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Advances in Paper-Based Analytical Devices

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Abstract

Microfluidic paper-based analytical devices (μPADs) are the newest generation of lab-on-a-chip devices and have made significant strides in both our understanding of fundamental behavior and performance characteristics and expansion of their applications. μPADs have become useful analytical techniques for environmental analysis in addition to their more common application as medical point-of-care devices. Although the most common method for device fabrication is wax printing, numerous other techniques exist and have helped address factors ranging from solvent compatibility to improved device function. This review highlights recent reports of fabrication and design, modes of detection, and broad applications of μPADs. Such advances have enabled μPADs to be used in field and laboratory studies to address critical needs in fast, cheaper measurement technologies.

INTRODUCTION

Microfluidic paper-based analytical devices (μPADs) have drawn significant attention in analytical chemistry since 2007 owing to their advantages over conventional methods, such as inexpensive production, portability, operational simplicity, miniaturization, compatibility with biomolecules, high speed, point-of-care detection, and minimal reagent consumption (1–4). μPADs provide an alternative platform for liquid transport via capillary forces without the need for external pumps (5). Also, multiplexed analysis can be performed by simply adding channels. Fabrication techniques include photolithography, wax printing, screen printing, inkjet printing, and plasma oxidation (6). The applications of μPADs have been widely presented in the fields of clinical diagnostics, environmental monitoring, and food safety assurance using electrochemical, colorimetric, fluorescent, immunological, and molecular detection methods (3, 7–12).

In this review, we discuss recent and impactful articles on μPADs and their applications for environmental, food safety, and medical monitoring. A number of previous reviews focusing on μPADs have been published, providing more thorough coverage of specific elements that readers are encouraged to consult (11, 13–18). Here, we focus on key publications that have shaped the field and present future perspectives and current challenges.

A BRIEF HISTORY OF PAPER-BASED DEVICES

Paper has been utilized for chemical measurements for centuries since litmus paper was used to measure pH in the 1700s (19). The field lay largely dormant until spot tests were used for metal ion detection with colorimetric ligands in the 1930s and 1940s (20). These paper tests pioneered the development of other analytical tools for on-site analysis. For example, Comer (21) developed the first bioactive paper for glucose analysis in urine in 1956. Paper was also used for chromatography for a significant period of time (22).

The next major development came in the form of lateral flow assays (LFAs). LFAs emerged in the 1970s for molecular detection, focusing on proteins. Nitrocellulose membranes were used as a substrate to transport fluids via capillary action. LFAs typically capture labeled antibodies and qualitatively and/or semiquantitatively detect biomarkers by observing a colored band (23). Serological LFAs, including pregnancy and HIV tests, appeared in the 1980s (24). LFAs are now applied in the fields of medicine, food, environment, and biodefense (25). Paper-based microfluidics became a popular research area when the Whitesides group published the first μPAD using photolithography as the patterning technique (1). μPADs are different from LFAs and litmus paper because they use chemical printing and/or cutting to define flow channels. Thus, it became possible to conduct multiplexed analysis using small sample volumes. Photolithography (26), plasma treating (27), wax printing (28), plotting (29), wax dipping (30), inkjet printing (31), flexographic printing (32), laser treatment (33), and stamping (34) were demonstrated for fabrication of μPADs. In addition, three-dimensional (3D) origami-based μPADs were fabricated by folding and stacking paper to control sample and reagent flow for glucose and protein detection (35). Finally, different detection methods were introduced, such as electrochemical detection (36), fluorescence (37), and chemiluminescence (38).

FABRICATION STRATEGIES FOR PAPER-BASED ANALYTICAL DEVICES

Microfluidic devices can be fabricated using any type of porous membrane that possesses the right combination of thickness, pore distribution, price, and absorption rate. The properties of the paper should be carefully considered to choose the best membrane substrate to construct the

microfluidic device. Hydrophobic barriers are created by patterning the hydrophilic membrane so that sample and/or reagents can flow through isolated regions. Patterning methods such as photolithography (39), printing (31), cutting (40), and chemical vapor deposition (41) have been used to define hydrophobic barriers. Again, the barrier selection is important, and careful consideration should be given to the nature of solutions to be used to ensure compatibility.

Photolithography

Photolithography was the first patterning method for production of µPADs (1). Photolithography is a high-precision method enabling formation of well-defined hydrophilic and hydrophobic areas on the paper. Photolithography is based on transferring a geometric structure from a photomask, which is expensive and time consuming to produce, to a light-sensitive photoresist impregnated in the paper. Asano & Shiraishi (26) reported a fast, inexpensive fabrication approach using a 3D printer to pattern hydrophilic and hydrophobic zones onto the paper without using a photomask. In this method, chromatography paper was cut to the desired dimensions and dipped in an octade-cyltrichlorosilane n-hexane solution for 5 min. Next, the modified paper was exposed to UV light through the photomask to yield hydrophilic zones (26). The fabrication method offers advantages such as disposability, cost-effectiveness, lightweight quality, simplicity, and a lack of expensive fabrication instrumentation.

Plasma Treatment

Plasma cross-linking is an alternative method for patterning channels. Li et al. (27) first employed plasma treatment to pattern µPADs in 2008. Hydrophilic channels were formed by hydrophobization of the filter paper with alkyl ketene dimer with curing to make hydrophobic barriers. The paper samples were placed between patterned metal masks and then treated with the plasma (27). The plasma treatment has fewer steps than photolithography (1). For this method, an important disadvantage is that expensive plasma oxidizers and a mask are required to generate barriers. In addition, it is not suitable for mass production and cannot be used with organic solvents (42).

Wax Printing

Wax printing has been widely used for fabrication owing to its advantages that include low cost, simple fabrication, rapidity, robustness, and lack of organic solvent consumption (28, 43). Wax printing forms hydrophobic barriers and hydrophilic channels by printing wax patterns on the paper surface and melting the wax through the paper. Although wax-printed channels work with aqueous solutions, including acids, bases, buffers, and glycerol, wax is not compatible with most organic solvents (28). In addition, wax printing requires heating, and wax printers have limited availability (44). Moreover, the nature of the wax may cause certain biological solutions with low surface tension to flow through the hydrophobic barriers because of the reduced surface energy of the barrier. Finally, wax printing has poor resolution of designed patterns compared to photolithography methods. Wax spreading should be evaluated correctly to create the channels of defined geometry (28).

Lu et al. (45) and Carrilho et al. (28) first introduced wax printing for µPAD fabrication in 2009. Three ways of wax patterning were developed by Lu et al. In the first approach, a wax pen was used to draw the desired pattern on a paper, and the paper was heated in an oven to allow melted wax to penetrate the paper. In the second approach, the pattern was designed using a computer and printed with an inkjet printer followed by painting with a wax pen and melting

in an oven. The third approach was based on direct utilization of a wax printer for printing the desired pattern on the paper (45). In another study, Carrilho and coworkers (28) proposed a cheap and simple technique to fabricate paper-based microfluidic devices. In this work, a commercially available solid wax printer was used to print wax onto Whatman No. 1 chromatography paper, and a hot plate was used to melt the wax into the paper. After this process was completed, hydrophobic barriers were created in paper that allowed the sample or reagents to flow along the channels.

Chiang et al. (46) reported a μPAD that was fabricated in a single step by 3D wax printing, as shown in **Figure 1a**. In this study, a custom-made extruder was used to print patterns with high resolution. The pattern resolution obtained with the method was slightly lower than the photolithographic method; however, the approach was more affordable than photoresist. Another advantage of the method is that predesigned masks and heating equipment are not necessary for formation of hydrophobic wax barriers. A new wax printing technique called laser-heating-wax-printing was developed to rapidly pattern wax barriers in paper using a CO_2 laser (47). Typical wax printing includes two steps: patterning paper with a wax printer and using a heater to melt wax into the paper. However, this laser heating process offers a single-step device fabrication, which consists of heating, melting, penetration, and solidification of wax.

