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Advances in paper-based point-of-care diagnostics

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ABSTRACT

Advanced diagnostic technologies, such as polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA), have been widely used in well-equipped laboratories. However, they are not affordable or accessible in resource-limited settings due to the lack of basic infrastructure and/or trained operators. Paper-based diagnostic technologies are affordable, user-friendly, rapid, robust, and scalable for manufacturing, thus holding great potential to deliver point-of-care (POC) diagnostics to resource-limited settings. In this review, we present the working principles and reaction mechanism of paper-based diagnostics, including dipstick assays, lateral flow assays (LFAs), and microfluidic paper-based analytical devices (μPADs), as well as the selection of substrates and fabrication methods. Further, we report the advances in improving detection sensitivity, quantification readout, procedure simplification and multi-functionalization of paper-based diagnostics, and discuss the disadvantages of paper-based diagnostics. We envision that miniaturized and integrated paper-based diagnostic devices with the sample-in-answer-out capability will meet the diverse requirements for diagnosis and treatment monitoring at the POC.

Contents

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1. Introduction

Annually, infectious diseases, including acquired immunodeficiency syndrome (AIDS), tuberculosis (TB) and malaria, cause approximately 15 million deaths, accounting for about 25% of deaths worldwide (Bissonnette and Bergeron, 2010; Morens et al., 2004). More than 95% of the deaths occur in developing countries due to the lack of cost-effective medical interventions (Lee et al., 2010; Yager et al., 2006). Diagnostics are commonly used to diagnose the cause of symptoms in patients, to monitor the efficacy of treatment, and to screen for potential diseases in asymptomatic but high-risk population (Hay Burgess et al., 2006). As such, diagnostics are of importance in the healthcare system and have a critical impact on decision-making clinically and epidemiologically. For example, malaria causes one child death in every 45 s and nearly one million infant deaths per year in Africa, despite the fact that this disease is both preventable and curable. Since immediate treatment is required upon the appearance of symptoms, rapid and specific diagnosis of malaria would be critical for the treatment and prevention (Mukhopadhyay, 2010). However, the diagnosis is often based on the clinical experience without any laboratory evidence in resource-limited settings. If a rapid test kit is affordable, simple-to-operate, and accurate to diagnose malaria, it can be a lifesaver.

Although advanced diagnostic technologies, such as polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA), have already been implemented in developed countries, they cannot be widely used in developing countries because of limited availability of laboratory infrastructure, skilled personnel and financial supports (Hay Burgess et al., 2006; Mabey et al., 2004; Martinez et al., 2010b; Yager et al., 2006, 2008). Compared with standard laboratory testing, point-of-care (POC) diagnostics are rapid, simple and inexpensive, and thus have great accessibility to resource-limited settings (Hart et al., 2011; Tabak, 2007). Therefore, POC diagnostics are essential to initiate and scale up on-site medical care for the prevention and control of infectious diseases (Hauck et al., 2010; Lee et al., 2010; Wang et al., 2010, 2013b).

Microfluidic technologies have been widely employed in developing POC diagnostics to address global health issues because of their evident advantages (Hawkins and Weigl, 2010). For example, microfluidic devices, coupled with different functional units (e.g., pumps, valves and reactors) and integrated into a miniaturized analytical system (Chin et al., 2007; Hawkins and Weigl, 2010), can manipulate small volumes of fluids (Whitesides, 2006). Therefore, microfluidic devices significantly reduce the consumption of samples and reagents, the complexity of operation procedures, and the length of assay time without compromising specificity and sensitivity (Chin et al., 2007; Laksanasopin et al., 2009; Whitesides, 2006). On the other hand, most microfluidic devices, which are made of glass, silicon, and polymers like poly(dimethylsiloxane) (PDMS) and poly (methylmethacrylate) (PMMA) (Martinez et al., 2010b; Nilghaz et al., 2012), require both complex fabrication processes and external instruments, thereby making them unsuitable for POC testing (Nilghaz et al., 2012). In contrast, paper-based microfluidics hold great potential to deliver POC diagnostics to developing countries in terms of being **a**ffordable, **s**ensitive, **s**pecific, **u**ser-friendly, **r**apid and robust, **e**quipment-free, and **d**eliverable to end-users (**ASSURED**) (Hawkins and Weigl, 2010; Lee et al., 2010; Martinez et al., 2010b). The detailed comparison between chip-based and paper-based devices is listed in Table 1.

Although there are some recent reviews on paper-based diagnostics (Parolo and Merkoçi, 2013; Yetisen et al., 2013), we here focus on the substrates and methods to design and fabricate paper-based microfluidics, the working principles and reaction mechanism of paper-based diagnostics, and most importantly, the latest advances in improving applications of paper-based diagnostics at the POC. We also discuss the challenges associated with paper-based diagnostics for improving their performance and accessibility.

2. Substrates, fabrication methods and working principles of paper-based diagnostic devices

Paper fabrication is one of the most important technologies in human history (Grifantini, 2009), which was born in the 2nd century AD in China (Rooz, 2010). Currently, paper (including membrane) based materials have been utilized for biochemical analyses, including dipstick assays, lateral flow assays (LFAs), and microfluidic paper-based analytical devices (μPADs) (Parolo and Merkoçi, 2013). Dipsticks refer to urine test strips at the beginning. They were developed by a Parisian chemist, Jules Maunmené, in 1850 and marketed by an English physiologist, George Oliver, in 1883. Subsequently, pH test strips were patented and commercialized in the 1920s (Foster and Gruntfest, 1937). Till 1956, the first latex agglutination assay developed by Plotz and Singer laid the technical basis of LFAs (Wong and Tse, 2009). Since then, the basic principle of LFAs has been further refined and widely used for rapid detection of infectious diseases. More recently, Whitesides et al. introduced paper-based microfluidics to fabricate low cost and simple μPADs, which were highlighted as one of 10 emerging technologies in 2009 by *Technology Review* (Grifantini, 2009). The substrates and fabrication methods of basic dipsticks, conventional LFAs and emerging μPADs, and their working principles are introduced as follows.

2.1. Paper substrates and alternatives

Nowadays, a variety of paper materials have been developed and two main kinds have been widely utilized to fabricate diagnostic devices for POC testing (Ballerini et al., 2012; Yetisen et al., 2013). One is cellulose fiber based materials, such as filter

Table 1
The comparison between chip-based and paper-based devices.

Comparison	Chip-based device	Paper-based device
Material	Glass, silicon, polymer, etc.	Paper and membrane
Manufacture	Channel fabrication and surface modification	Hydrophilic channels and hydrophobic barriers
Driving force	Pump	Capillary force and evaporation
Result analysis	Reader	Reader or visual detection

