadegh et al.,

Table 1
Major fabrication methods for paper-based microfluidic devices.

Fabrication techniques	Patterning agents	Advantages	Limitations	Minimal barrier line width (μm)
Photolithography (Costa et al., 2014; Deng et al. 2014; Wu et al., 2014)	Photoresist (e.g., SU-8)	High resolution of microchannel with sharp barrier	High-cost, require expensive instruments; need more washing steps to remove non-polymerized photoresist; fragile when bending	200 ± 5
Ink jet printing (Hu et al., 2012; Wang et al., 2014b)	Polystyrene, AKD, methylsilses-quioxane	Only require simple printing equipment; fast, mass production (< 10 min); cheap reagent patterning (AKD)	Require heating step after deposition; need customized ink jet printers	500
Wax printing/dipping (Li et al., 2014b; Santhiago et al., 2014; Wang et al., 2014b)	Wax	Fast, simple fabrication process (< 5–10 min); mass production	Require heating step and expensive wax printer.	650 ± 70
Screen printing (Kong et al., 2014; Li and Liu, 2014)	Wax, conductive materials (e.g. silver ink)	Simple process fabrication, easy to fabricate electrode on paper substrate	Low resolution of microchannel with rough barrier	337 ± 36
Laser patterning (Chitnis et al., 2011; Katis et al., 2014)	Wax or silicone	High resolution	Extra step to create hydrophilic channel	62 ± 1
Plasma treatment (Kao and Hsu, 2014; Li et al., 2008)	AKD	Low cost chemical reagents	Need photomask for different pattern	300–500
Cutting (Abbas et al., 2013a; Cassano and Fan, 2013; Glavan et al., 2013)	No need	Simple, fast fabrication process, no need patterning reagent, very low cost	Not precisely control microchannel size, dependent on paper-materials	45–300

2015; Yetisen et al., 2013). Multilayer patterning in paper microfluidic channels evolved from wax-printing technique capable of controlling the penetration depth of melted wax (through heating time) to double-side printing of patterns (Fig. 2C–i). The development of 3D paper analytical devices has been reported by several research groups (Ge et al., 2012b; Lewis et al., 2012; Li and Liu, 2014; Liu and Crooks, 2011; Martinez et al., 2008a). Generally, the fabrication of 3D microfluidic devices involves the combination of many 2D microfluidic layers stacked together using alternating layers of patterned paper and double-sided tape. Holes are made between layers through the tape to create hydrophilic connections and the whole system was packed to allow fluid flow and distribution to different detection areas (Fig. 2C–ii) (Costa et al., 2014; Jayawardane et al., 2015; Noh and Phillips, 2010; Thom et al., 2014).

A fabrication method named Origami, derived from the traditional Japanese art of folding paper, has also been demonstrated by Crooks and co-workers (Liu and Crooks, 2011; Liu et al., 2012; Scida et al., 2013). Different parts of the microfluidic system are first fabricated in different positions in a unique piece of paper, and then the paper is folded to allow contact of specific zones and channels. In this assembly method, adhesive tape was avoided to minimize contaminations and nonspecific adsorption as well as to speed up the assembly process. However, these microfluidic devices have received negative feedback from practical diagnostic industry due to reproducibility issues and manual fabrication procedure.

3.1.2. *Flow control systems*

Liquid handling systems require the ability to stop, redirect or control the speed of fluid flow as well as to manage and separate sample mixtures. Unlike conventional microfluidic devices based on pressure-driven flow that use pumps, automated valves or pneumatic control systems (Au et al., 2011; Desai et al., 2012), paper-based microfluidics provide an alternative non-suction flow by generating fluid transport through capillarity (Kumar et al., 2015; Zhang et al., 2015b). Depending on practical functional applications such as transport, mixing and detection, various methods have been developed to control fluid flow in paper-based biosensors.

Toley et al. have reported the development of a paper microfluidic device with fluid-control valve actuation that can operate by movable paper strips and fluid-triggered expending element (sponge) (Toley et al., 2015). Xiao et al. have reported the development of a paper device with fluid-control valves that can operate by a magnetic cantilever triggered by a simple integrated circuit (Li et al., 2013). Magnetic valves can be turned “on” or “off” to allow fluid flow with programmable timing capability. However, the integrated circuit and an electromagnet in the device might increase fabrication cost and the complexity of the device. Barry et al. have developed 2D paper networks that used lateral flow and have the ability to reconfigure them to enable programming of multi-step reagent delivery sequences (Lutz et al., 2011). This paper network uses multiple fluid inlets to control the arrival time of each fluid to the detection zone or shut off each fluid source in a time sequence (Fig. 3A). The system is expected to provide a platform for autonomous multi-step fluidic processing. Time control of liquid movement through the channel can also be achieved by using water-soluble barrier materials such as sucrose or trehalose that increase resistance to fluid flow (Jahanshahi-Anbuhi et al., 2014; Lutz et al., 2013). The flow rate is mainly determined by the barrier thickness, and increasing this factor leads to a reduction in flow rate.