Pen Plotting

Although photolithography can produce small structures, the cost of related tools is undesirable for most applications. Plotting is an alternative method for fabricating devices. Nie et al. (29) presented a μPAD fabricated with a one-step plotting technique to decrease manufacturing production costs. The method is based on creating hydrophobic channels on paper during evaporation of the solvent from the pen. It is not possible to form channels with high reproducibility and resolution when drawing them by hand, but automated systems can improve this at the added cost of the equipment. Additionally, μPADs fabricated with this method are not suitable for using some organic solvents.

Pen plotting has some advantages compared to inkjet printing, such as reutilization of the pen cartridge with new reagents. Also, it is compatible with different substrates such as filter paper and nitrocellulose membranes. TPENs can also be used with most organic solvents, allowing for a broader range of chemical reagent inks. Finally, viscosity modifiers or surfactants are not required to plot inks onto paper.

Wax Dipping

While wax printing is attractive, it only coats the surface of the fibers and requires a wax printer. Manufacturing of wax printers has been discontinued. A novel wax dipping technique was reported to fabricate μPADs by Songjaroen et al. in 2011 (30). In this technique, an iron mold was produced with a laser cutter and was placed on Whatman No. 1 paper to protect the hydrophilic channel. The assembly was submerged into melted wax at 120–130°C for 1 h. After the assembly was cooled, the magnet was removed, leaving hydrophilic channels in the paper. This process does not require complicated and expensive apparatus or chemical compounds. Nechaeva et al. (48) modified the wax dipping method for fabricating μPADs to determine hydrogen sulfide in fuel oil samples (**Figure 1b**). After fabrication, the device was coated with Parafilm M. Next, the system was placed between two glass slides and heated in an incubator for 5 min to melt Parafilm M and paper layers together for creating hydrophobic and hydrophilic zones. Although wax dipping has some advantages, it is subject to some of the same issues as wax printing, such as the lack of organic solvent and surfactant compatibility.
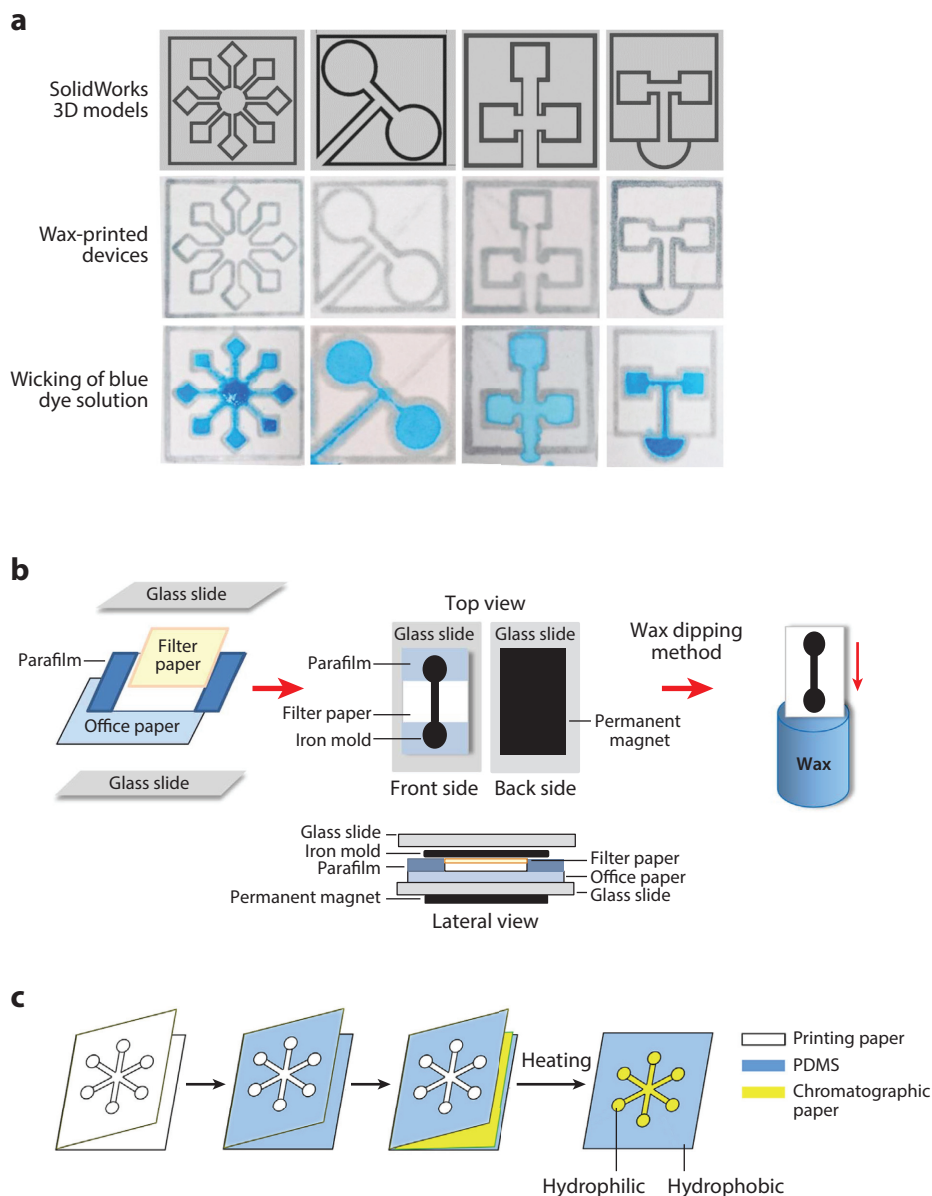
Figure 1

(*a*) Photographs of the 3D wax-printed μPADs. Adapted with permission from Reference 46. Copyright 2019, Elsevier. (*b*) Fabrication of a paper-based analytical device with modified wax dipping method. Adapted with permission from Reference 48. Copyright 2018, Elsevier. (*c*) Fabrication of μPAD by a folded paper mask. Adapted with permission from Reference 55. Copyright 2019, Elsevier. Abbreviation: PDMS, polydimethylsiloxane.

Inkjet Printing

Maejima et al. (31) introduced direct inkjet printing in 2013. This method makes hydrophobic patterns using an inkjet printer and a UV light source for curing. The UV curable ink used to pattern microfluidic channels consisted of a hydrophobic acrylate composition, including nonvolatile organic compounds. Hydrophobic barriers were generated after the ink penetrated into the paper followed by curing of the polymer with a UV light source for 60 s. This method is reproducible and capable of printing on a paper substrate as well as adding reagents for biochemical detection. It should also be noted that the ink in the cartridge cannot be replaced, and various organic solutions cannot be kept in the ink box for a long time due to the adverse effect of the organic solvent on the printer (49).

Flexographic Printing

Olkkonen et al. (32) published the first example of flexographically printed μPADs in 2010. Solutions of polystyrene in toluene or xylene used as printing inks were applied to an anilox roll to form hydrophobic barriers. The viscosity of inks used in flexographic printing is lower than the viscosity of screen printing ink. The plate and impression roll were rotated for penetrating the ink into the paper and patterning hydrophobic and hydrophilic channels. The advantages of flexographic printing are that polystyrene does not need heat treatment and is biocompatible. The other advantage is that it is capable of transferring reagents to the paper in a single roll-to-roll process (32). However, lower-resolution hydrophilic channels are attained by the flexography technique compared to the photolithography technique (50). Developments regarding this technique are still in progress, and further optimization studies are needed.