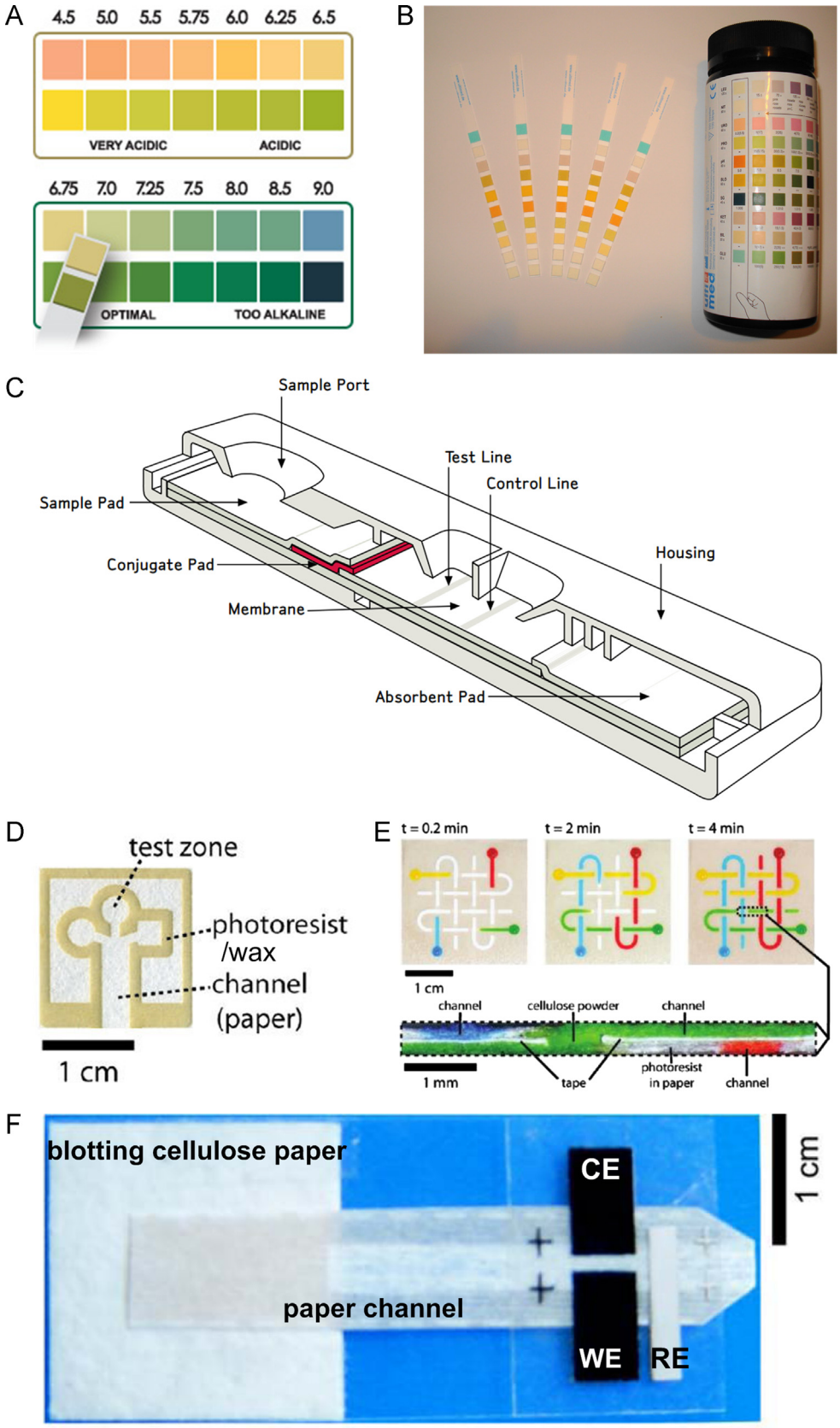

Fig. 1. Three kinds of paper-based diagnostic platforms: dipsticks (A–B), lateral flow test strips (C), and microfluidic paper-based analytical devices (μPADs, D–F). (A) Diagnostic pH test strips (http://www.phionbalance.com). (B) Urine test strips (http://www.omegairl.com). (C) A typical schematic view of a lateral flow test strip, including complex and sandwich formats (Millipore, 2009). (D) A typical two-dimensional (2D) μPAD (Martinez et al., 2010b). (E) A typical three-dimensional (3D) μPAD (Martinez et al., 2008b). (F) A typical microfluidic paper-based electrochemical device (μPED) (Nie et al., 2010b).

paper and chromatography paper, which are the major substrates of dipsticks and μPADs (Pelton, 2009). The other is nitrocellulose membrane, which is the key material for LFAs (Millipore, 2009; Wong and Tse, 2009). Cellulose, which is a linear chain macromolecule composed of hundreds of glucose units (O'Sullivan, 1997), is fibrous, hydrophilic, biodegradable, and insoluble in water and most organic solvents. Nitrocellulose is produced by partial nitration of cellulose. Nitration strengthens the porous property of cellulose and changes cellulose from hydrophilic to hydrophobic. Both cellulose fibers and nitrocellulose membranes are porous materials. Porosity, together with surface chemistry and optical properties of these materials, is critical for the preparation of paper-based diagnostic devices (Pelton, 2009). Surface chemistry has an impact on molecule or particle immobilization, non-specific adsorption, and color expression in diagnostic assays. Porosity and the surface chemistry together determine wet properties of these materials, and thus affect the behavior of fluid on/in the device. Moreover, the optical properties of these materials can influence the accuracy of colorimetric or fluorescent readouts (Pelton, 2009). For example, many commercial paper materials are added with fluorescent molecules (i.e., optical brightening agents) in order to make them appear white. However, such modification can lead to a high background in fluorescence-based diagnostic assays. To this end, polymeric additives are selectively added in the papermaking process to adjust the properties of these materials. For example, some rewetting agents (e.g., surfactants) have been added to fabricate nitrocellulose membranes (Wong and Tse, 2009).

In addition, some other paper substrates and alternatives have been explored to develop paper-based diagnostic devices. Glavan et al. (2013) silanizated cardstock paper with a fluorinated alkyltrichlorosilane to obtain omniphobic R^F paper and then utilized the paper to fabricate pressure-driven open-channel microfluidic devices. Similar to PDMS-based microfluidic systems, these devices exhibit comparable performance in the control of fluid flow. In addition, these devices are inexpensive, lightweight, and disposable. In another study, Han et al. (2013b) employed a soft paper/polymer composite to fabricate reconfigurable three-dimensional microfluidic devices on benchtop. Additionally, some alternative materials, such as flexible film (Focke et al., 2010), cotton yarn (Safavieh et al., 2011), cotton cloth (Nilghaz et al., 2012) and vegetable parchment (Yan et al., 2012), were used as a matrix to fabricate microfluidic diagnostic devices.

2.2. Dipstick assays

Dipsticks, such as pH test strips and urine test strips, are simple to design, easy to manufacture and convenient to use. Generally, pH test strips are manufactured by soaking a piece of filter paper into a mixture of acid–alkali indicators with a certain concentration ratio. After dried, the paper is impregnated with detection regents. When an unknown sample is dispensed on the paper, the detecting regents react with the analyte (H^+) and develop a color. By referring to a standard indicator card, the pH value of the solution can be indicated and thus the concentration of H^+ is semi-quantified. Further, pH test strips (Fig. 1A) have been used to measure the level of pH in urine or saliva to monitor diet intake and to obtain the general health information of human body (Young and Young, 2010). Color indicators can be added to detect lead acetate, potassium iodide, etc. (http://www.gelifesciences.com) as well. Similarly, urine test strips (Fig. 1B) have been designed to detect metabolic products in urine, which have become basic diagnostic tools to indicate pathological changes. For instance, urinary metabolic products (e.g., protein, glucose, and salt) from patients with nephritic or diabetic diseases can be detected using a standard urine test strip.

2.3. Lateral flow assays (LFAs)

Lateral flow test strips are typically composed of a nitrocellulose membrane, sample pad, conjugate pad, wicking or absorbent pad and backing pad (Millipore, 2009) (Fig. 1C). The absorbent pad provides a driving force based on capillary effect, and the backing pad provides a certain mechanical support to the device. Nitrocellulose membrane is the most popular and important material in LFAs because it provides a platform for both reaction and detection during the assay (Millipore, 2009). Capturing molecules, e.g., antibodies, can be deposited on the nitrocellulose membranes to form test and control lines by electrostatic interaction, hydrogen bonds and/or hydrophobic forces (Millipore, 2009). Each two adjacent components overlap with each other by a small part in order to coordinate the fluid flow. When the assay is performed, a small volume of sample is applied onto the sample pad. The sample pad is pretreated with a buffer (e.g., to adjust pH) to improve the performance of or compatibility with the other components used in the assay (Wong and Tse, 2009). The mixture migrates along the pad and then carries conjugated particles, which are preloaded onto the conjugate pad, along the nitrocellulose membrane. Various particles have been employed in LFAs for their unique optical, electronic, and/or structural properties, such as gold nanoparticles (AuNPs) (Daniel and Astruc, 2004) and upconversion nanoparticles (UCNPs) (Lin et al., 2012).