Translation of several conventional microfluidic devices that exploit adjacent flow streams onto 2D paper network has been intensively explored in literature over the last 5 years (Fu et al.,

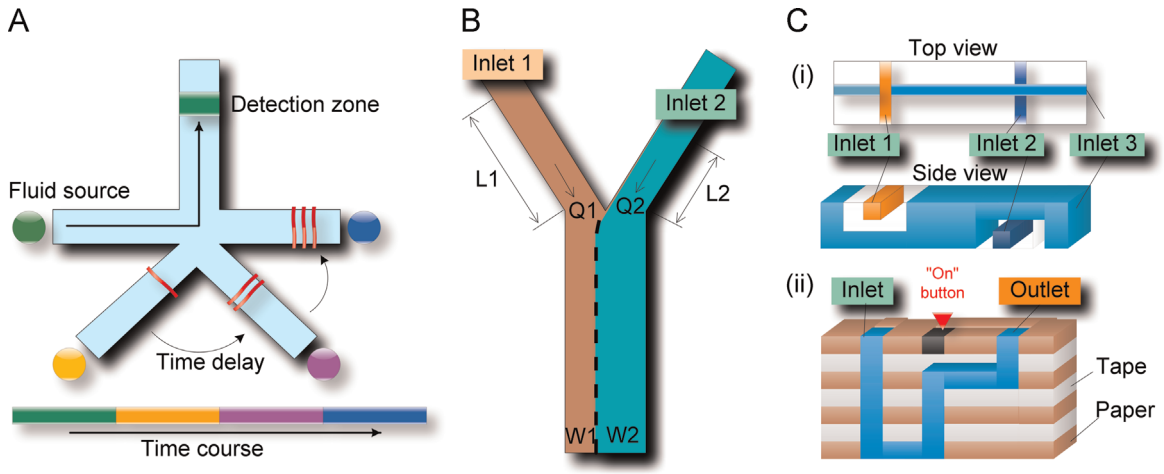

Fig. 3. Flow control in paper-based microfluidic sensor. (A) Time-controlled fluid delivery in 2D paper network. Each colored fluid can arrive to the "detection zone" at a different time course (Lutz et al., 2013). (B) Flow control and diffusion in a 3D structure Y-shape paper-based device. Alternating the inlet length (L) induces fluid flow rate changed (Q) and results in varying fluid width ($^{W2}/_{W1}$) ratio at downstream (Osborn et al., 2010). (C) Distribution of fluid flow in both vertically and laterally without mixing using (i) capillary wicking in 3D microfluidic paper analytical devices (Martinez et al., 2008a) and (ii) the ballpoint pen 'on' buttons allows fluid to wick from one channel to the other vertically in microfluidic channel (Martinez et al., 2010).

2011a; Kauffman et al., 2010; Kinahan et al., 2015; Osborn et al., 2010). These inexpensive and simple paper-based devices perform hydrodynamic focusing, diffusion-based analyte extraction from a complex mixture, micro-mixing, and rapid (single or serial) dilutions. A modified Y-shape device has been optimized to control the inter-diffusion zone by simply changing inlets position or their flow rate (Fig. 3B). Extraction of a desired species is implemented by simply cutting a region of interest from the device after flow has stopped. Niedl et al. have demonstrated a system in which chemical fluids stored inside responsive hydrogel reservoirs can be released, dissolved and mixed together upon external stimulation such as temperature. A cascade of chemical reaction can be implemented in paper-based microfluidic without the need of external pump (Niedl and Beta, 2015).

Martinez et al. fabricated 3D μPADs that enabled the user to design the structure of the channels, control the fluid flow, and control the device functions as shown in Fig. 3C-(i) (Martinez et al., 2010). With an "on" button fabricated by utilizing the gap distance between the adjacent layers of papers, hydrophilic material such as cellulose powder was used to fill the gap allowing fluid flow through. Thus, by using a standard narrow object such as a ballpoint pen, the gap could be closed and the devices could be manually programmed by users (Fig. 3C-(ii)). Recently, a versatile point-of-care μPAD platform, developed by Wei et al. can control fluidic flow in the microchannel in the absence or presence of target sample. In turn, an "on" or "off" color signal was generated and traveled to the observation spot (Wei et al., 2015). Li et al. described a reconfigurable switch for controlling capillary wicking in paper-based channels by cutting a channel into two parts and then manually separating or joining them to control the capillary flow (Li et al., 2008). Alternatively, Noh and Phillips programmed the flow rate of fluids by modulating the wettability of paper channels, thus controlling the time required for fluids to wick to different locations within a μPAD. Programmable μPAD have introduced a different, practical method for controlling capillary flow in paper-based devices (Noh and Phillips, 2010).

3.1.3. *Separation and preconcentration of complex samples*

Real-world samples are usually complex mixtures of different molecules, solvents and other compounds. For a number of spectroscopic analytical techniques that can be used without specific receptors or receptor-ligand interactions; the sensor ability to detect a target analyte depends on how the analyte can be effectively extracted from the mixture. For increased accuracy and sensitivity it is necessary that biosensors integrate the ability to process and separate mixtures in order to decrease the background signal prior to analysis. Two important properties of paper can be designed to enable sample separation: surface chemistry and the porous structure. In non-functionalized cellulose paper, anionic molecules or particles can easily move under the effect of both capillary action and repulsion forces of the cellulose hydroxyl groups. On the other side, cationic molecules tend to adsorb directly onto paper through electrostatic interactions, which prevent their displacement. To overcome this problem and provide more flexibility for sample movement and separation, different groups have proposed a number of solutions that can be classified into three major strategies: the use of immobilized receptors to specifically capture the molecule of interest and extract it from the mixture (affinity separation), the use of a filtration membrane (size exclusion), and separation by taking advantage of the physical–chemical properties of the target molecule such as diffusion capabilities or electrostatic interactions (Fig. 4).