Laser Treatment

Laser treatment was adapted for fabricating μPADs in 2011 by Chitnis et al. (33). To treat the surface of parchment paper, a CO_2 laser cutting/engraving machine was used to change the wettability of the surface. Huang et al. (51) proposed a modified laser printing process to generate high-quality multilayer circuits on paper substrates. The approach used silver nanowire (AgNW) ink patterned with a solvent-free transfer method. At first, a heat roller was applied to the paper to form a polyimide film. Then, the patterned circuits were printed onto the paper to make the bottom toner sheet using a laser printing machine whose toner includes thermoplastic polymer powders mixed with carbon black or coloring compounds. Next, the polyimide film was coated with AgNWs that have excellent electrical and mechanical properties. This step was followed by a hot laminating treatment, resulting in the formation of the bottom AgNW circuit layer because the polyimide film adheres to the paper to attach AgNWs (51). Finally, an additional printing and coating process was employed to obtain multilayer 3D structured circuits. High-efficiency and solvent-free properties of the process are the advantages of this modified laser printing technique. In contrast to inkjet printing, paper wetting was not necessary, enhancing resolution and performance of the electronic circuits. Although silver-based inks have good conductivity and printability performance, they are expensive and have limited shelf life. Furthermore, the overall fabrication process shown in this work is complicated.

Stamping

Curto et al. (34) first introduced stamping for producing μPADs. In this technique, a polydimethylsiloxane (PDMS) stamp containing indelible ink as a modifying agent was applied to

a filter paper to create the microfluidic pattern. Although the technique is suitable for low-cost mass production, it has drawbacks, including low resolution of structures and the requirement for various shaped stamps (34). A related suspended-droplet mode microfluidic paper-based device (SD-μPAD) that relies on stamping and wetting coupled with gravity as the driving force was proposed by Sun et al. (52). Such devices can be rapidly and cost-effectively fabricated in large scale with a new microcontact printing approach that utilizes a Teflon contact printing stamp. At first, a Teflon stamp was fabricated with a thermo-molding method. For fabrication of the device, the Teflon stamp was applied onto a PDMS-coated glass slide followed by peeling off to attain a thin liquid PDMS layer on the surface of the stamp. After that, the liquid PDMS was allowed to cover the gaps within the pattern on the stamp in a vacuum chamber. The stamp was applied onto a piece of paper and cured. Once the Teflon stamp was removed from the surface of the paper, superhydrophobic patterns and a hydrophilic region were formed on the paper chip. The major advantage of the method is the ability to retain, deliver, and mix liquid samples in channels of the SD-μPADs owing to the difference in surface tension (52). The disadvantage is the need to make a new stamp for each new pattern, which can take time and add cost.

Paper-Based Device Configurations

μPADs can be fabricated in 2D or 3D formats. Whereas wax printing, inkjet printing, photolithography, and plasma treatment are typically used to fabricate 2D μPADs, stacking or folding the patterned paper along a vertical axis is used to fabricate 3D μPADs. The reproducibility and sensitivity of the microfluidic devices can be improved by the 3D structure of the sensor at the cost of more complicated fabrication.

3D Origami and Kirigami Paper-Based Devices

The first 3D μPADs were fabricated by patterning, cutting, and then stacking individual paper layers, resulting in a time-consuming fabrication process. Liu & Crooks (35) reported the use of folding following traditional origami methods as a faster, easier method for creating 3D devices. A wide range of devices can be produced in this way. Another advantage of 3D origami devices is that electrodes can be combined together on paper to create multiplexed assays (13). Using similar concepts, Sun et al. (53) proposed a novel rotational microfluidic paper-based device that prevents colorimetric reagent from diffusing in the detection zone, enhancing the accuracy and performance of the multiplexed colorimetric detection. The rotational paper-based device consisted of detection and auxiliary layers generated from wax printing. When assembled, the detection layer consisted of four detection zones and a sample reservoir. This novel rotational paper-based device offers a low-cost and user-friendly 3D paper-based platform for on-site analysis (53).

A paper-based immunochromatographic assay using 3D devices was developed by Weng et al. (54). After the channel pattern was printed onto chromatography paper using a wax printer, hydrophobic and hydrophilic zones were created using a heating process. Next, the patterned paper was folded based on origami principles to obtain a 3D fluidic structure with five zones, including a sample loading zone, two transportation zones, a detection zone, and an absorbent zone. This vertical flow assay is advantageous relative to LFAs in terms of fast analysis and reduced sample/reagent consumption (54).

Xie et al. (55) reported a novel method for fabrication of μPADs utilizing a folded paper mask, as shown in **Figure 1c**. A paper mask was patterned via an inkjet printer and cut along the design. Uncured PDMS was applied to coat the folded paper mask, followed by placing a chromatographic paper between the folded paper mask to obtain a sandwich structure. Thus, PDMS was able to

penetrate the chromatographic paper from both sides simultaneously, transferring the pattern from the paper mask. A curing process was then employed to generate a μPAD using the hydrophobic nature of the PDMS to prevent fluid leakage. The advantages of this technique include cost-effectiveness, simplicity, green chemistry, lack of high-tech tools, and fast fabrication.

Wang et al. (56) reported a paper-based microfluidic device that integrates an electromagnetically actuated valve and an adjustable time controller as an electric switch to detect multiple tumor biomarkers. The μPAD consists of a fluid-delivery channel, a timing channel, and a movable timing unit. The fluid delivery was time controlled to automate the system. The time controller includes a timing channel with two adjustable conductive iron bands, which trigger a magnetic valve with a connected fluid flow to the iron band, resulting in chemiluminescent immunoassay reactions. The flow can be manually controlled by manipulating the position of iron bands along the timing channel. With this method, carcinoembryonic antigen (CEA) in serum samples was successfully analyzed in 16 min.

Wax printing provides mass production of PADs as a result of its two-step process during fabrication. Although wax printing has been widely used for fabrication due to its advantages that include low cost, easy and fast fabrication, and lack of organic solvent consumption, wax printers have limited availability. In addition, wax printing has disadvantages such as low resolution and instability of patterns upon heating at high temperatures. To overcome the penetration issue of wax on paper, 3D origami devices have been designed with multilayer structures. The additional dimension enables flow control, improved sensitivity, and multiplexed detection.

Detection Devices

After fabricating devices, it is important to generate a readout. Although some μPADs can be analyzed with the naked eye, many give better results if some sort of readout instrument to improve accuracy and/or store data is used. Common readout devices include electrochemical and optical systems, with numerous variants being reported.

Optical Readout Devices

Im et al. (57) developed a novel smartphone-assisted, paper-based, optical biosensor for simple, cheap, and accurate detection of glucose and lactate in cell culture media (**Figure 2a**). After the sample was dropped onto the detection zone of the PAD, glucose and lactate reacted with horseradish peroxidase, resulting in the formation of a colored product in the presence of H_2O_2. Then, the PAD was placed in a smartphone-embedded camera to capture the image of the detection zones via a mobile application, followed by quantifying the analyte concentrations based on color intensity.

Chen et al. (58) designed a rapid colorimetric detection method using a competitive enzyme-linked immunosorbent assay (ELISA) on a μPAD for ketamine analysis used in the fight against drug abuse. An Android smartphone with a custom smartphone app and Image J software was utilized as an optical imaging device to analyze the test results in a portable, recordable, and transferable manner. With the addition of 3,3′,5,5′-tetramethylbenzidine (TMB) to the paper-based device, a colored product was formed for ketamine-based horseradish peroxidase in clinical oral samples.