There are two formats, i.e., sandwich and competitive (or inhibition) formats, for LFAs (Wong and Tse, 2009). In the sandwich format, the conjugated particles react with the analyte of interest (if exists) to form particle–analyte complexes, and then the complexes continue to migrate along the fluid flow. The complexes are captured at the test line via the interaction between analytes and corresponding capturing molecules. The obsessive conjugated particles, which are free of analytes, can exceed the test line. These particles are then captured by another type of capturing molecules and thus form a control line. In the competitive format, the conjugated particles can react with capturing molecules deposited at both test and control lines. As such, the analyte competes for the binding sites with the capturing molecules at the test line, leading to non-aggregation of conjugated particles at the test line. In the absence of analyte, conjugated particles can be captured at both test and control lines. Both sandwich and competitive format assays can be used for qualitative and quantitative detection of proteins and nucleic acids in previous studies (Wong and Tse, 2009). In general, sandwich format assays are utilized for an analyte with multiple antigen epitopes, while competitive format assays are designed to detect an analyte with a single antigen epitope (Wong and Tse, 2009).

2.4. Microfluidic paper-based analytical devices (μPADs)

Paper is hydrophilic and porous, thus providing a natural platform for fabricating microfluidic channels. Microfluidic paper-based analytical devices (μPADs) are pioneered by Whitesides et al. at Harvard University in 2007 (Mao and Huang, 2012). They developed both two-dimensional (2D) (Fig. 1D) and three-dimensional (3D) (Fig. 1E) μPADs by patterning paper with a variety of assay designs (Nilghaz et al., 2012), in which μPADs are mainly based on capillary force to drive aqueous fluid movement (Martinez et al., 2010b). 2D μPADs are made by patterning physical or chemical hydrophobic boundaries to form microchannels on paper (Cassano and Fan, 2013; Grifantini, 2009). Recently, various approaches, including cutting, photolithography, plotting, inkjet etching, plasma etching, wax printing, etc., have been proposed to create channels and barriers in paper (Martinez et al., 2010b). The dimensions of the resulting channels together with the characteristics of paper and ambient conditions (temperature and humidity) can affect the wicking rate of fluid (Martinez et al., 2010b). The reagents required for biochemical reactions can be immobilized

Table 2
A summary of dipstick assays, LFAs and μPADs.

Platform	Material	Fabrication method	Mechanism		Example	Analyte	Detection limit	Reference
Dipstick assays	Filter paper	Soak	Chemical reaction	Acid–alkali reaction	pH Test paper	H^+	10^{-14} M	http://www.gelifesciences.com
				Precipitation reaction	Lead acetate test paper	H_2S	N/A	
				Redox reaction	Potassium iodide test paper	Oxidizing agent, e.g., Cl_2, Br_2	N/A	
LFAs[a]	Lateral flow test strip[a]	1. Dispense 2. Dry 3. Assembly 4. Laminate 5. Cut	Biological reaction	Antigen–antibody reaction	Home pregnancy test paper	hCG[a]	20 mIU/mL	http://www.early-pregnancy-tests.com
				Nucleic acid hybridization	Nucleic acid test paper	HIV-1[a] DNA fragment	0.1 nM	Hu et al. (2013)
μPADs[a]	2D μPAD	Pattern physical or chemical hydrophobic barriers on chromatographic paper	Biochemical or electrochemical reactions		Blood glucose test paper	Glucose	4 mM	http://www.diabetes.co.uk
					ELISA test paper[a]	Rabbit IgG	54 fmol/zone	Cheng et al. (2010)
	3D μPAD	Stack layers of patterned paper			Liver function test paper	Transaminase	53 U/L (ALT[a]) and 84 U/L (AST[a])	Pollock et al. (2012)

[a] LFAs: lateral flow assays; μPADs: microfluidic paper-based analytical devices; a lateral flow test strip is made from a nitrocellulose membrane, sample pad, conjugate pad, wicking or absorbent pad and backing pad; hCG: human chorionic gonadotropin; HIV-1: human immunodeficiency virus type 1; ALT: alanine aminotransferase; and AST: aspartate aminotransferase.

on paper with different patterns (e.g., four-leaf clover) by hand dispensing or inkjet printing (Martinez et al., 2010b). Functional chemical or biological molecules can be immobilized on paper by physical absorption, chemical coupling, and carrier-mediated (e.g., gold particles) deposition (Pelton, 2009; Sicard and Brennan, 2013). When the reagents are dried, the paper-based devices can be used for biochemical analyses. 3D μPADs are produced by stacking layers of patterned paper in such a way that channels in adjacent layers of paper connect with each other (Martinez et al., 2008b). Both 2D and 3D μPADs can serve as a substrate for filtering samples, performing chromatographic separations, and taking biochemical reactions (Martinez et al., 2010b). Compared with 2D μPADs, 3D μPADs have several potential advantages due to their capability to incorporate complex networks of channels, thus providing multiple functionalities (Martinez et al., 2010b).

Microfluidic paper-based electrochemical devices (μPEDs) are one category of μPADs with electrodes. In principle, the reported digital results of μPEDs are electrochemical signals instead of colorimetric signals commonly used in typical μPADs. The electrodes used in μPEDs are prepared from conducting inks (carbon or metal) by screen-printing (Nie et al., 2010b), inkjet-printing (Kit-Anan et al., 2012), or pencil-drawing (Dossi et al., 2013) on paper or plastic. A typical electrode-based sensing unit comprises of three electrodes, including a working electrode (WE), a counter electrode (CE) and a reference electrode (RE) (Fig. 1F) (Nie et al., 2010b). The working electrode is biologically or chemically modified in order to achieve sensitive and specific sensing function. The specificity of μPEDs is similarly achieved by the methods used in paper-based colorimetric assays, such as antibody–antigen reaction, nucleic acid hybridization reaction, or enzymatic reaction (see Section 3). Different from colorimetric assays, these reactions used in μPEDs are mainly redox reactions, either direct or indirect. To ensure sufficient sensitivity of the electrochemical reactions, materials such as AuNPs, carbon nanotubes and/or grapheme nanosheets, are employed to modify the working electrode (Lu et al., 2012; Wang et al., 2012a; Yan et al., 2012).

3. Reaction mechanism of paper-based diagnostics

Various paper-based diagnostics have been designed to detect pH value, urine metabolites, blood glucose, liver function, hormones, infectious agents, etc. According to the reaction mechanism, these tests can be categorized into chemical, biological and electrochemical reactions (Table 2).

3.1. Chemical reaction: color change

Most chemical reactions with color change can be achieved on paper, such as acid–alkali reaction, precipitation reaction, redox reaction and enzymatic reaction, etc. These reactions generally involve a one-step procedure. Taking litmus test strips as an example, litmus is a purple water-soluble mixture with color change when the pH value ranges from 4.5 to 8.3. The solution of litmus changes to red under acidic conditions and to blue under alkaline conditions. Therefore, litmus red and blue test strips are produced for the detection of alkaline and acid conditions, respectively. pH test strips can be dispensed with several compounds to exhibit different color changes in response to different pH values. Semi-quantitative detection of H^+ concentrations of solutions can then be achieved by grading the pH values of solutions from 1 to 14. In addition, more sensitive pH test strips with precision of 0.1 and 0.01 have been developed (http://preclaboratories.com). These pH test strips have a narrow testing pH range and they can be combined to quantitatively indicate pH value ranging from 1 to 14.

3.2. Biological reaction

3.2.1. Antigen–antibody binding

Antigen–antibody binding based immunoassays detect either antigen or antibody present in a clinical sample. Home pregnancy test strips have been one of the most successful diagnostic paper-based immunoassays so far. It measures a hormone, human chorionic gonadotropin (hCG), in urine from pregnant women. hCG is a heterodimeric glycoprotein with α and β subunits. α subunit is identical to that of some other hormones (e.g., luteinizing hormone), while β subunit is unique to hCG. Home pregnancy test strips just make use of β subunit and contain three kinds of antibodies, i.e., anti-hCG antibody, monoclonal antibody (MAb) and immunoglobin G (IgG). Anti-hCG antibody, conjugated with colored particles, can specifically recognize and bond with hCG in the sample. MAb and IgG can bond to hCG and anti-hCG antibody, respectively, thus forming the test and control lines. Since the reactions between these molecules cannot be observed by the naked eye, signal molecules need be employed to indicate whether the reactions occur or not. This idea has been used to measure tumor markers, e.g., primary hepatic carcinoma (Yang et al., 2011), and to diagnose infectious diseases, e.g., AIDS (Van den Berk et al., 2003). Additionally, ELISA has also been realized on paper-based devices (Apilux et al., 2013; Cheng et al., 2010; Liu et al., 2011b).