Separation of blood components is one issue that has attracted interest on μPADs-based separation. Despite their size averaging 7 μm, red blood cells can diffuse through a membrane with pores of 2.5 μm due to their deformability. The use of membrane with smaller diameter has allowed the separation of blood cells from plasma. Songjaroen et al. used this concept by combining pasting a blood separation membrane and a paper strip with overlapping regions. Then iron mold was used to form hydrophobic channels by wax dipping (Songjaroen et al., 2012). After deposition of a blood sample on the membrane area, blood cells were trapped inside, allowing plasma to travel to the detection area through a patterned microchannel. However, in addition to being relatively expensive, membranes with pore size smaller than 2.5 μm tend to slow down the flow and thus increase the processing time. To overcome this problem, another research group combined the use of a separation membrane with agglutinating antibodies (anti-A, B) (Yang et al., 2012). The deposition of whole blood on a μ-PAD functionalized with anti-A, B results in the formation of large red *blood* cell aggregates that are easily filtered using membranes with large-diameter pores, while allowing faster flow and separation blood serum. However, as in any other paper-based separation, the extraction of the components from paper could be a daunting task. Another interesting separation strategy is to take advantage of the sample chemical properties and its interaction with the paper

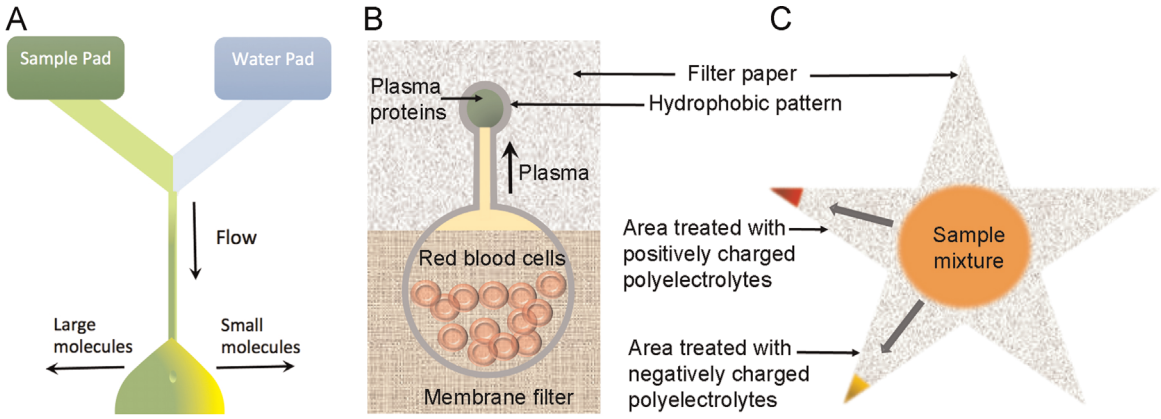

Fig. 4. Paper-based separation of complex mixtures. (A) Size based separation on filter paper, mixture of protein (blue) and small molecule (yellow) in the left sample pad and H_2O in the right. Small molecule separates in right zone (adapted from (Osborn et al., 2010)). (B) Separation of plasma from whole blood by using combination of filter paper and blood separating membrane. Plasma protein detected by bromocresol blue (BCG) (green) (adapted from (Xiong et al., 2012)). (C) Separation of dyes by treating fingers with cationic (PAH) and anionic (PSS) chemicals (adapted from (Abbas et al., 2013a). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

matrix. The use of the diffusion coefficient of molecules has been reported by Osborn et al. (Osborn et al., 2010). Large molecules tend to diffuse slowly as compared to small molecules. Based on this fact, the group developed a two dimensional microfluidic paper-based H-filter to separate proteins and small molecules. The design consists of a modified Y device with a geometry that has been optimized to broaden the inter-diffusion zone (Fig. 4.B–i). Extraction of desired species from a mixture is implemented by simply cutting a region of interest from the device after flow has stopped. However, this technique would be difficult to implement for molecules that have good affinity to paper, and it is limited by the ability to extract the separated product and overcome adsorption. Other sample properties such as solubility in a given solvent can also be used for separation (Carvalhal et al., 2010).

Using a different strategy, Abbas et al. showed that filter paper can be effectively used to separate complex mixtures by a differential surface functionalization and a simple geometrical design (Abbas et al., 2013d). They prepared a star shaped filter paper and treated each arm a different concentration of poly (allylamine hydrochloride) (PAH), or poly (sodium 4-styrenesulfonate) (PSS), thus creating a gradient of surface charge. The components of different mixtures migrate in different arms of the paper star and concentrate at different distances. In addition to obtaining efficient separation of mixtures, this approach enables a subsequent preconcentration of the separated molecules at the tip of the arms with a remarkable preconcentration factor of 10^9. Such increase in sample concentration enabled attomolar detection limits using surface-enhanced Raman scattering. The pre-concentration of molecules at the tips is mainly driven by a fast evaporation in a relatively small area, leading to the accumulation and easy extraction of molecules.

Another preconcentration method was reported by Moghadam et al. by implementing isotachophoresis on paper (Moghadam et al., 2014). In this nonlinear electrophoretic technique and under the effect of a constant current, sample ions migrate and accumulate in different zones between leading and trailing electrolytes. The migration depends on the electrophoretic mobility of the molecules. This method achieved a pre-concentration factor of around 10^3 and an effective extraction of 60% from 100 μL sample volume. More recently, single cell separation of microalgae from mixture was shown using paper-based device. The carbon- powder pores were printed on paper with specific diameter and depth to retain only a single cell. The procedure also showed that micro algal colonies could be grown from this separated single cell. Hence, this technique offered better separation as compared to labor intensive conventional methods Chen et al. (2015). In another study, a technique for separation of cells was proposed using chemotaxis of cancer cells. Single layer of wax-patterned paper impregnated with a cell-compatible hydrogel was made. One layer contained cancer cells in hydrogel while other layers contained blank spots and spots with hydrogel alone. Then different gradients of oxygen concentration were used as chemotaxant. Cells having high metastatic potential showed more chemotaxis. Thus this method showed new way to differentiate cells on basis of their chemotactic behavior but the limitation associated with this technique is its inability to detect single cell migration needs to be improved (Mosadegh et al., 2015). A similar but simpler approach was proposed by Walsh et al. in which they made a gradient of chemoattractant on paper to cause cells to move (Walsh et al., 2015). Owing to its importance, research in separation and preconcentration research on μ-PADs is expected to significantly increase over the next few years.