Electrochemical Readout Devices

The Whitesides group (59) developed a portable electroanalytical platform by combining a commercially available handheld glucometer and μPADs for detection of cholesterol and ʟ-lactate in
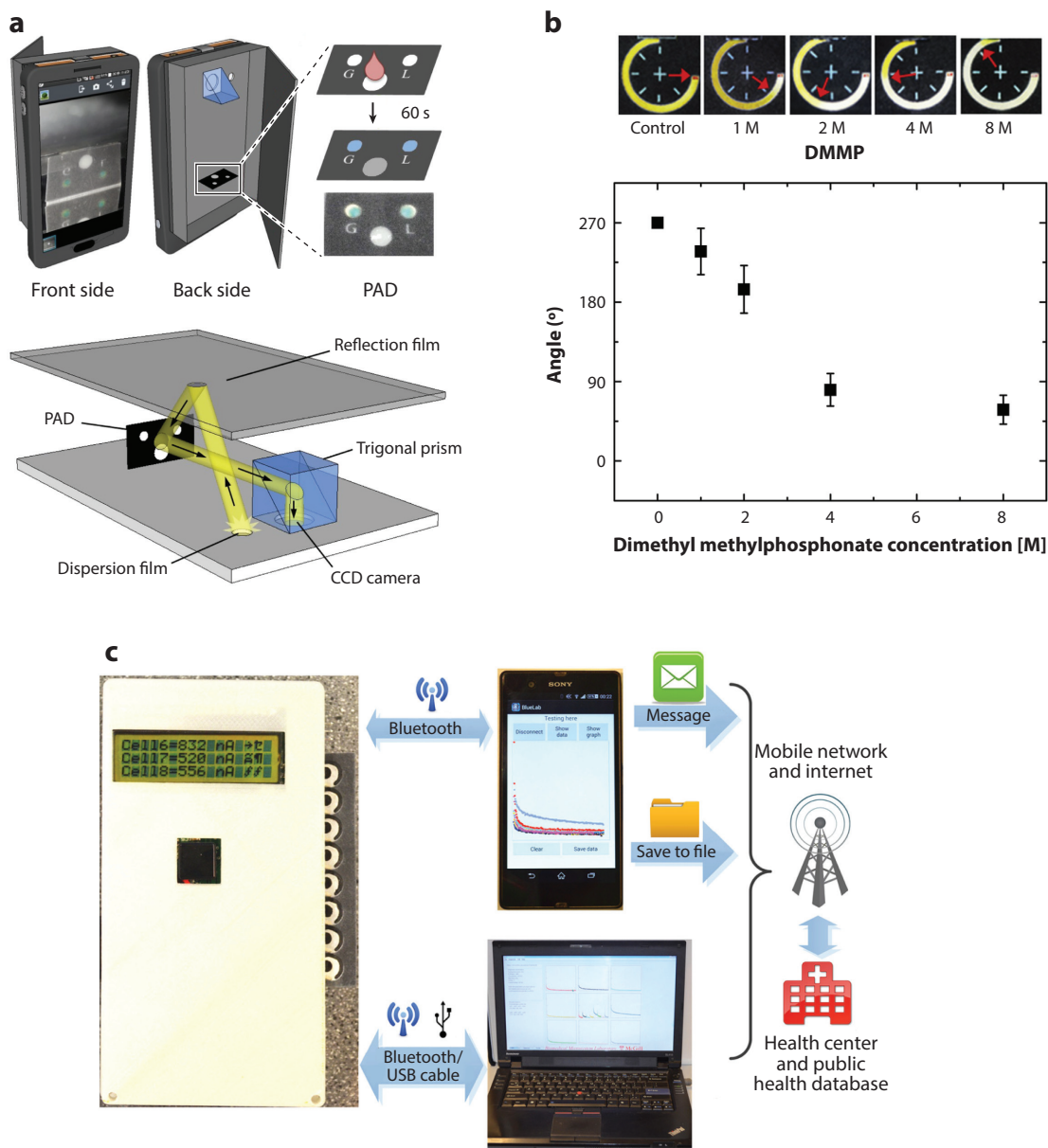
Figure 2

(*a*, *i*) Data acquisition from smartphone-based PAD. (*a*, *ii*) Principle of optical readout via the smartphone-based PAD. Adapted with permission from Reference 57. Copyright 2016, Elsevier. (*b*) Angle-based readout of DMMP in a foldable µPAD. Adapted with permission from Reference 68. Copyright 2018, Elsevier. (*c*) Wireless data transfer of a portable µPAD diagnostic platform with a multichannel potentiostat. Adapted with permission from Reference 60. Copyright 2016, AIP Publishing LLC. Abbreviations: CCD, charge-coupled device; DMMP, dimethyl methylphosphonate; PAD, paper-based analytical device.

human plasma and ethanol in food samples. The electrochemical μPADs (ePADs) consisted of microfluidic channels, electrodes constructed with graphite ink, and electrical wires printed with silver ink. A drop of sample was applied to the dry μPAD placed in the glucometer. Next, the electrochemical readout was read on the glucometer LCD. The study represented the first example of combining new μPAD methods with existing portable potentiostats.

Zhao & Liu (60) proposed an electrochemical microfluidic paper-based immunosensor array using a portable eight-channel potentiostat with a Bluetooth module for multiplexed detection of HIV and hepatitis C virus (HCV) (**Figure 2b**). The reaction zones were patterned on paper using wax printing, while the electrode system was printed on the reaction zones using pen plotting. Carbon and Ag/AgCl inks were used to fabricate the working, counter, and reference electrodes, respectively. The electrochemical readout on the potentiostat was triggered by opening the PC software or smartphone app. When an output signal was obtained, it was transferred to a remote site via its wireless communication. This integration of paper-based microfluidic sensors and mobile equipment enables users to perform inexpensive, portable, real-time, simultaneous detection of HIV and HCV (60).

Another example of electrochemical readout is the paper-based bipolar electrode-electrochemiluminescence system developed to detect glucose by Chen et al. (61). This system includes a rechargeable lithium battery, a smartphone used for imaging the electrochemiluminescence signal, a μPAD made with carbon ink screen printing, an electronic circuit model, and an instrument container. Microfluidic channels were fabricated by wax screen printing. The distance between the smartphone CMOS camera and anode was optimized to obtain a clear image of electrochemiluminescence emission. Using a smartphone decreased the detection platform cost relative to charge-coupled device cameras or photomultiplier tubes. The detection limits for glucose in phosphate buffer solution and artificial urine samples were found as 0.017 mM and 0.030 mM, respectively, over a concentration range of 0–5.0 mM, which were lower than those attained employing paper-based devices with detection techniques such as electrochemical, colorimetric, and three-electrode electrochemiluminescence (62).

Colozza and coworkers (63) developed a wearable ePAD for fast detection of mustard agents [bis(2-chloroethyl) sulfide] as aerosolized chemical weapons. Filter paper was used to support the ePAD, and all reagents were loaded onto the origami sheets, providing a reagent-free origami biosensor. Mustard gas compounds inhibit the enzyme choline oxidase; detection was achieved by measuring the enzymatic product, H_2O_2, at the screen-printed electrode, which was modified with carbon black/Prussian blue nanocomposite. In the case of a terrorist attack, these wearable sensors offer integration with drones, military uniforms, and portable devices, allowing an accurate, real-time alarm system for a mustard agent.