3.2.2. Nucleic acid hybridization

Compared with antigen/antibody (protein) testing, nucleic acid testing (NAT) is more suitable for early detection of genetic and infectious diseases (Craw and Balachandran, 2012; Yu et al., 2012). Typically, a NAT assay requires two types of oligonucleotide probes, i.e., detector probe and capture probe. Detector and capture probes are both complementary with target nucleic acid sequence, while the detector probe is used to combine with a tag to make the reaction visible or measurable. The tag can be colored particles (e.g., AuNPs) for colorimetric assays (Hu et al., 2013), or electroactive molecules (e.g., thionine) for electrochemical measurements (Lu et al., 2012). Since genetic and infectious diseases can be determined by a fragment of gene-specific nucleic acids, NAT based strips promise a great potential for rapid and reliable diagnosis.

3.2.3. Functional nucleic acid-based reaction

Functional nucleic acids, including DNAzymes, aptamers and aptazymes, are nucleic acids which have functions not limited to nucleic acid hybridization (Liu et al., 2009). Most DNAzymes possess enzyme activity in the presence of specific ions, and this mechanism can be utilized for sensing given metal ions by measuring the enzymatic activity. Similar to antibodies, aptamers are essentially nucleic acid molecules that can specifically bind a wide range of proteins (Liu and Lu, 2006). A combination of DNAzymes and aptamers results in aptazymes. They have been employed for colorimetric sensing metal ions and biochemical molecules in LFAs (Liu et al., 2006; Mazumdar et al., 2010). The mechanism of their signal detection relies on target analyte-induced cleavage of nanoparticle conjugates or disassembly of nanoparticle aggregates, which is captured at the test line for color development on lateral flow test strips.

3.3. Electrochemical reaction

Electrochemical detection can be achieved on the basis of both redox reactions and non-redox reactions (Han et al., 2013a). Redox reactions are involved in electrons transfer between molecules or particles (e.g., enzyme and nanoparticles), while non-redox reactions are related with the changes of electrical properties, such as impedance, resistance, conductance, and potential (Han et al., 2013a). Since electrochemical detection possesses features such as high sensitivity and selectivity, low cost, and portability, it has been extensively applied in various assays, thus providing an alternative detection scheme for paper-based diagnostics (Dungchai et al., 2009; Pei et al., 2013). The most successful example of electrochemical detection is the blood glucose meter and test strip for diabetic patients that constitute about 5% of the world's population (Heller and Feldman, 2008). For example, a total of about 6 billion electrochemical strips were produced in 2007, which surpassed the amount of any other assays (Heller and Feldman, 2008). The glucose meter is in essential an amperometer, and it measures the quantity of an electroactive species as a result of the oxidation of glucose by reagents stored in the test strips (Nie et al., 2010a). Briefly, the test strip is impregnated with glucose oxidase and other components (e.g., ferrocyanide). When a drop of blood is added, glucose oxidase catalyzes the oxidation of glucose, and the glucose meter quantifies the electrons generated by the oxidation and correlates them to the level of glucose in blood (Nie et al., 2010a). More detailed information about the principle and development of glucose meters can be found in *Chemical Reviews* (Heller and Feldman, 2008; Wang, 2008). Moreover, a recent review has discussed the capabilities and limitations of current μPEDs (Maxwell et al., 2013).

Besides the above mentioned reactions, there are chemiluminescent (Ge et al., 2012; Wang et al., 2012b), electrochemiluminescent (Yan et al., 2013) and photoelectrochemical reactions (Wang et al., 2013a) used for paper-based diagnostics. These reactions can be regarded as an integration of two or more of chemical, biological, and/or electrochemical reactions.

4. Advances in development of paper-based diagnostics

For a qualitative detection, colorimetric changes in paper-based diagnostic assays can be visualized by the naked eye to yield a yes/no answer. In contrast, imaging analysis by a handheld reader or a cell phone (Martinez et al., 2008a), or visual estimation by the naked eye (by comparing the color change to a predetermined score chart) (Dineva et al., 2005) is used to report quantitative readouts. However, both qualitative and quantitative strategies suffer from low sensitivity and poor accuracy. Additionally, the performance of paper-based diagnostics can be enhanced by reducing complexity and increasing functionality such as the integration of sample pretreatment and result analysis. Here, we present the state-of-the-art advances in improving paper-based diagnostics for POC applications.

4.1. Sensitivity

For a number of analytes, conventional paper-based diagnostics do not have enough sensitivity for clinical applications. For example, in current LFAs, various strategies have been widely employed to increase their sensitivity, such as enzyme-based (He et al., 2011; Parolo et al., 2013a) and metal ions (gold or silver)-based (Rohrman et al., 2012) signal enhancement. Enzyme- or metal ion-based enhancement strategies have two steps to fulfill its reaction, i.e., firstly detection and then amplification. These two strategies can improve the detection limit up to hundreds of folds. Liu et al. improved the sensitivity in LFAs by adopting horseradish peroxidase (HRP) to enhance the color intensity in the presence of HRP substrate, 3-amino-9-ethyl-carbazole (AEC), which lowered the detection limit from 0.5 nM to 50 pM (Mao et al., 2009). They further optimized the conjugation of HRP and thiolated DNA to AuNPs by adjusting their immobilization sequence and adding sodium dodecyl sulfate (SDS), which lowered the detection limit by 1000 times (Fig. 2A) (He et al., 2011). On the other hand, Parolo et al. (2013a) tested three different HRP substrates, i.e., AEC, 3,3′-diaminobenzidine