3.2. Surface activation and biofunctionalization

Cellulose paper is mainly composed of a homopolymer of (1→4)-β-glucopyranose linked through acetal bonds and forming long β-1,4-glucan chains. The hydroxyl groups of these chains make the paper hydrophilic with a negative charge. As a result, paper can only adsorb cationic molecules. The immobilization of biomolecules that have usually an overall negative charge or citrate-capped nanoparticles require the use of crosslinkers or intermediate surface coating. To allow the immobilization of a variety of molecules and particles and enable biosensing applications on paper, a large number of methods have been developed (Fig. 5). Three of the most commonly used approaches are described below.

3.3. Adsorption

In this type of functionalization, a material is adsorbed on paper using electrostatic and Van der Waals forces. This method offers a simple and inexpensive way to functionalize complex paper sensors. It can be achieved by dip coating in which paper is simply dipped in the solution of interest for a short time and is then dried to yield the desired property. For example, Songjaroen et al. used wax to form hydrophobic patterns on paper using an iron mold (Songjaroen et al., 2011). Layer by layer deposition is the second major adsorption technique used to functionalize paper. Different compounds can be used to make hydrophobic or cationic zones on the paper by alternating cationic polymers with anionic

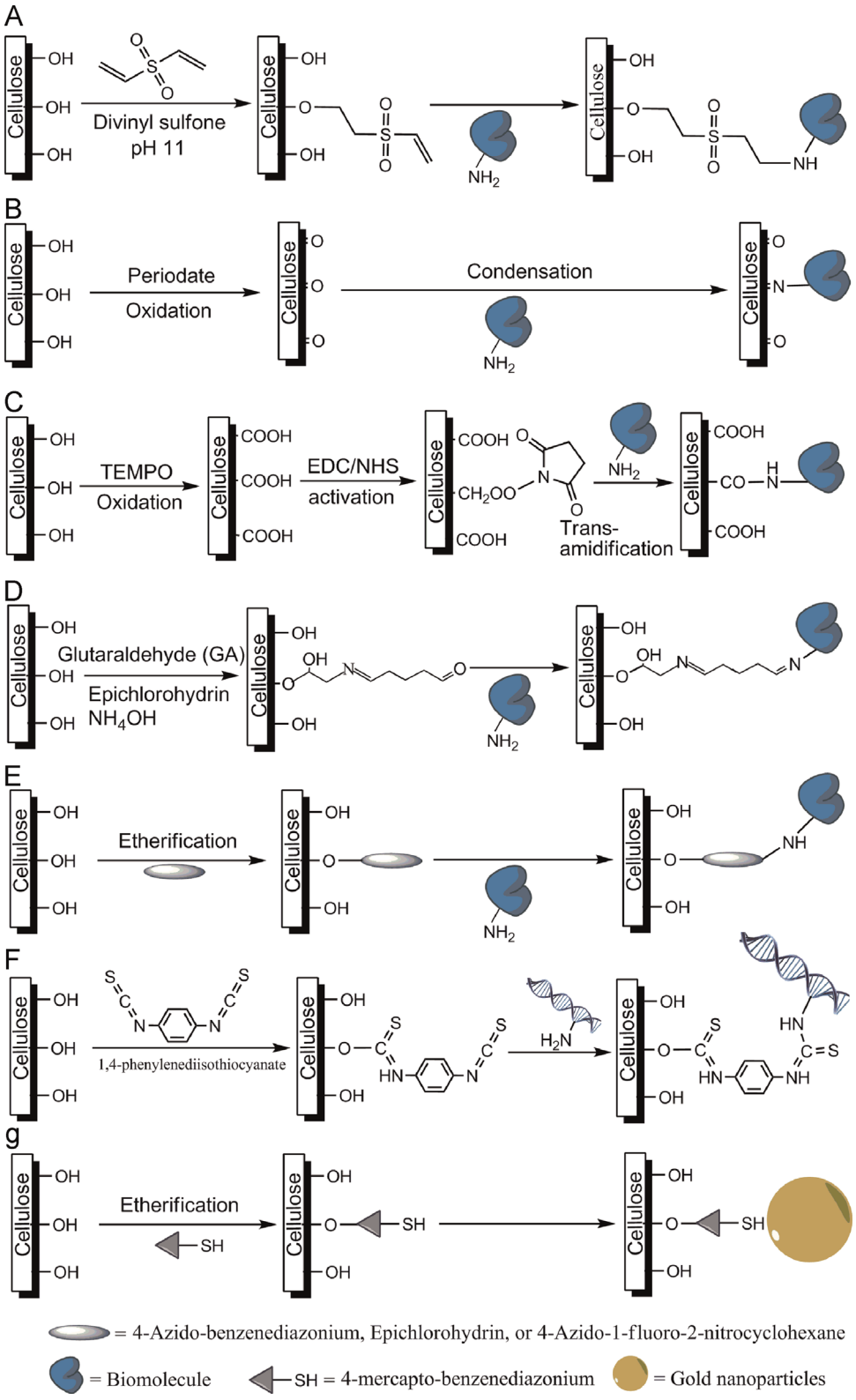

Fig. 5. Scheme of different surface functionalization methods of cellulose paper.