Instrument-Free Readout Devices

Although instruments can help improve accuracy and sensitivity of μPADs, they can increase cost and add to the necessary components for point-of-care measurements. Several methods have been developed that enable readout without instruments. One such approach is distance-based detection where the length of a colored bar on a μPAD can be used to quantify the analyte of interest (64–66). Using distance helps eliminate bias errors common to hue or intensity measurements. Also, distance-based devices can be combined with portable analytical devices. For example, a microfluidic distance readout sweet hydrogel–integrated paper-based analytical device (μDiSH-PAD) as a real-time analysis platform was fabricated by Wei et al. (67) to monitor different targets. Glucose was formed by enzyme glucoamylase due to the presence of the target in the analyte with the aid of the hydrogel. As glucose moved along the hydrophilic barrier via capillary action, H_2O_2

was generated by GOx and reacted with 3,3′-diaminobenzidine (DAB), turning brown by reaction with horseradish peroxidase. This technique is promising for real-time, selective, and sensitive clinical and environmental monitoring of trace targets using the target-responsive aptamer hydrogel system (67).

Lee et al. (68) recently proposed a foldable μPAD for detection of an acetylcholinesterase (AChE) inhibitor called dimethyl methylphosphonate (DMMP) based on a semiquantitative analysis with the naked eye (**Figure 2c**). Three-dimensional μPADs were folded along a dashed line, and all reagents were placed on the paper device to carry out the sequential enzymatic reactions. As a result of the reaction of H_2O_2 and a chromogenic indicator, the angle of color formation was obtained on the ring-type paper channel. The scale bars were added at 45° intervals to enable reading the angle of yellow area on the channel more accurately. The platform offers semiquantitative analysis of chemical warfare agents and organophosphate pesticide within 20 min.

APPLICATIONS

Because of their inherent advantages of speed, low cost, ease of use, and disposability, μPADs have found use in many applications. Much of the early work focused on medical applications and global health, but numerous other areas emerged, including food safety, environmental monitoring, and forensics.

Medical Applications

The US Centers for Disease Control and Prevention (CDC) reported that half of adults in the United States are affected by chronic diseases, accounting for 75% of all health care costs (69). Cardiovascular diseases are a leading cause of death, followed by infectious diseases, according to the World Health Organization (WHO) (70). Infectious diseases, including acute respiratory infections, malaria, HIV, and tuberculosis, cause more than 95% of mortality in developing countries (71). Cancer has also become a major threat to human life. The WHO reported that over 14 million people were diagnosed with cancer in the world in 2012. It is estimated that 22 million people will have cancer annually in the next 20 years (72). It is possible to improve patient condition and/or prolong the life span of cancer patients with early detection (73). Complex biological samples such as blood, tissue, urine, and saliva are used to detect cancer, diabetes, cardiovascular disorders, chronic lung disease, and infectious diseases in medical diagnostics. μPADs as point-of-care diagnostic tools have garnered attention because of their advantages of cost-effectiveness, portability, and simplicity. Biological samples can flow via capillary action due to the porous nature of paper, making paper a good alternative to traditional diagnostic techniques (18, 74).

Noiphung et al. (75) developed a novel PAD to determine blood type. Due to the cross-reactivity of different blood types, the specific blood type should be matched between donors and recipients. If not matched properly, a potentially fatal hemolytic transfusion reaction occurs. Traditional techniques for blood typing either require complicated steps or are insensitive and expensive (76, 77). In this study, both wax printing and wax dipping methods were used to fabricate the blood typing μPAD. A blood separation membrane was attached to paper using wax dipping for plasma separation. The device had better performance with twofold antibody immobilization and 1:2 sample dilution. The distance of red blood cell movement relative to the plasma separation in the channel was calculated to evaluate the blood type. During testing of real samples, the accuracy was compared to the traditional slide test technique and found to be 92%, 85%, 89%, 93%, and 96% for A ($n = 12$), B ($n = 13$), AB ($n = 9$), O ($n = 14$), and Rh ($n = 48$) typing, respectively. The developed μPADs were stable at 4°C for 21 days. Although the assay takes 10 min, which is
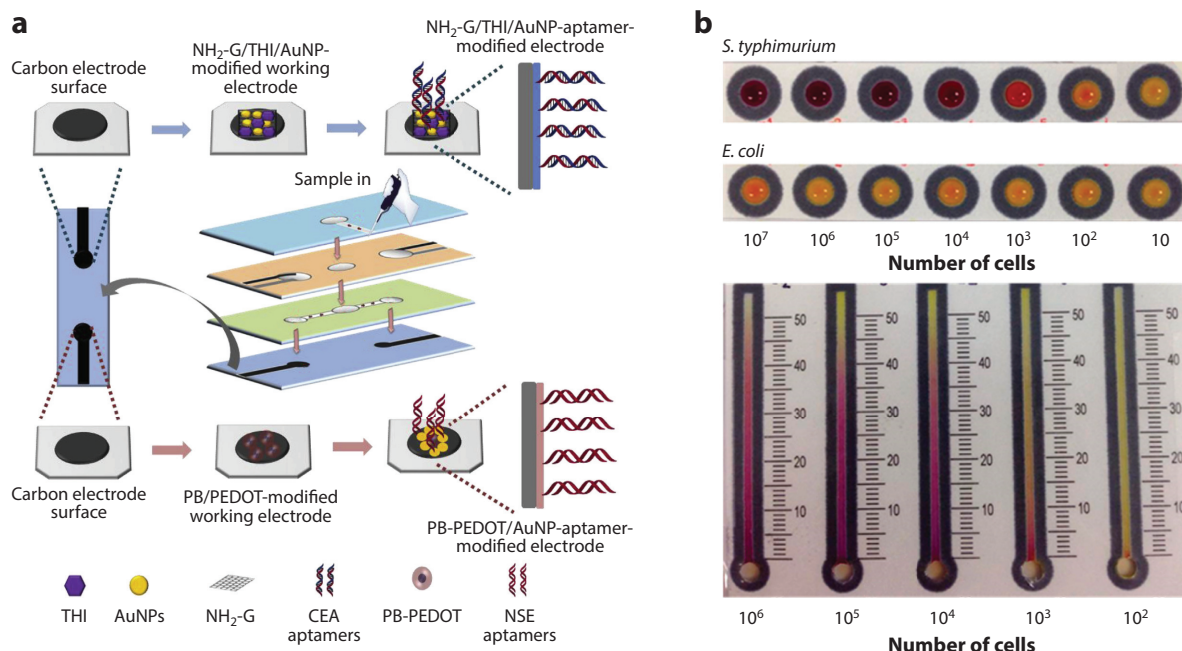
Figure 3

(*a*) Electrochemical paper-based aptasensor for multiplexed cancer biomarker analysis. Adapted with permission from Reference 78. Copyright 2019, Elsevier. (*b*) Well-array devices and chemometers for *Salmonella typhimurium* and *Escherichia coli* detection. Adapted with permission from Reference 4. Copyright 2017, American Chemical Society. Abbreviations: AuNP, gold nanoparticle; CEA, carcinoembryonic antigen; NSE, neuron-specific enolase; PB/PEDOT, Prussian blue/poly(3,4-ethylenedioxythiophene); THI, thionin.

longer than the slide test technique, further confirmation is not necessary for reverse ABO blood grouping using the proposed method.