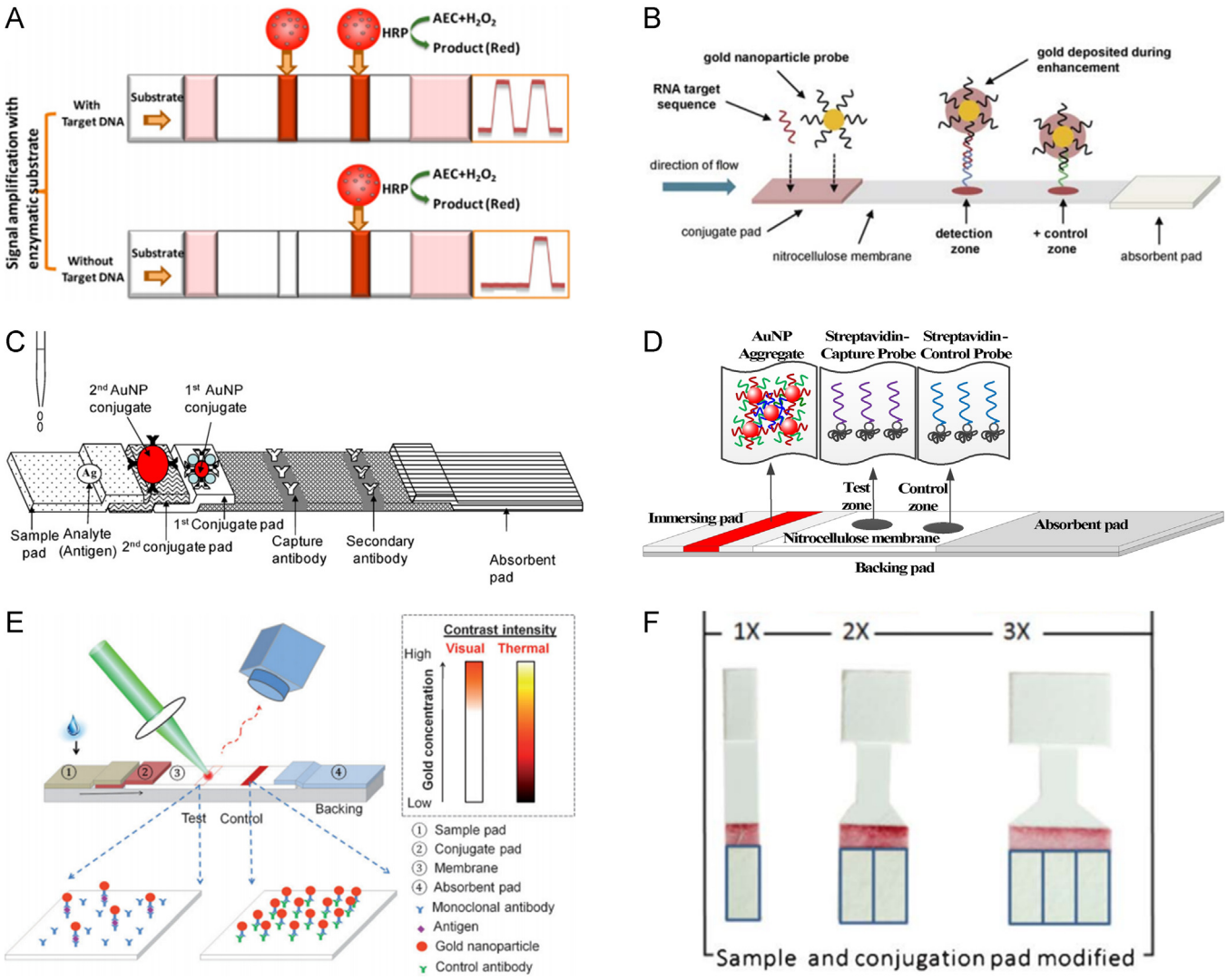

Fig. 2. General methods to improve sensitivity in lateral flow assays (LFAs). (A) Enzyme-based signal enhancement (He et al., 2011). (B) Gold-or silver-based signal enhancement (Rohrman et al., 2012). (C) Dual gold nanoparticle (AuNP) conjugate-based signal amplification (Choi et al., 2010). (D) Oligonucleotide-linked AuNP aggregates-based signal enhancement (Hu et al., 2013). (E) Thermal contrast (Qin et al., 2012). (F) Architecture modification (Parolo et al., 2013b).

tetrahydrochloride (DAB) and 3,3′,5,5′-tetramethylbenzidine (TMB), and found that TMB was more suitable than the other two. Rohrman et al. (2012) presented a strategy to improve the detection of HIV RNA using gold and silver enhancement (Fig. 2B). The underlying mechanism may be due to the fact that metallic ions (gold or silver) are reduced and deposited on the surface of AuNPs, thus increasing the size and optical extinction (Rohrman et al., 2012).

Recently, colored particle conjugation or aggregation has been employed to improve the sensitivity in LFAs with integration of detection and amplification procedures, i.e., amplification and detection are performed at the same time (Choi et al., 2010; Liu et al., 2011a; Tang et al., 2009). This strategy can improve the detection limit from several folds to several 10-fold. Choi et al. (2010) developed a method for signal amplification by using two kinds of AuNP conjugates, in which one AuNP conjugate was used to develop color and the other to enhance the color development (Fig. 2C). By optimizing the combination of two AuNPs, the detection limit of troponin I was as low as 0.01 ng/mL, which was 100-fold more sensitive than that in the conventional LFAs. Hu et al. (2013) successfully developed improved nucleic acid LFAs by using oligonucleotide-linked AuNP aggregates, which hold great potential to detect a broad range of nucleic acids. Its corresponding detection limit was as low as 0.1 nM with almost 3-fold signal amplification (Fig. 2D). In addition, Qin et al. (2012) improved the sensitivity of LFAs to 36-fold by using thermal contrast (Fig. 2E). The underlying mechanism is that metal nanoparticles generate heat in the presence of optical stimulation (Govorov and Richardson, 2007). Upon stimulation, the surface plasmon at the metal–dielectric interface of metal nanoparticles transfers from an excited state to a ground state, thus releasing heat (Qin et al., 2012). By modifying the dimension of test strips, Parolo et al. (2013b) improved the sensitivity by 8-fold (Fig. 2F). Accordingly, the volume of sample and the amount of reagents deposited on test strips were enlarged, thus increasing the amount of target–label complexes at the test line, which was confirmed by using mathematical simulations.

4.2. Quantification

Clinical diagnosis often requires quantitative measurements of proteins, nucleic acids, and other biomarkers. However, visual colorimetric measurements by the naked eye is not sufficient for the quantitative purpose due to the variation in visual perception of color among end-users and under different lighting conditions (Dungchai et al., 2009). In order to achieve quantitative analysis in paper-based diagnostics, cameras or scanners are used to record the color intensity due to its relationship with the amount of

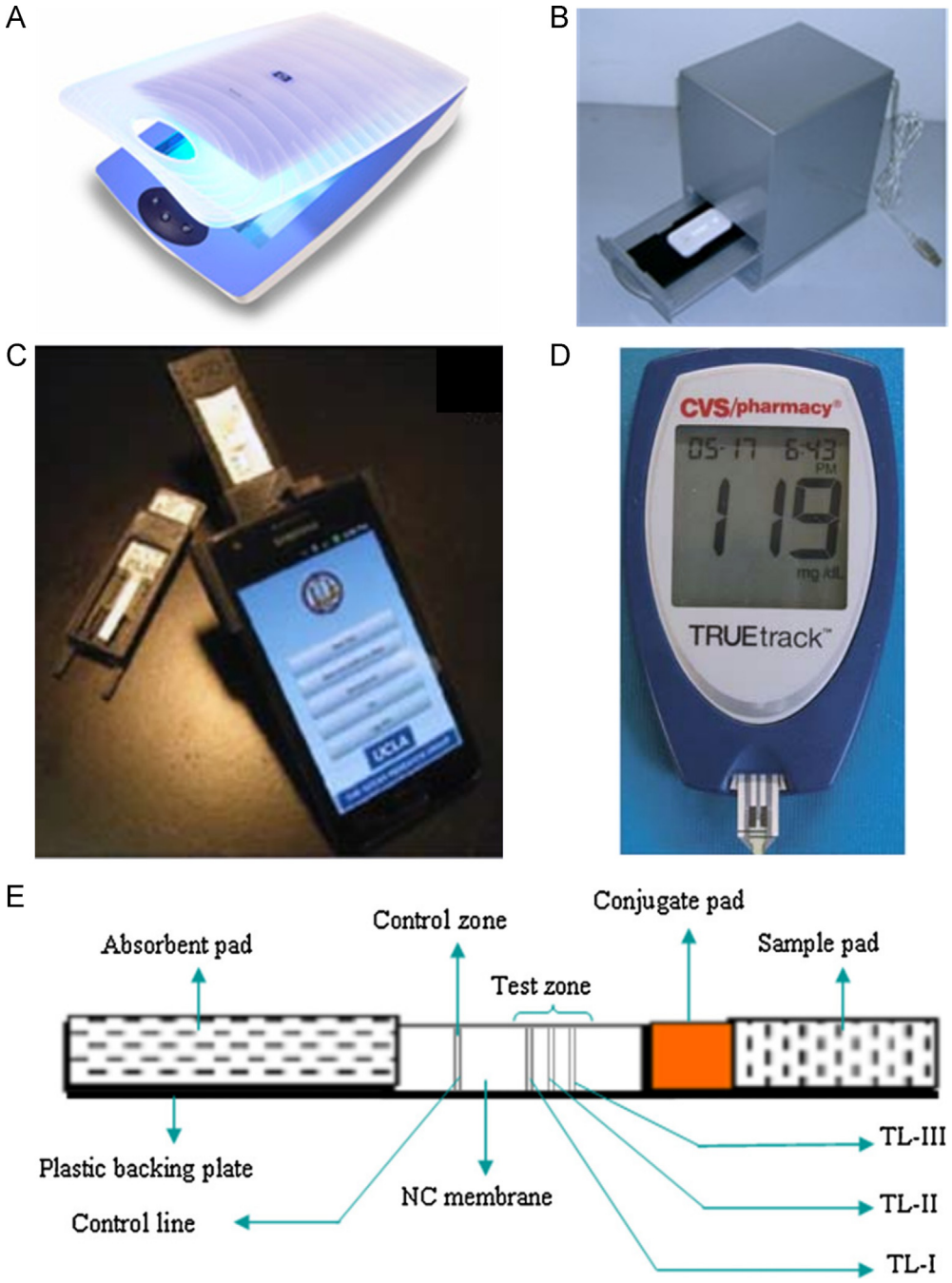

Fig. 3. Typical methods to achieve quantitative detection of analytes. Optics-based analysis: (A) scanner-based analysis (Glynou et al., 2003), (B) reader-based analysis (Mao et al., 2009), and (C) mobile phone-based analysis (Mudanyali et al., 2012). (D) Electrochemistry-based analysis: glucose meter- or analogous reader-based analysis (Nie et al., 2010a). (E) Naked eyes-based analysis (Zhang et al., 2012).