material (Li et al., 2012; Xing et al., 2007). The third technique of modifying paper by adsorption is ink jet printing. This technique is relatively expensive as compared to the aforementioned methods, but it produces more accurate patterns on paper. A consumer inkjet printer has already been used to form a layer of silver nanoparticles on paper (Yu and White, 2010). The printing was accomplished by filling the ink cartridges with nanoparticle solution and printing the desired pattern, which allows repetitive printing of patterns on the same spot. A similar technique was utilized with biomolecule (bio ink) loaded ink cartridges (Khan et al., 2010; Xu et al., 2005), and with polymers (Elsharkawy et al., 2014; Xu and Enomae, 2014). The main issue associated with this

type of printing is that repeated printing can cause contamination, and the biomolecule activity can be affected by high temperature printing. Other adsorption techniques can combine multiple components. Zinc oxide (ZnO) nanoparticles have been used to functionalize paper for the immobilization of immunoglobulins (IgGs). ZnO forms a good interface for IgGs immobilization because of its high isoelectric point, as compared to biological molecules that usually have a low isoelectric points (Khatri et al., 2014).

3.4. Covalent modification

The available functional groups on cellulose paper include a backbone of hydroxyl groups and the reducing end of the cellulose ring. Ultimately these groups are fairly unreactive, however they can be chemically modified to introduce more reactive groups. Hydroxyl groups in the cellulose backbone are mainly involved in these chemical reactions. The availability of these hydroxyl groups and reactivity varies form one carbon position to other. Based on the involvement of carbon atoms in intramolecular hydrogen bonding, the OH group of carbon at position 3 (C3) is the least reactive while most reactive ones are at C2 and C6 (Credou and Berthelot, 2014). Chemical functionalization provides better binding of biomolecules to paper surface by covalent bonding, but often these processes involve a number of steps and can compromise the paper properties.

Different attempts have been made to chemically modify paper in order to immobilize biomolecules and nanoparticles. Paper can be oxidized to produce carbonyl or carboxyl groups, which can then covalently bind with amine terminated molecules such as proteins or DNA. Carboxyl groups can be introduced by oxidation of OH group at C6 by using sodium bromide (NaBr), sodium hypochlorite (NaClO) and (2,2,6,6-tetramethyl-piperidin-1-yl)oxyl free radical (TEMPO). These treatments have been used for the immobilization of enzymes (Karra-Châabouni et al., 2008). Another way to introduce carboxyl groups without oxidation is by etherification of hydroxyl groups of cellulose. First, the OH group was activated by treatment with NaOH and was then reacted with monochloroacetic acid (Ibrahim et al., 2013). Photoreactive paper functionalized with fluoro-2-nitro-4-azidobenzene (FNAB) has been produced for the immobilization of proteins. The advantage of this technique is that different biomolecules can be attached irrespective of their functional groups (Bora et al., 2006). Amine groups were introduced on paper by using tosyl chloride to activate OH groups and bonding them with triethanolamine (Diekmann et al., 2003). Other oxidation methods by which we can activate the surface of paper include the epichlorohydrin reaction and reaction with to glycidyl methacrylate (GMA) to yield epoxy groups (Tyagi et al., 2009). The bioaffinity property of biomolecules can also be used to functionalize paper. This bioaffinity is important because there is a large family of proteins which have cellulose binding domains. Recombinant technology was also used to produce recombinant biotin with cellulose binding domains h. zu0gcz (Bodelón et al., 2014).

Paper or microfibrillated cellulose has also been modified by esterification of the OH groups of cellulose with COOH groups. Different azo dyes have been reported to immobilize on paper by this method (Isaad and El Achari, 2011). Cationic nitrocellulose paper can be made by esterification of hydroxyl groups using nitric acid in the presence of a strong acid such as phosphoric acid or sulfuric acid (Kennedy and Quinton, 2000). Other cationic polymers that increase biomolecule binding efficiency include (polyamide-epichlorohydrin (PAE) and polyvinyl amine (PVA)) (Geffroy et al., 2000). Cen et al. showed that paper can even be modified to make it antibacterial. In this work, paper was modified with N-hexyl bromide poly (4-vinylpyridine) group. First, paper was activated by UV to form copolymer of 4-vinyl pyridine (4VP). Then, it was quaternized with hexyl bromide (Cen et al., 2003).

Cellulose fibrils can also be reacted with polyamide-epichlorohydrin (PAE) and polyvinyl amine (PVA); derivatives to produce hydrophobic cellulose esters. (Geissler et al., 2014). Beta cyclodextrin, a cyclic oligosaccharide of glucose, has also been used in improve hydrophobicity as it can be chemically attached to cellulose by using a citric acid cross linker (Dong et al., 2014). In addition to the aforementioned chemical modifications, other methods that are being employed for paper functionalization were described in detail by Heinze and Kalia (Heinze and Liebert, 2001; Kalia et al., 2014).

3.5. Entrapment

Bioactive paper can also be produced by entrapping biomolecules on paper. In an interesting study, sol–gel procedure was used to entrap enzymes and their substrates in different layers (Hossain and Brennan, 2011). First, an enzyme-loaded sol–gel was layered on paper by ink jet printing, followed by forming layer of substrate in sol–gel. The two layers were separated by a layer of silica. This resulted in a stabilized enzyme matrix that remained viable for 2 months at 4 °C and for two weeks at room temperature. In another study, bioactive enzymes and gold nanoparticles were encapsulated by sol–gel method and paper was coated in this sol–gel solution (Luckham and Brennan, 2010). Wang et al. entrapped an enzyme between different layers of polymer (poly L-arginine) and silica. These multiple layers were used to ensure proper stability and preserve the activity of the enzyme (Wang et al., 2014a). A combination of adsorption, covalent attachment and entrapment can also be found in literature.