Wang et al. (78) developed a paper-based electrochemical aptasensor to simultaneously monitor CEA and neuron-specific enolase (NSE) for early diagnosis of lung cancer (**Figure 3*a***). Among immunoassay techniques, label-free electrochemical methods have drawn attention for the selective and sensitive detection of cancer biomarkers. Wax printing was used to create microchannels, and screen printing was used to fabricate a three-electrode system. To improve the sensitivity of the aptasensor, amino functional graphene (NH$_2$-G)/thionin (THI)/gold nanoparticle (AuNP) and Prussian blue (PB)/poly(3,4-ethylenedioxythiophene) (PEDOT)/AuNP nanocomposites were synthesized for modifying the working electrodes. CEA and NSE aptamers were added to the (NH$_2$-G)-THI-AuNP-modified and the PB/PEDOT-AuNP-modified electrodes, respectively. In the final configuration, the screen-printed counter and reference electrodes were placed in one layer, and the screen-printed working electrodes were positioned in a separate layer. Next, the layers were combined using double-sided tape. Sample was added to the microchannel and, on reaching the screen-printed electrode system, CEA and NSE were detected using a multichannel potentiostat. The aptasensor demonstrated linear behavior in ranges of 0.01–500 ng mL^{-1} and 0.05–500 ng mL^{-1}, with detection limits of 2 pg mL^{-1} and 10 pg mL^{-1} for CEA and NSE, respectively. The typical level in a human serum sample for diagnosis of lung cancer is 5 ng mL^{-1} for CEA and 15 ng mL^{-1} for NSE.

Boonyasit et al. (79) developed an affinity paper-based electrochemical impedance device (PEID) for cardiovascular risk assessment. Monitoring inflammatory markers such as C-reactive

protein (CRP) is important in individuals at risk of coronary disease (80). The PEID was fabricated using screen printing. Cytidine 5′-diphosphocholine sodium salt dihydrate (CDP-choline) was immobilized on the electrode surface. Finally, the electrochemical impedance measurements were conducted using a single frequency value (100 Hz). The phosphocholine-modified screen-printed electrodes were utilized to detect CRP in blood samples and demonstrated a good sensitivity to CRP levels within 0.005–500 mg L^{-1}, with a detection limit of 0.001 mg L^{-1}. The developed PEID has advantages, including reduced assay time, from measuring only one frequency value via electrochemical impedance and low price due to the nonnecessity of antibody utilization on the electrode.

Foodborne Pathogen Detection

Foodborne pathogens, such as *Salmonella*, *Streptococcus* spp., *Escherichia coli*, and Norovirus, are transmitted to humans through consumption of contaminated food. Annually, 48 million cases of foodborne illness arise in the United States, with 3,000 cases resulting in death according to the CDC (81). In 2018, 53,000 pounds of standard meat products and 207 million eggs were recalled because of suspected *Salmonella* contamination (82). Viral foodborne outbreaks associated with Norovirus account for 37% of total outbreaks, whereas *Salmonella* is responsible for 34% of bacterial foodborne outbreaks (83). Ideally, food products need to be monitored at every stage in food production until distribution to the final customer. Therefore, early detection of foodborne pathogens is necessary to assure food quality and prevent foodborne disease.

The Henry group (4) developed a paper-based platform with immunomagnetic separation for detection of *Salmonella typhimurium* in bird feces and whole milk samples (**Figure 3***b*). For fabrication of the analytical devices, well-array and distance-based sensors were constructed using wax printing. *S. typhimurium* bacteria were separated from the sample using an *S. typhimurium* antibody conjugated to magnetic beads. Then, the bacteria were incubated with a biotin conjugated anti-*Salmonella* antibody. Next, β-galactosidase conjugated to streptavidin was added to create an enzyme-modified system. In the last step, the bacteria were quantified with chlorophenol red-β-D-galactopyranoside (CPRG) on paper devices due to the enzymatic reaction between CPRG and β-galactosidase, yielding a red-colored product. The color intensity in well-array sensors was captured with a smartphone and analyzed with Image J, whereas quantification with the distance-based device was done by measuring the length of the colored band. The detection limits obtained with distance-based and well-array paper devices were comparable. After inoculation of *S. typhimurium* in samples, the detection limits were found to be 10^5 CFU g^{-1} and 10^3 CFU mL^{-1} for bird fecal samples and whole milk samples, respectively, without culturing. Also, the method was selective toward *S. typhimurium* and showed no interference from *E. coli*. The proposed method has advantages over conventional culture methods, such as portability, reduced assay time, and ability to detect *S. typhimurium* in complex sample matrices.

Suaifan and coworkers (84) developed a fast, label-free μPAD for detecting *Staphylococcus aureus*. Since *S. aureus* bacterium is a top five foodborne pathogen, early detection is important to prevent foodborne illnesses (85). A gold electrode was generated using a gold-coated self-adhesive tape and a paper support. Magnetic nanobead-peptide probes were developed and immobilized on the gold biosensor via Au-S linkage, while unattached magnetic nanobeads were removed with an external magnet. The sensing mechanism was based on application of *S. aureus* proteases resulting in dissociation of a magnetic nanobead-peptide complex. Although the color change could be observed with the naked eye for qualitative detection, it was necessary to analyze color changes using ImageJ software for quantitative detection. The developed biosensor was successfully applied to detect *S. aureus* in pure broth culture and inoculated food products with detection limits of 7 CFU/mL and 40 CFU/mL, respectively.
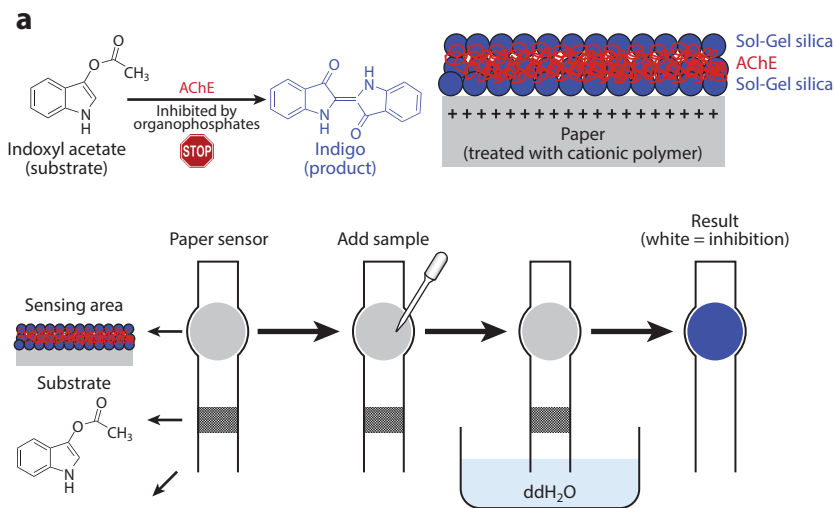
Environmental Applications

Environmental pollution is a rising concern in need of extensive monitoring because of the wide range of effects it can have on human, plant, and animal well-being. Environmental contaminants can take on a variety of forms, including physical, chemical, biological, and radiological (86). Detecting pollutants and following remediation efforts are priorities due to the ability of pollutants to transport into water, soil, and air. Upon human exposure, pollutants can cause health effects that include but are not limited to cancer, respiratory diseases, kidney disease, and nervous and skeletal damage (87, 88). Similarly, environmental pollution can have significant ecological impacts. Studies have shown that air pollution in particular can disrupt plant and aquatic life, causing a cascade effect of environmental disruption (89, 90). Among environmental contaminants of interest, pesticides, heavy metals, aqueous-based compounds such as perchlorate, various phenolic compounds, volatile organic compounds, fluorinated compounds, and pharmaceuticals are of particular concern (91). Because of the large abundance of environmental contaminants in food, water, air, and soil, it is critical to have cost-effective sensing platforms that are also reliable and reproducible. μPADs offer an alternative analytical technique to traditional methods that are inexpensive, effective, and suitable for in-field analysis of environmental pollutants (92). Herein, methods for detecting environmental contaminants with μPADs are discussed, and specific examples of each classification of contaminant are provided.