analyte. A desktop scanner or commercially available portable strip reader (e.g., DT1030) can be used to obtain the images of assay results, and the intensity of test zones can be analyzed by image-processing (Fig. 3A and B) (Glynou et al., 2003; Mao et al., 2009). In addition, a hand-held optical colorimeter was developed for quantifying colorimetric assays in μPADs by the analysis of the transmission of light through paper (Ellerbee et al., 2009). Bond et al. (2013) investigated the use of spectrophotometric measurement of blood spotted on chromatography paper as a low cost ($<$ \$0.01/test) alternative to the HemoCue method (a recommended standard-of-care in resource-limited settings).

Thanks to the rapid increase in the coverage and usage of mobile phones (especially smart phone) worldwide, mobile phones promise potential tools in diagnostics and telemedicine (Martinez et al., 2008a). Martinez et al. (2008a) used a camera phone as a probable detector for digitizing the results of paper-based colorimetric assays, which was comparable to desktop scanner, portable scanner and digital camera based quantification. Additionally, camera phone can transfer the assay results from an on-site operator to an off-site laboratory for result interpretation by a trained medical professional. Further, Ozcan et al. developed a compact and light digital rapid-diagnostic-test (RDT) reader platform and manually attached it to the existing camera unit of a mobile phone (Fig. 3C) (Mudanyali et al., 2012). It was demonstrated that the mobile phone-based RDT reader worked well with various LFAs for detection of malaria, TB and HIV, thus providing real-time detection/quantification of the

target analytes. A recent review has detailed the use of mobile phone based techniques for POC diagnostics (Zhu et al., 2013).

Electrochemistry-based detection has also been used for quantification in paper-based microfluidic assays. Dungchai et al. (2009) used photolithography to make a μPAD and then employed screen-printing technology to prepare electrodes on the device. They further demonstrated the device with the ability to determine glucose, lactate and uric acid in biological samples based on oxidase enzyme reaction. Whitesides et al. coupled simple electrochemical μPADs with a commercial glucose meter to rapidly quantify the amount of compounds relevant to human health (e.g., glucose, cholesterol, lactate and alcohol) in blood or urine (Fig. 3D) (Nie et al., 2010a). Yu et al. used collapsible μPAD and screen-printed electrodes (SPEs) to create simple, low cost, disposal devices for detection of DNA with an excellent analytical performance (Lu et al., 2012). The detection limit for target nucleic acid was as low as 0.2 aM. On the other hand, a barcode detection strategy was developed to semi-quantify the concentration of analyte by the naked eye, in which the concentrations of analytes were expressed as the number of test lines (Fig. 3E) (Cho and Paek, 2001; Fang et al., 2011; Fung et al., 2009; Zhang et al., 2012).

4.3. Simplification

Efforts have been made to simplify the fabrication procedure and detection protocol of paper-based diagnostics. For example, to simply the fabrication procedure, Phillips et al. recently published a simple method for assembling 3D μPADs using spray adhesive to permanently bond multiple layers of paper, thus avoiding tedious alignment and assembly steps (Lewis et al., 2012). Further, Martinez et al. presented a new method for fabricating 3D μPADs using toner as a thermal adhesive to bond multiple layers of patterned paper (Schilling et al., 2013). The fabrication process is rapid, involves minimal equipment (a laser printer and a laminator), and can produce complex channel networks with dimensions down to 1 mm. One the other hand, Cassano and Fan (2013) reported a μPAD fabrication method by simple craft-cutting and lamination. The constructed devices using this method have been exploited for simultaneous detection of bovine serum albumin (BSA) and glucose in synthetic urine with colorimetric assays. Liu and Crooks (2011) reported a method based on the principles of origami (paper folding) to prepare 3D μPADs. The required reagents were deposited on a single sheet of flat paper using photolithography, and the paper was then folded by hand to assemble 3D μPAD, which seems promising for low cost and simple applications at the POC (Fig. 4A).

To simplify the detection protocol, Khan et al. (2010) developed a diagnostic paper with three arms treated with different antibody solutions (A, B and D) for instantaneous ABO blood typing. Martinez et al. developed a fully enclosed μPAD, which could prevent embedded microchannels from contamination and avoid evaporation (Schilling et al., 2012). Cheng et al. (2010) developed a paper-based ELISA (P-ELISA) which requires a less volume of sample and reagents, simpler equipment, and less turnaround time than conventional ELISA. Liu et al. (2011b) developed a portable 3D μPAD for performing ELISA (Fig. 4B). All the reagents required for analysis were first stored in a dry form within the device and then dissolved in a buffer and delivered to the test zones. The test strip was movable; it was moved sequentially to desired points to complete a specific reaction required