3.6. Transduction systems

Cellulose paper is an organic material with no significant optical, electrical or chemical properties that can be used for transduction. Hence, any application for sensing requires the integration with a transducer which could be colorimetric, electrochemical or other materials capable of converting a bio/chemical reaction that occurs on the paper into a visible or a measurable signal.

3.6.1. Colorimetric transduction

Visual color formation or color change due to molecular interactions is often the easiest and most cost-effective detection system. The color shift in the visible range can be produced by a number of different phenomena and many types of reactions can be used for the development of colorimetric sensors. Enzymatic reactions can yield products that absorb in the visible range, which is commonly used in a variety of ELISA techniques and other enzyme and nanozyme-based colorimetry (Cheng et al., 2010;Hossain and Brennan, 2011; Luckham and Brennan, 2010). A change in color can also be obtained chemiluminescence (Gámiz-Gracia et al., 2005; Liu et al., 2014; Wang et al., 2012; Yu et al., 2011), by a change of pH in a solution, chemical reactions (Bui and Abbas, 2015), chemical transitions in molecules (Brockgreitens et al., 2015), supramolecular self-assembly _ENREF_125 (Abbas et al., 2013b), or by the assembly/aggregation of nanoparticles (Abbas et al., 2012b). Abundant literature is also available on colorimetric detection using chromogenic chemical reactions. A number of these reaction can be obtained with a variety of metal ions interactions and chromogenic compounds. An example is the detection of Fe (III) in industrial waste solution using μPADs (Apilux et al., 2010). In this study ascorbic acid and 1, 10-phenanthroline was added to paper and then Fe (III) was added to the test zone. Ascorbic acid caused the reduction of Fe (III) to Fe (II), which in turn yielded a chromogenic complex with 1,10-phenanthroline.

Simultaneous colorimetric detection of glucose, ketone in urine and nitrite in saliva was also reported (Klasner et al., 2010). The reacting molecules for each analyte were immobilized on paper and produced chromogenic products. Glucose was detected by an enzymatic reaction with glucose oxidase. Ketone reacted with glycine to produce imine derivatives, which then form color complexes with sodium nitroprusside. Finally, nitrite was converted to nitrous acid by using citric acid. This nitrous acid forms diazo compound in presence of sulfanilamide which then produced a colored product by forming a complex with *n*-(*1*-naphthyl) ethylenediamine. A very selective and sensitive method with limit of detection of 1 ng/mL for copper ions was also reported (Chaiyo et al., 2015). The mechanism for color development was based on thiosulfate mediated catalytic etching of silver nanoplates (AgNPls) in the presence of copper ions. This yields a change in color from pink to transparent depending on concentration of copper ions. For the detection of phenolic acid (important antioxidants), a simple color reaction between phenolic acids and Fe^{3+} was exploited to make another paper based colorimetric sensor (Xiang et al., 2015). The paper substrate was functionalized with Fe^{3+} and the color reaction was developed upon interaction with the analyte. The method showed a micromolar detection limit. Colorimetric methods were also developed for the detection of proteins using bromophenol blue to stain proteins (Wang et al., 2010). The unique idea proposed in the study was the self-calibrating ability of the system. This method showed that simple, inexpensive, semi quantitative and self-calibrating device could be produced.

3.6.2. Plasmonic transduction

Plasmonic sensors can be developed either in propagative mode using continuous metal wires or thin films on a transparent substrate (Abbas et al., 2011), or in localized mode known as localized surface plasmon resonance (LSPR), which requires the use of noble metal nanoparticles. Given the fibrous and opaque nature of paper, only LSPR has so far been used for paper-based plasmonic sensors. All of these sensors take advantage of two important LSPR properties:

i. Plasmonic colorimetry: Localized surface plasmon resonance (LSPR) is the collective oscillation of conduction electrons at the surface of noble metal nanoparticles, under the excitation of electromagnetic radiations. The assembly or aggregation of gold nanoparticles results in the coupling of plasmonic fields surrounding each particle, and the generation of enhanced field and sensitivity in the inter-particle space, called plasmonic hotspots (Abbas et al., 2013c; Abbas et al., 2012a). At the macroscale level, this phenomenon translates into a change in light absorbance and thus a change in color of the nanoparticle solution (Abbas et al., 2012a). The use of plasmonic colorimetry in biosensing mainly involves the detection of a target that directly or indirectly causes nanoparticle aggregation. This strategy has been largely used in lateral flow assays (Hu et al., 2014; Sun et al., 2014).
ii. Surface-enhanced Raman scattering (SERS): The use of plasmonic nanoparticles with Raman spectroscopy results in a remarkable enhancement of the collected signal from a target analyte, ranging from 10^4 to 10^{12} folds. This phenomenon has been first reported in 1974 (Fleischmann et al., 1974), and has since been used in abundant literature dealing with sensing, and using a variety of nanoparticles such as silver nanoparticles (Yu and White, 2010), gold nanorods (Lee et al., 2011; Tian et al., 2012) and gold nanospheres (Ngo et al., 2012). SERS is based on the combination of Raman spectroscopy with LSPR, and thus it is an interfacial phenomenon. As a result, SERS analysis is only effective for the detection of molecules adsorbed on nanoparticles or present in their vicinity (< 5 nm). Best results are obtained with resonant molecular structures containing a benzene ring, or molecules containing amine or thiol functional groups capable of attaching to gold nanoparticles. While paper provides better SERS enhancement as compared to other substrates due to its 3-dimentional fibrous matrix (Lee et al., 2010), it fails to provide high reproducibility for the same reasons and its quantitative capabilities are limited.