Organic Particulates and Pesticide Detection

Enzymatic and immunoassays have been frequently used for pollutant detection, particularly of pesticides, and their implementation onto paper has allowed analysis to be user friendly. One common assay format relies on the inhibition of AChE by organophosphates (**Figure 4a**). Nouanthavong et al. (93) fabricated nanoceria-coated μPADs to detect organophosphate-based pesticides. The nanoceria reacts with the H_2O_2 produced by the AChE enzyme system, causing a color change from colorless to yellow. The H_2O_2 concentration is inversely proportional to the pesticide concentration, yielding a decrease in the colorimetric output seen on the paper sensor when pesticide is present. The study looked specifically at methyl paraoxon and chlorpyrifos oxon pesticides and was able to achieve limits of detection of 18.3 ng/mL and 5.3 ng/mL, respectively, both of which are either better or comparable to previously reported findings. Other reports of devices that use the AChE assay for pesticide detection include the development biocompatible dipstick-like sensors and 3D colorimetric sensors, including foldable and multilayer paper-based devices (94–96). Additional methods for pesticide or related detection using either immunoassays or enzymatic reactions use supplementary detection techniques. Examples include the development of a time-resolved fluorescent immunoassay sensor for simultaneous detection of six chemical pesticide residues and a 3D origami electrochemical enzymatic biosensor for quantification of pesticides in surface waters (97–99).
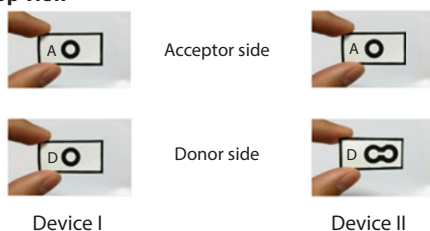
Volatile compounds such as ammonia are difficult but important to quantify because of risks to human health associated with them. Phansi et al. (100) developed a membraneless gas-separation sensor that uses colorimetric intensity quantification techniques (**Figure 4b**). The sensor is composed of three paper layers and is carefully fabricated to allow guided diffusion of the volatile compounds between layers. The diffusion pushes the analyte to the acceptor reservoir where it interacts with the reagents, resulting in a colorimetric reaction. The device is one of the first reports of a gas-liquid separation performed in a paper-based microfluidic device.

For detecting nonvolatile compounds, new nanoscale techniques such as molecularly imprinted polymers (MIPs) and nanoparticles have been utilized. Qi et al. (101) created a rotational paper-based device for multiplexed fluorescent detection of the phenolic compounds 4-nitrophenol and
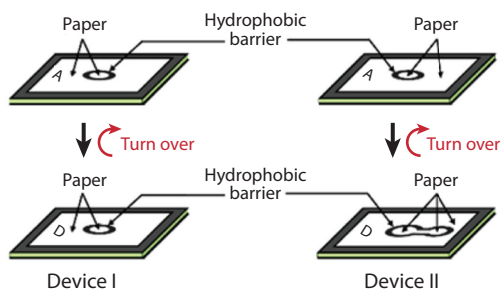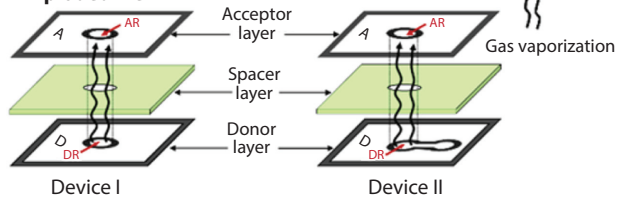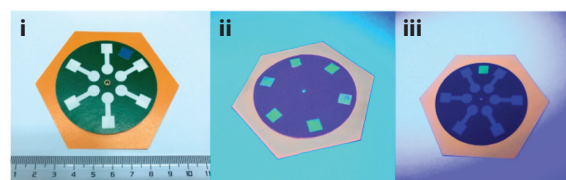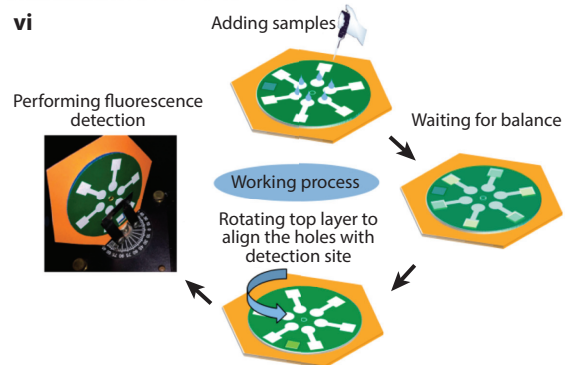
a

Indoxyl acetate (substrate) →[AChE, Inhibited by organophosphates, STOP]→ Indigo (product)

Sol-Gel silica
AChE
Sol-Gel silica
Paper (treated with cationic polymer)

Paper sensor → Add sample → → Result (white = inhibition)

Sensing area

Substrate

ddH$_2$O

b

Top view

Acceptor side
Donor side

Device I   Device II

3D view

Paper   Hydrophobic barrier   Paper

Turn over   Turn over

Paper   Hydrophobic barrier   Paper

Device I   Device II

Exploded view

Acceptor layer
Spacer layer
Donor layer

Gas vaporization

Device I   Device II

c

i   ii   iii

vi

Adding samples

Performing fluorescence detection

Working process

Waiting for balance

Rotating top layer to align the holes with detection site

2,4,6-trinitrophenol using quantum dots (QDs) and MIPs (**Figure 4*c***). The device consists of three layers: a sample layer, a middle layer, and a detection layer. On the detection layer, the MIPs were covalently bound to glass fiber paper impregnated with the QDs, stabilizing the fluorescent signal emitted by the QDs. Without any analyte bound, the sensor fluoresces. Upon binding, the fluorescence is quenched. It was determined that the MIP aided significantly in analyte recognition and fluorescent quenching, creating a better linear correlation. An initial sample volume of 5 μL was added to the sample layer, flowed through the device, and reacted with the QD-MIP framework. Once the reaction was complete, the device was rotated 60° to expose the detection reservoir and the fluorescent measurement was taken. High sensitivity and selectivity were achieved, with limits of detection for 4-nitrophenol and 2,4,6-trinitrophenol of 0.097 mg/L and 0.071 mg/L, respectively.

Similarly, Kong et al. (102) created a 3D sensor using MIPs for the detection of bisphenol A, an environmental contaminant that leaches from plastic and can cause adverse health effects (**Figure 5*a***). The MIP membranes and $ZnFe_2O_4$ nanoparticles were immobilized on paper layer A. The targeted analyte was added in various concentrations to the detection zone and allowed to incubate. On paper layer B, TMB was loaded to a corresponding detection zone, and layer A was placed on top of layer B followed by the addition of H_2O_2 and buffer under optimized conditions, which induced a color change detectable to the naked eye. Other detection techniques for organic contaminants that utilize nanomaterials are starting to become popular among researchers because they can be tuned for sensitivity and selectivity and allow for detection limits in the nanomolar and picomolar ranges. For example, Alvarez-Diduk et al. (103) created a sensor that uses embedded fluorescent graphene QDs with a turn-off response for sensitive detection of certain phenolic compounds.