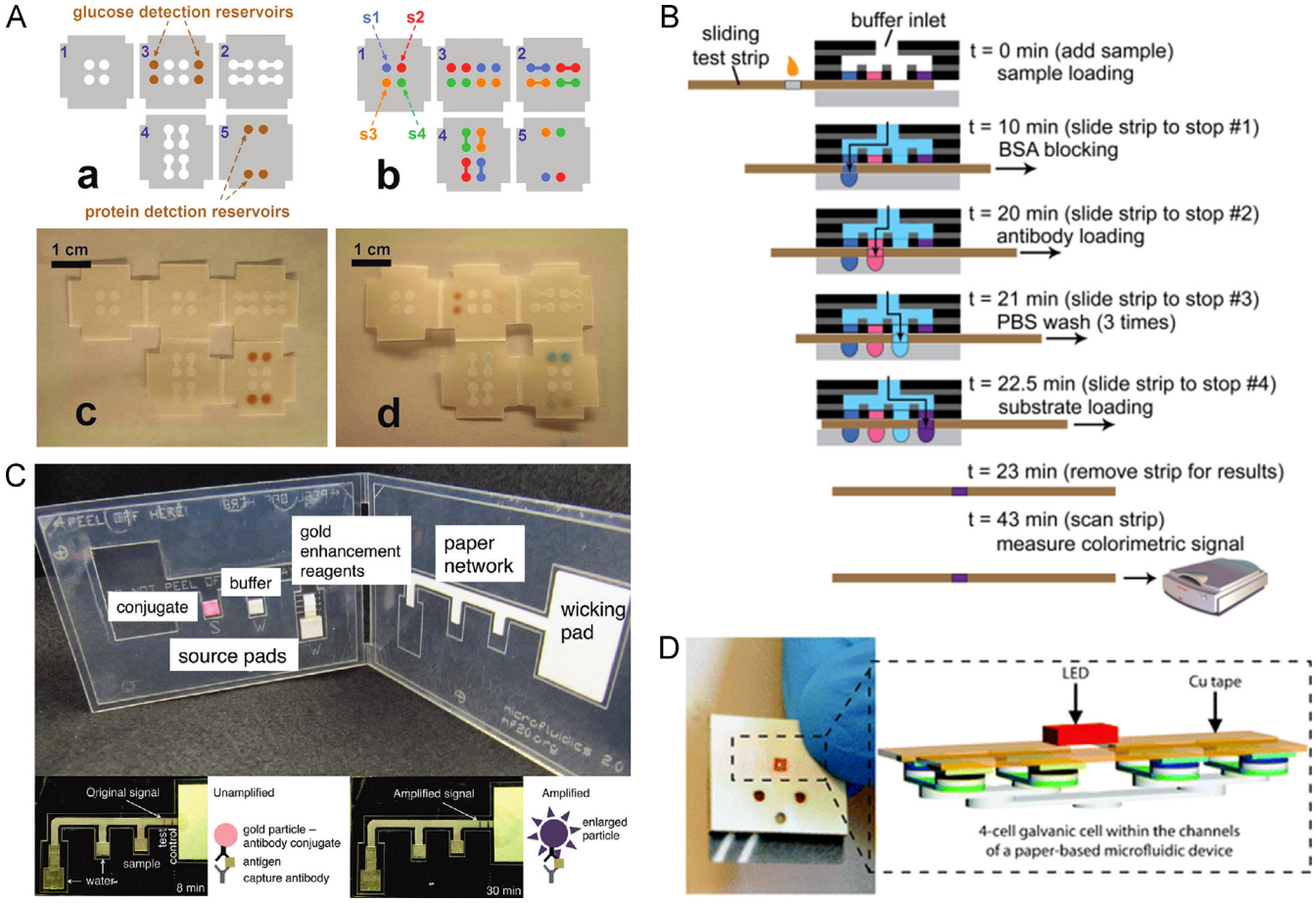

Fig. 4. Representative methods to simply the fabrication procedure and detection protocol of paper-based diagnostics. (A) A 3D μPADs fabricated by origami (paper folding) (Liu and Crooks, 2011). (B) A disposable 3D μPAD for enzyme-linked immunosorbent assay (ELISA) (Liu et al., 2011b). (C) 2D paper network (2DPN) format assay (Fu et al., 2012). (D) A self-powered μPAD for on-chip fluorescence assay (Thom et al., 2013).

in ELISA. Via the movement of the test strip, the ELISA procedure was significantly simplified. Recently, Apilux et al. (2013) reported a paper-based device for automating the multi-step procedure of sandwich-type ELISA. Their main strategy is to use separate channels to perform different steps used in ELISA.

On the other hand, Yager et al. developed 2D paper networks (2DPNs), which experimentally and theoretically demonstrated the capability of paper-based devices to carry out multi-step chemical procedures that are hard to perform in current LFAs (Fu et al., 2010b, 2011b; Kauffman et al., 2010; Lutz et al., 2011). Further, Yager et al. performed gold-based signal amplification in the 2DPNs (Fu et al., 2010a, 2011a, 2012). Since the distances between each inlet and the detection pad were different, the arrival time of solutions in the inlets was different, enabling sequential reaction and signal enhancement by adding the reagents simultaneously on different inlets (Fu et al., 2010a). This assay was further simplified by using a 2DPN card to store the gold enhancement solution (Fu et al., 2011a) or dry reagents (Fu et al., 2012) (Fig. 4C). For nucleic acid testing, Govindarajan et al. (2012) reported a low cost μPAD for POC extraction of bacterial DNA from raw viscous samples using microfluidic origami. As demonstrated, *Escherichia coli* with a bacterial load as low as 33 CFU mL^{-1} was reliably extracted from pig mucin (simulating sputum) and subsequently detected.

Additionally, electronic components, such as power sources and capacitors, play a significant role in paper-based diagnostic devices. Researchers have made efforts to integrate paper-based diagnostic devices with power sources and capacitors. For example, Phillips et al. developed a μPAD with multiple galvanic cells (also termed as fluidic batteries) integrated directly into the microfluidic channels (Thom et al., 2012). These fluidic batteries provided power for on-chip devices (e.g., a UV LED) and made an on-chip florescence assay possible (Fig. 4D). Such a strategy could make μPADs independent of externally powered readers. They subsequently reported two general designs for these fluidic batteries via connection in series and/or in parallel to provide predictable and tunable sources of power (desired current and time) for on-chip assays (Thom et al., 2013). Liu and Crooks (2012) developed a battery-powered electrochemical sensing platform with an electrochromic display for POC diagnostics. They employed an integrated metal/air battery that powered both the electrochemical sensor and electrochromic readout. Zhang et al. (2013) developed a 3D origami μPAD with a stable, environment-friendly and noble metal-free (Ag/Ag^{+}) primary battery ($Cl|FeCl_3|NaCl|AlCl_3|Al$) for driving luminal ($Ru(bpy)_3^{2+}$) electrochemiluminescence system to detect glucose.

Yu et al. developed a novel microfluidic photoelectrochemical paper-based analytical platform, which integrated an internal chemiluminescent light source, a paper supercapacitor, and an external digital multi-meter (DMM) (Ge et al., 2013; Wang et al., 2013a, 2013c). The paper supercapacitor was formed via a screen-printed carbon working electrode and counter electrode. It could collect and store the photocurrents generated from the paper sample zone under an internal chemiluminescent light source for 1 min. Once the switch was turned off, the supercapacitor instantaneously released the stored electrical energy through the DMM and produced