The anisotropic and non-homogeneous structure of paper fibers results in inhomogeneous adsorption of nanoparticles and random formation and distribution of plasmonic hot spots. unlike in other nanostructured substrates, SERS data collected from nanoparticle-impregnated paper can be significantly dependent on the position of the laser spot ($\sim 5\ \mu m$) used for analysis. reproducibility Is probably the main challenge facing paper-based SERS analysis today, and the development of new impregnation methods such layer by layer printing will certainly help achieve a uniform sensitivity (Wang et al., 2014c). despite this limitation, paper-based SERS offers one of the best detection limits in analytical chemistry, reaching values down to the attomolar level (Abbas et al., 2013a; Polavarapu et al., 2014).

3.6.3. Electrochemical transduction

Electrochemistry is the second most used transduction system after colorimetry for paper-based sensing. It mainly consists on the deposition of planar electrodes on a paper substrate for elective detection of electrogenic reactions. Beside rapid and specific detection, the most attractive feature of electrochemical transduction is the facile implementation into robust, portable, and miniaturized devices with digital reading. The transduction mechanism can be performed in different modes including cyclic voltammetry, chronoamperometry, square wave voltammetry and potentiometric measurements (Lei et al., 2012; Lisak et al., 2015; Shiroma et al., 2012). Usually, the fabrication of electrochemical sensing devices on paper requires the deposition of conductive inks or metals on the paper network to form conductive electrodes (Fig. 6) (Ge et al., 2014; Nie et al., 2010). A wide range of methods have been used to fabricate electrodes on paper including screen printing, stencil printing, sputtering deposition, metallic wire tape and nanoparticle growth (Adkins et al., 2015). The material could be different from metal source or mixture with carbon, graphite, and metallic nanoparticles for deposition on (de Araujo and Paixao, 2014; Kong et al., 2014; Renault et al., 2014; Santhiago et al., 2014). However, its high roughness and the fibrous nature of paper cause some limitations in electrode quality and performance.

In addition to generating electrons, the incorporation of specific enzymes and their substrate can electrochemically generate species that emit light by chemiluminescence and electrochemiluminescence (ECL) (Forster et al., 2009). Liu et al. have reported a self-powered origami 3D fluidic paper analytical device for the detection of adenosine using aptamers as the sensing probe, incorporated with electrochemical readout using a digital multimeter (Liu et al., 2012). This novel fabrication strategy overcomes the need of direct printing of the electrodes onto the channel architecture, which would render the channels hydrophobic owing to the binder present in the carbon ink. Zhang et al. introduced a self-powered μPAD device with carbon nanotube modified paper as electrodes and hollow channel for fluid transport. Hg^{2+}-specific aptamer functionalized on AuNP modified carbon nanotube electrode provide high sensitive Hg^{2+} detection at picomolar level. Importantly, the μPAD device can be self-powered using an integrated glucose/O_2 biofuel cell with Pt modified carbon modified electrode system which provides portability and point-of-care testing device in remote areas (Zhang

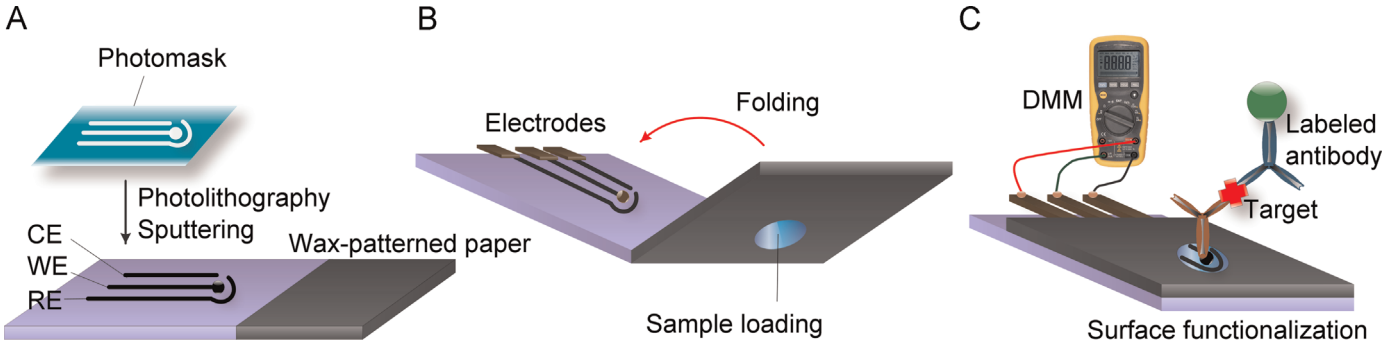

Fig. 6. Electrochemical sensor on paper-based microfluidic. (A) Fabrication process of paper-based electrochemical sensor with integrated working electrode (WE), counter electrode (CE) and reference electrode (RE) using standard photolithography and metal sputtering (Shiroma et al., 2012). (B) 3D electrochemical analytical device with electrode surface functionalization (enzyme, antibody, receptor, oligonucleotide, bio-marker) for two or more transducer signal generation (Rattanarat et al., 2014) (C) Bioassay on 3D paper-based electrochemical sensor with digital multimeter (DMM) reader (Lu et al., 2012).

et al., 2015a). Unlike colorimetric sensors, chemiluminescence and electrochemical techniques offer the ability to eliminate background interference in portable microfluidic devices (Liu and Zhang, 2015; Wang et al., 2015). Delaney and Gao have fabricated a paper-microfluidic with electrochemiluminescent detection based on the reaction of tris(2,2′-bipyridyl)ruthenium (II) (Ru(bpy)) for the detection of 2-(dibutylamino)ethanol, nicotinamide adenine dinucleotide and carcinoembryonic antigen on a 3D microfluidic origami device (Delaney et al., 2011; Gao et al., 2015). When a potential is applied, the generated signal can be detected by placing the detection zone close to the lens of a camera phone. The detection limit was achieved in the femtomolar level with this approach.