Heavy Metals and Inorganic Contaminants

Similar approaches used to detect organic contaminants have been used to detect heavy metals, which are of significant concern due to their toxic effects in both humans and animals (88). Several methods for detecting heavy metals on μPADs have been reported, including but not limited to colorimetric, electrochemical, and fluorescence (105). Sun et al. (53) created a rotational device for multiplexed detection of Ni^{2+}, Cu^{2+}, and Cr^{4+} with limits of detection of 4.8, 1.6, and 0.18 mg/L, respectively (**Figure 5*b***). The rotational design improves detection performance by preventing reagents from diffusing into other areas of the device. Li et al. (106) designed a 3D multiplexed colorimetric sensor for detection of Fe^{3+}, Ni^{2+}, Cr^{4+}, Cu^{2+}, Al^{3+}, and Zn^{2+}. The device uses two pieces of paper and is engineered to create eight individual flow pathways to prevent random reagent diffusion. The limits of detection for all metal ions were approximately an order of magnitude lower than previous reports. The multiple paper layers allowed for multiple reactions between pretreatment and detection reagents to occur, yielding sensitive and selective simultaneous detection of metal ions.

A common problem with heavy metal detection is the limit of detection, as it is often present in concentrations <10 ppm. To combat this issue, researchers have begun implementing

a

+ BEE + EGDMA

Bulk polymerization

TMB+

oxTMB ← •OH

TMB ← H₂O₂

α-Sheet   β-Sheet   ZnFe₂O₄   BPA   AA

b

Adding colorimetric reagent

i

Rotating

Adding sample

ii

Rotating

Measuring color intensity

Blank   Ni   Cu   Cr

iv

iii

Colorimetric reaction

c

• Hg²⁺ in samples

2 min

Optical fiber analysis

Pt nanoparticles

TMB substrate

TMB

oxTMB

nanomaterials into the sensing platform, tapping into the nanomaterial's fluorescent and colorimetric properties upon binding with the metal of interest. Wu et al. (107) developed a fluorogenic turn-on probe for sensitive and selective detection of Cu^{2+} ions, with a limit of detection of 0.41 pM. With an increasing concentration of Cu^{2+}, the intensity of the fluorescence emission increased. Chen et al. (108) developed a sensor for quantitative determination of Hg^{2+} that utilized platinum nanoparticles and TMB (**Figure 5c**). The oxidation of TMB by the platinum nanoparticles induces a blue color change, but when Hg^{2+} is present, the reaction is inhibited, yielding a decrease in colorimetric response that is directly proportional to Hg^{2+} concentration. Much like the work done with pesticides, researchers have begun exploring with some success ion-imprinted technology on paper for heavy metal detection (109). The ion-imprinted technology has allowed for lower limits of detection while also remaining sensitive and selective for a variety of heavy metal ions. Qi et al. (110) developed a 3D multiplexed fluorescent sensor for quantitative detection of Cu^{2+} and Hg^{2+} ions, with linear ranges of 0.11–58.8 µg/L and 0.26–34.0 µg/L, respectively.

Much of the analysis done for environment contaminants using paper-based devices has been on waterborne contaminants (**Figure 4a**) (104). These include previously mentioned contaminates such as pesticides, heavy metals, and phenolic compounds, to name a few. Additional contaminants include nitrates and phosphates. Wang et al. (111) fabricated a paper-based device for electrochemical detection of nitrite. Utilizing the thin-layer diffusion mass transport of paper-based electrodes and the implementation of graphene nanosheets and gold nanoparticles, fouling-resistant and portable detection of nitrite was achieved. Cinti et al. (112) created a paper-based screen-printed electrochemical sensor for quantification of phosphate ion in river water. The sensor is first impregnated with the necessary reagents, followed by printing of the electrodes. The electroanalytical platform has high reproducibility, long storage times, and a limit of detection of 4 µM. However, there have been significant strides in monitoring contaminants found in soil, air, and other matrices. Ryan et al. (113) were able to create an electrochemical paper-based probe to monitor TNT concentrations found in soil. The soil samples were treated with a solution of glycol/choline chloride and then analyzed with the µPAD. In short, the working electrode was adhered to a strip of filter paper, which acts as an active transport mechanism for the TNT to reach the working electrode for analysis. Owing to the porous properties in the paper, interfering ions of different sizes such as methyl parathion can be accounted for.

Due to the complicated nature of environmental samples, analyte matrices are often a large obstacle. Matrix effects can affect the direct analysis of a specific analyte. For example, soil is composed of a complex matrix of nonvolatile to volatile compounds that can bind and interfere with detection. Ueland et al. (114) describe how humic acids in soil can disrupt the colorimetric detection of dangerous explosive residues found in soil. Generally, environmental samples have to undergo a detailed and often complex sample preparation to extract the analyte before analysis. Further adding to the problem, each environmental sample has a slightly different matrix, causing the sample preparation to be challenging. To address the challenges of complex matrixes, samples undergo digestion protocols that involve degrees of concentrated acids, ultrasonic frequencies, and high temperatures (115–117). To make environmental screening more user friendly and compatible for field analysis, development of less-aggressive and mild extraction procedures is critical. To combat issues such as matrix effects and low sample concentration, researchers have begun developing preconcentration techniques into their µPADS. Phan et al. (118) implemented ion concentration polarization in a µPAD that was able to preconcentrate the analyte prior to reaching the detection zone. Briefly, the microfluidic paper is impregnated with a polymer matrix of choice that can undergo ion-selective transport when exposed to a DC electric field. The device uses a combination of electroosmosis, ion selective transport, and electrophoresis transport

forces to concentrate the targeted analyte in a predetermined section of the paper that can then be subjected to detection with the addition of appropriate detection agents.

CONCLUSIONS AND FUTURE DIRECTIONS

In this review, we described the recent advances in paper-based microfluidic devices that can be used for environmental and point-of-care diagnostics. Methods of fabrication and detection modes were also discussed in detail. The research presented here proves that μPADs can be successfully applied for analysis of simple and complex systems with minor alterations in the device design, sample pretreatment, and fabrication methods. Despite the advances in paper-based microfluidic technology, there are several issues that remain. Analysis of real samples continues to pose a problem, as their complex matrices and need for pretreatment make them challenging for μPAD analysis. The stability and shelf life of the devices, particularly those that use biological assays for analysis, are additional topics of consideration. With paper-based devices starting to be used for multiplexed analysis, matters of selectivity, sensitivity, and limits of detection are still challenges. Continued efforts to address the needs of the field will allow μPADs to expand their usage as a standard analytical technique that can be implemented in a variety of traditional and nontraditional settings.

While there has been enormous growth in the field of μPADs, many challenges and opportunities lie ahead. First, most research in this field is based in academic laboratories around the world. Few examples of commercialization have occurred and even fewer have succeeded. Commercial growth has been hampered by the lack of a clear market with a reasonable value that will provide an acceptable return on investment relative to the existing lateral flow assay market. To make a lasting impact, inroads to key markets in either developed or developing economies are required to translate the technology out of the academic setting. To achieve this goal, the hurdles outlined above must be crossed. Beyond commercial growth, there remains significant opportunity to grow the fundamental science of the field as well. For example, the vast majority of studies discussed in this review use cellulose-based porous materials because they are inexpensive and widely available. However, other porous materials exist and may provide additional benefits if one considers the material science behind the flow and assay. There is also a need to continue making easier-to-use devices. Current devices are either inherently simple but have limited assay complexity or are complex to operate but capable of carrying out complex operations. Finally, the majority of research in the field has focused on human health applications. We have only begun to describe uses for fields such as food science, agriculture, and environmental analysis. In particular, merging μPADs with the field of citizen science where ordinary individuals collect data and report to a centralized database will provide unique information about the world around us and what we are exposed to on a daily basis.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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