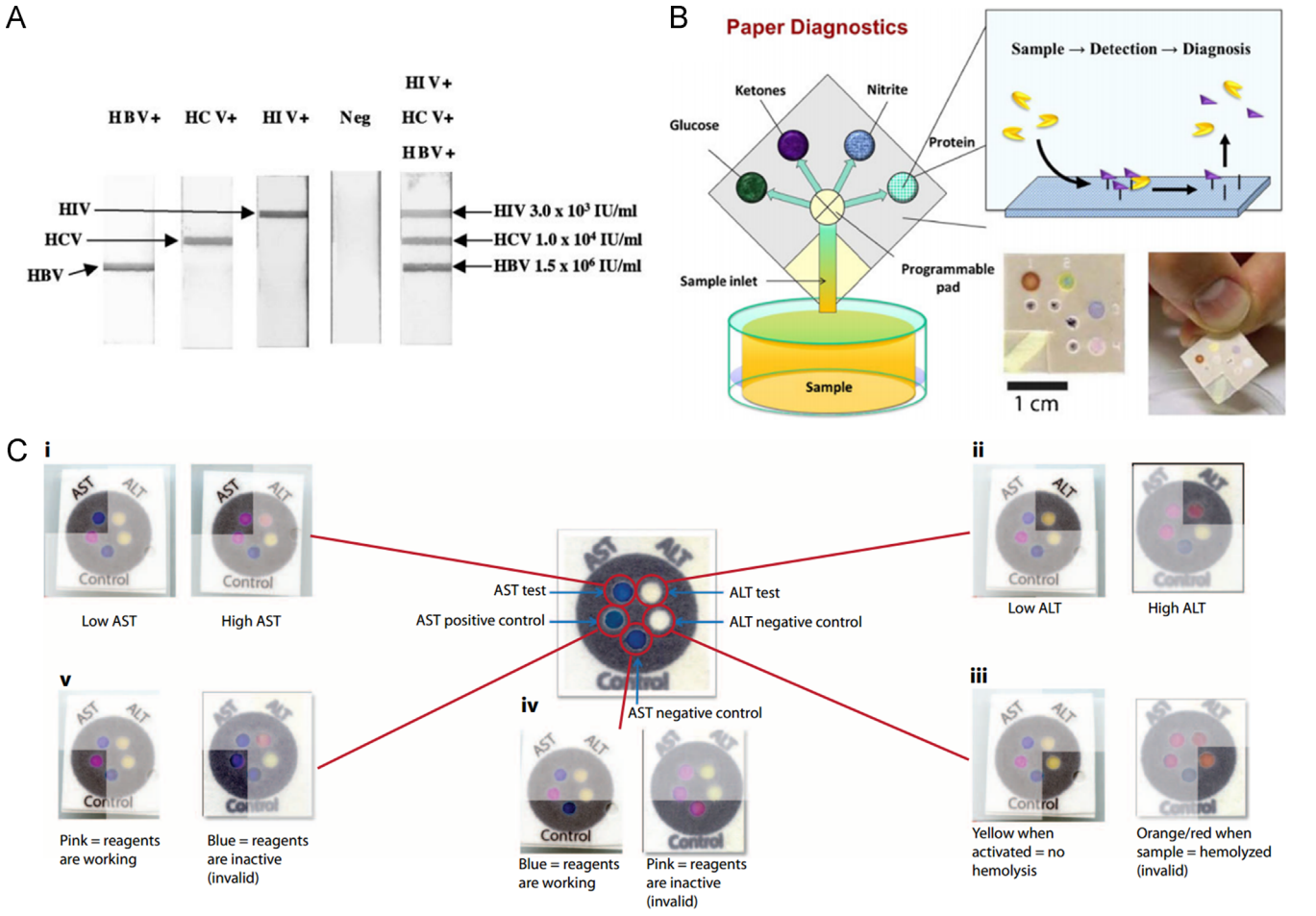

Fig. 5. Multifunctional paper-based diagnostics with multiple detection or sample pretreatment. (A) Simultaneous visual detection of multiple viral amplicons based on a LFA (Dineva et al., 2005). (B) A programmable μPAD for urinalysis (Novak et al., 2013). (C) A multiplexed transaminase test strip (Pollock et al., 2012).

an about 13-fold-amplified and DMM-detectable current, which was more sensitive than the direct photocurrent measurement.

4.4. Multi-functionalization

One approach for multi-functionalization is to simultaneously detect multiple analytes. Dineva et al. (2005) developed a LFA with three test lines for visual detection and identification of multiple nucleic acid amplicons, i.e., hepatitis B virus (HBV) DNA, hepatitis C virus (HCV) RNA, and human immunodeficiency virus type 1 (HIV-1) RNA at the same time (Fig. 5A). In addition, they overcame the low sensitivity of LFAs by using detector probes labeled with multiple colored particles, and employed a standard chart with assigned intensity scores of 1–5 for semi-quantitative detection. Compared with LFAs, μPADs have inherent advantages in multiple detections due to their 2D or 3D structures. For example, Martinez et al. (2010a) developed a programmable (post-fabrication) 3D μPADs for multiple patterns of fluid flow. Further, they used the device for urinalysis, with which the end-user could choose to run a single or multiple colorimetric assay(s) for the detection of glucose, proteins, ketones and nitrite (Fig. 5B) (Novak et al., 2013).

Another approach for multi-functionalization is to integrate sample pretreatment, separation, reaction and detection. Yang et al. (2012) developed a μPAD that separated blood plasma from whole blood using the meshwork of paper fibers to entangle agglutinated red blood cells and then performed a colorimetric assay for plasma glucose. Further, they developed a simple, rapid, low cost POC diagnostic test for sickle cell diseases by running the hemoglobin solubility assay in paper. Due to the entanglement effect of the meshwork of paper fibers, the polymerized hemoglobin was prevented from diffusing through the paper, while the soluble hemoglobin was free to wick through the paper (Yang et al., 2013). The migration distance of soluble hemoglobin from the center of the blood stain and corresponding normalized color intensity showed significant differences between normal, sickle cell trait and sickle cell disease blood samples. Whitesides et al. developed a 3D μPAD for detection of two enzymatic markers of liver function (alkaline phosphatase, ALP, and aspartate aminotransferase, AST) and total serum protein (Vella et al., 2012). The device can perform the entire procedure of sample preparation and qualitative detection. A mobile phone was then used to digitize the color intensity and to send the result to medical professional for off-site analysis, thus giving a quantitative readout. Based on this strategy, many other biomarkers (e.g., alanine aminotransferase, ALT) could be measured as well. Further, they demonstrated a major progress to make the device for rapid, semi-quantitative measurement of AST and ALT from a fingerstick whole-blood specimen for low cost, POC liver function testing (Fig. 5C) (Pollock et al., 2012). With a well clinical applicability, the device has been optimized for visual readout in AST and ALT ranges comparable to current cutoffs used clinical management decisions for HIV and TB treatment monitoring (Pollock et al., 2012).

5. Disadvantages of paper-based diagnostics

Paper-based analytical devices provide cost-effective solutions for POC diagnostics. They provide end-users with an ideal preliminary screening tool for healthcare. Such devices are simple, inexpensive and useful (Whitesides, 2013). However, paper-based diagnostics need to be further improved in terms of clinical performance. As reported, existing paper-based analytical devices show varying specificity and sensitivity (Pike et al., 2013), which may cause false-negative and false-positive results. Home pregnancy tests may yield false-negative results and lead to unsafe sex, contraceptive non-adherence, and a higher rate of sexually transmitted infections and pregnancy (Rahman and Berenson, 2013). On the other hand, false-positive home pregnancy testing results can result in significant patient anxiety and unnecessary interventions (Nakhal et al., 2012). POC glucose meters also have varying levels of clinical performance and accuracy (Watkinson et al., 2012). Using inappropriate glucose meters may overestimate glucose levels, which leads to inappropriate insulin dose adjustment (Perera et al., 2011). OraQuick At-Home HIV test, a FDA-proved POC HIV test, has a 92% sensitivity and a 99.98% specificity (Arnold, 2012). In theory, it can cause one false negative in every 12 HIV-infected individuals.

Paper-based diagnostic devices are criticized for varying sensitivities and specificities, which may be mainly due to the following four reasons. First, varying detection methods and diverse substrates can be coupled in different combinations to prepare testing devices. The detection methods vary from optical to electrochemical sensing. For a given detection method, a range of substrates can be assessed to reach an optimal sensitivity and specificity, as well as to reduce cost. For example, a range of Hi-Flow Plus nitrocellulose membranes (from HF075 to HF240, Millipore) with different flow speeds can be used in LFAs, depending on the requirements for sample volume, assay time, specificity and sensitivity (Millipore, 2013). In general, the quicker the capillary flow speed is, the higher the specificity is, and then the lower the sensitivity is (Millipore, 2009). By comparing membranes HF240 with HF180, Mao et al. (2009) found that the signal of the test line on HF240 was significantly higher than that on HF180. Second, there exists variation in reporting results with the naked eye among end-users, especially for colorimetric detection-based LFAs. The subjective judgment from operators and differences in the illumination setting can lead to controversial readouts, especially when the detection signal is close to threshold. Third, the robustness of testing devices can greatly affect the performances. The used reagents such as enzymes, antibodies or antigens should withstand harsh environmental conditions during storage, shipping and testing (Then and Garnier, 2013). Temperature and humidity, which affect the migration speed of liquid and the recognition between molecules, in addition to the long-term stability of reagents, can lead to varying signals. Fourth, batch-to-batch variation is another well-known challenge which needs to be addressed to ensure reproducibility for POC testing (Abe et al., 2010; Li et al., 2011). Therefore, the sensitivity and specificity of paper-based diagnostics need to be further improved for wide applications in clinical or home settings.

6. Conclusion and future perspectives

Paper-based microfluidic devices have been widely employed to develop POC diagnostics due to their low cost and easy scale-up in manufacturing. With the interfacing between materials science and biomedical engineering, paper-based diagnostics are becoming simpler, more sensitive, more accurate, and multi-functional. When combining paper diagnostics with mobile phone based optical detection, telemedicine plays an important role in improving healthcare services in resource-limited settings (Wang et al., 2011). However, the potential of paper-based diagnostics will not be maximized until other aspects of POC diagnostics are achieved on paper, such as sample pretreatment (Govindarajan et al., 2012), plasma separation (Carvalhal et al., 2010), nucleic acid isolation and amplification (Craw and Balachandran, 2012). These aspects are of importance for paper-based NAT, which are superior to conventional immunoassays in terms of specificity and sensitivity. We believe that theoretical simulation and optimization can make a great contribution to the development of paper-based diagnostics (Fridley et al., 2013). We also envision that the fully integrated paper-based NAT with the

capability of achieving sample-in-answer-out capability will be a big step forward in improving patient care at the POC.

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