Many other research groups investigated the fabrication of 3D electrochemical μPAD using wax-patterned paper and screen-printed electrodes for multiplexed immunoassays (Wu et al., 2015). The 3D μPAD provided good correlation between the concentration of tumor markers and the ECL peak intensity over a wide dynamic range that covered most of the levels found in human plasma and serum. Sensitive DNA detection was developed based on the amplification effect of graphene modified on gold-paper working electrode (Au-PWE) and further amplification upon DNA sandwich hybridization.

A significant research is currently directed towards the modification of the electrodes with functional materials (Li et al., 2014a), and the combination of two or more transduction schemes (Rattanarat et al., 2014). The hyphenation of electrochemical μPAD with commercial analytical devices such as glucometers, blood analyzer systems and enzyme-based biosensors for the food industry has opened a great potential for field-testing platforms. Extensive development and increased industrial translation is expected in this field over the next few years.

4. Challenges and perspectives

Future development of paper-based analytical devices is driven by the convergence of a number of technological trends observed over the last few years: (i) The shift in healthcare paradigm towards personalized and preventive medicine and active patients opens up new demand for home-health monitoring and self-testing; (ii) The broadening of the possibilities of printing technologies, enabling a rapid deposition on paper of a variety of materials including metals, nanoparticles and biomolecules; (iii) the generalized use of smartphones or mobile communication devices including in low resource countries, which provides the opportunity to perform imaging and image analysis on site using disposable devices, and (iv) the rise of sustainability awareness in science, technology and business, which promotes the use of bio-based environment-friendly renewable materials such as cellulose.

To take advantage of this favorable environment, paper-based analytical devices need to overcome a number of challenges including:

1. *Performance*: The development of new strategies for sample pre-concentration is a major direction in μPADs research. Owing to the usual use of naked-eye detection or hand-held devices, the inherent limitations of μPADs (sensitivity, limit of detection) can only be overcome by an effective separation and pre-concentration process to allow the analysis of minute analyte concentrations. A few papers only were published so far on this issue but more are expected in the future. Multiplex detection is another major need for paper-based assays. The ability to detect multiple targets in a single assay will immensely improve the usability and cost-effectiveness of paper-based tests.
2. *Readouts*: There are a number of applications where quantification is not a major need. When qualitative detection (presence or absence of an analyte or pathogen) is the main information, visual detection with the naked eye would be sufficient. This is particularly true in food safety monitoring, where the presence or lack of salmonella or listeria for instance will directly result in product recall. However, in many other applications, the concentration of the analyte is the main information, and this ultimately will require digital reading by electrochemical devices, LED readers, smartphones or hand-held spectrometers. Such integration is important for medical diagnostics or food spoilage where the effect of a molecule or a microbe depends on its concentration. Despite the relative increase in cost with the integration or use of readers, this development will be necessary to make paper-based sensing an accurate and reliable technology. Recent advances in paper-based batteries add another step towards the simplification of μPADs readers (Nguyen et al., 2014).
3. *Stability of biological components:* Although μPADs are usually disposable devices, their effective commercialization requires a minimum stability to preserve the biological activity during transport and storage. One of the major directions to address this issue is the development of new and robust recognition units that could replace natural antibodies. Aptamers and artificial receptors have successfully been used, yet their onerous development conflicts with the simplification goal of paper devices (Consden et al., 1944). Nanozymes (nanoparticles with enzyme-like activity) are another promising direction to replace natural enzymes that could be instable in harsh environments (Consden, 1948). In addition to artificial alternatives for biological molecules, the use of lyophilization can help preserve biological components until use.
4. *Paper engineering*: The functionality of cellulose paper depends

on its structural, physical and chemical properties, which in turn are related to the fabrication process of paper. Filter paper currently commercialized was designed for filtration activities. Customized paper for μPADs can be developed by engineering the structure, composition or surface chemistry of cellulose fibers, or by combining cellulose with optically/electrically active materials. Such hybrid materials would provide a matrix with intrinsic transduction properties.

5. *Printing biosensors:* The ultimate route for μPADs simplification and commercial viability is to be able to perform all the design and fabrication steps using a printing process. A number of reports already demonstrated the feasibility of printing nanoparticles (Yu and White, 2013), electrodes (Määttänen et al., 2013), biomoleclues (Khan et al., 2010; Voskuhl et al., 2014), and a variety of polymers (Wang et al., 2014a) but more work is needed to integrate the different functionalities and overcome the challenges related to ink development and stabilization.

5. Conclusion

In this review, we describe the basic concepts and highlight the major technological and engineering developments performed over the last decade in the field of paper-based bio/chemical sensors. The fundamentals in paper microfluidics are described and different approaches of device fabrication, functionalization, and fluid control and transduction modes are critically discussed. The review provides the key challenges facing paper-based sensing today and the expected research and development trends over the next few years. It is clear from this incomplete review that paper-based sensing and diagnostics will move beyond its initially targeted use as a cost-effective screening tool for resource-limited environments. Due to new advancements in nanotechnology and materials engineering, paper-based sensing is becoming a serious alternative for rapid and affordable diagnostics, field testing for food safety and environmental control, and self-monitoring for personalized medicine.

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